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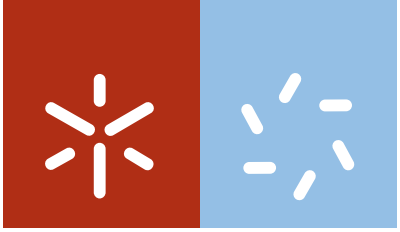
Plant-Based Pesticides:
Potential of Apiaceae Essential Oils
Rose Marie Oliveira Ferreira de Sousa
UMinho| 2016

Universidade do Minho
Escola de Ciências

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Plant-Based Pesticides: Potential of Apiaceae Essential Oils

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Professor Doutor Manuel Fernandes Ferreira
e da
Professora Doutora Ana Cunha

junho de 2016

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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Abstract

The development of biopesticides follows a strong rising trend over the last two decades, and a continued growth of the global biopesticides market is forecasted. This phenomenon is largely supported by the growing awareness to environmentally friendly food production and the introduction of restrictive regulations for pesticides. In light of their multiples advantage over synthetic pesticides, biopesticides appear economical, eco-friendly and less prone to the development of resistance, and hence are expected to provide more environmentally sustainable methods of biocontrol (Czaja et al., 2015). In fact, biopesticides have been integrated in several pest and vector management programs. As a complementary approach or alternative to synthetic pesticides, phytochemicals, namely essential oils (EOs) and volatile compounds are incorporated into crop protection products, as well as repellent formulations (Regnault-Roger et al., 2012). The best market opportunities have been assigned to EO-based pesticides, owing to the great availability, GRAS status, chemical simplicity, relatively low toxicity to vertebrates and specific mode-of-action of their constituents (Isman & Akhtar, 2007; Shaaya & Rafaeli, 2007).

In this context, the Apiaceae (=Umbelliferae) family, which comprised economically important plants species, presents a wide variety of bioactive compounds being also an important source of EOs and volatile compounds distributed by several distinct chemicals classes. Aware of their traditional use, their relatively large worldwide production and the bioactive potential described by many authors, we proposed to perform an appraisal of the biopesticidal potential of well-known Apiaceae species, whose experimental approach, results and discussion are here reported. In a few words, this work consisted in: (1) the characterization of EOs extracted from different plant parts of four species [*Anethum graveolens* (dill), *Cuminum cyminum* (cumin), *Foeniculum vulgare* var. *vulgare* (bitter fennel), *Petroselinum crispum* (plain leaf parsley)] and (2) the toxicological evaluation of some EOs and individual constituents, using as target organisms a mosquito (*Anopheles atroparvus*), a lepidopteran (*Pseudaletia unipuncta*) and a freshwater snail (*Radix peregra*). The anti-nematode properties of bitter fennel EOs and two phenylpropanoids against *Meloidogyne javanica* was also evaluated.

EOs were isolated by hydrodistillation and chemically characterized by GC and GC-MS. Quantitative data were obtained using GC-FID and the identification of constituents was achieved with the help of spectra libraries and determination of their KI. The overall identification of their content ranged between 88 and 99.5%. The EOs content (ranging between 0.10-3.45%) and chemical profiles were highly variable depending of the organ type, developmental stage and origin of the material. The development of dill umbels from the pre-flowering to the ripe fruit stage was accompanied by a significant increase of (S)-carvone (from 4 to 61% of the EO content) and a reduction of α-phellandrene and dill ether contents. Bitter fennel infrutescences and leaves plus stems EOs were mainly composed by estragole (64 and 28%, respectively), followed by α-phellandrene, α-pinene and fenchone. We also identified intra-individual

variations in the cultivated population of bitter fennel. Concerning cumin EOs, all fruit samples and the commercial EO were mainly constituted by cuminaldehyde (28-39 %), followed by variable amounts of *p*-mentha-1,4-dien-7-al, *p*-mentha-1,3-dien-7-al, β-pinene, *p*-cymene and γ-terpinene. EOs from parsley were the most complex of the four species, being characterized by a high percentage of hydrocarbon monoterpenes and several phenylpropenes (myristicin, apiole, elemicin and/or allyl-tetramethoxybenzene).

The activity of EOs and individual compounds was assessed against *A. atroparvus* (3rd instar larvae), *P. unipuncta* (eggs and larvae) and *R. peregra* (eggs, juveniles and adults). Bitter fennel EOs were also tested against eggs and juveniles of *M. javanica.* Whenever possible, mortality (%) was recorded for a range of optimized concentrations or at different times of exposure, and the lethal parameters for 50 % and 90% (lethal concentrations, LC_s, and/or lethal times, LTs) were estimated by Log-Probit analysis. EOs from parsley, cumin and bitter fennel (leaves more stems) were highly active towards the snail (both eggs and adults) and the mosquito larvae. Estimated LC_{50} against adult snails varied from 13.7 to 46.5 mg L^{-1} (48 h), and from 16 to 143 mg L^{-1} (24 h) against the mosquito larvae, with parsley fruit EO being the most dose-effective one in both situations. A short-time exposure (8 h) to bitter fennel EOs was also effective for snail control. Regarding assays performed on *P. unipuncta*, dill, cumin and parsley EOs, as well as the compounds (S)-carvone, cuminaldehyde and myristicin, showed satisfactory egg hatching inhibition, and acute contact toxicity to the 4th instar larvae (24 h-LC_{50} ranging from 108 to 197 µg cm^{-2}). Dill, cumin and bitter fennel EOs and some of their constituents were effective by fumigation (≥80%). In addition, the EO from parsley fruits, *trans*-anethole and cuminaldehyde displayed significant feeding deterrence and growth inhibition (FDI and GI >70%), when performing the no-choice assay with treated corn leaves, causing significant negative impact on the metabolism of this lepidopteran. Parsley fruits EO was of great efficacy against *P. unipuncta*, acting as ovicide, larvicide (by contact), feeding deterrent and growth inhibitor. Its constituents myristicin and apiole (purified by CC) showed similar effectiveness in several assays. Besides, we distinguish the phenylpropanoid *trans*-anethole (a constituent of bitter fennel EOs) for its acute insecticidal, nematicidal and molluscicidal activities, being more active than its isomer (estragole).

The present study demonstrates that EOs from the four studied Apiaceae species, along with some of their major compounds, have moderate to strong anti-insect and molluscicidal properties. EOs and compounds showed distinct performances depending on the type of assay, the exposure period, the tested organism and/or its developmental stage. In general, the reproductive organs (umbels-bearing fruits and eventually ripen fruits) were optimum sources of EOs containing the highest percentage of biologically active compounds (except bitter fennel). As a whole, the results demonstrates that the four plant species are promising sources of botanical biopesticides in the context of the biochemical control of phytophagous insects and vectors of human and animal diseases.

Resumo

O desenvolvimento de biopesticidas tem progredido consistentemente ao longo das duas últimas décadas e as previsões apontam para uma tendência de crescimento contínuo do mercado global destes produtos. Este fenômeno é explicado essencialmente por uma maior consciencialização para a produção de alimentos de um modo sustentável e pela introdução de regulamentações mais restritivas ao uso de pesticidas. Em virtude das suas múltiplas vantagens relativamente aos pesticidas sintéticos, os biopesticidas afiguram-se económicos, ecológicos e menos propensos ao desenvolvimento de resistências constituindo, portanto, métodos de biocontrolo ambientalmente mais sustentáveis (Czaja et al, 2015). Com efeito, os biopesticidas têm sido integrados em vários programas de gestão de pragas e vetores. Como abordagem complementar ou alternativa aos pesticidas sintéticos, os fitoquímicos, nomeadamente óleos essenciais (OE) e seus constituintes voláteis, são incorporados em produtos de proteção das culturas bem como em formulações repelentes (Shaaya & Rafaeli, 2007; Regnault-Roger et al, 2012). Existe uma grande oportunidade de mercado para os pesticidas à base de OEs ou seus constituintes, em virtude da sua grande disponibilidade, da sua classificação GRAS, da simplicidade estrutural das moléculas, da relativa baixa toxicidade para os vertebrados e modos de ação específicos (Isman & Akhtar, 2007; Shaaya & Rafaeli, 2007).

Neste contexto, a família Apiaceae (= Umbelliferae), que compreende espécies de plantas economicamente importantes, apresenta uma ampla variedade de compostos bioativos, sendo ainda uma importante fonte de OEs e compostos voláteis pertencentes a diversas classes químicas. Com fundamento nas suas utilizações tradicionais, na importante produção mundial e no potencial bioativo descrito por muitos autores, propusemos realizar uma avaliação do potencial biopesticida de espécies de *Apiaceae* bem conhecidas. Em resumo, este trabalho consistiu na: (1) caracterização dos OEs de diferentes partes de planta de quatro espécies [*Anethum graveolens* (aneto), *Cuminum cyminum* (cominho), *Foeniculum vulgare* var. *vulgare* (funcho amargo), *Petroselinum crispum* (salsa)] e (2) na avaliação toxicológica de alguns OEs, bem como de alguns constituintes, usando uma espécie de mosquito (*Anopheles atroparvus*), um lepidóptero (*Pseudaletia unipuncta*) e um caracol de água doce (*Radix peregra*) como organismos alvo. As propriedades anti-nemátode dos OEs de funcho amargo e dois fenilpropanoides contra *Meloidogyne javanica* foram igualmente avaliadas.

Os OEs foram isolados por hidrodestilação e caracterizados quimicamente por GC e GC-MS. Os dados quantitativos foram obtidos através de GC-FID sendo a identificação dos constituintes concretizada com base na consulta de bibliotecas espectrais e na determinação dos KI. A identificação abrangeu 88 a 99,5% do conteúdo total. Os teores em OE (compreendidos entre 0,10 a 3,45% w/w) e os perfis químicos variaram consideravelmente em função do órgão, do estágio de desenvolvimento e da origem do material. O desenvolvimento de inflorescências de aneto desde a prefloração à maturação dos frutos foi acompanhado por um aumento significativo de (S)-carvona (de 4 a 61% do teor dos OEs) e uma redução dos teores de α-felandreno e anetofurano. Por seu lado, os OEs de infrutescências e caules do funcho amargo eram maioritariamente constituídos por estragole (64 e 28%, respetivamente), seguida de α-felandreno, α-pineno e fenchona. Também

identificamos a ocorrência de variações intra-individuais na população cultivada de funcho amargo. Em relação aos OEs do cominho, todas as amostras de frutos e o OE comercial foram maioritariamente constituídos por cuminaldeído (28-39%), e quantidades variáveis de *p*-menta-1,4-dien-7-al, *p*-menta-1,3-dien-7-al, β-pineno, *p*-cimeno e γ-terpineno. De entre as quatro espécies os OEs de salsa foram os mais complexos, sendo caracterizados por uma elevada percentagem de hidrocarbonetos monoterpénicos e vários fenilpropenos (miristicina, apiole, elemicina e/ou alil-tetrametoxibenzeno).

Posteriormente, foi investigada a atividade dos OEs e alguns compostos individuais sobre *A. atroparvus* (larvas de terceiro ínstar), *P. unipuncta* (ovos e larvas) e *R. peregra* (ovos, jovens e adultos). Os OEs do funcho amargo foram ainda testados contra ovos e jovens de *M. javanica*. Sempre que possível, a mortalidade (%) foi registrada para uma gama de concentrações otimizadas ou em diferentes tempos de exposição, sendo os parâmetros letais para 50% e 90% (concentrações letais, CLs e/ou tempos letais, TLs) estimadas por análise Log-Probit. Os OEs de salsa, cominho e funcho amargo (folhas e caules) foram extremamente ativos contra as formas embrionária e adulta do caracol, assim como nas larvas de mosquito. As CL_{50} estimadas para os caracóis adultos variaram entre 13,7 a 46,5 mg L^{-1} (48 h), e entre 16 a 143 mg L^{-1} par as larvas do mosquito, sendo evidenciada a maior eficácia do OE de frutos de salsa na dose-resposta, contras ambos os organismos. A exposição de curta duração (8 h) aos OEs do funcho amargo foi também eficaz no controlo dos caracóis. Nos ensaios realizado com *P. unipuncta*, os OEs de aneto, cominho e salsa, bem como os compostos (S)-carvona, cuminaldeído e miristicina mostraram acentuados efeitos inibitórios na eclosão dos ovos, e uma aguda toxicidade por contacto sobre as larvas no quarto instar (as CL_{50} às 24 h variaram entre 108 a 197 μg cm^{-2}). Os OEs de aneto, cominho e funcho e alguns dos seus constituintes foram eficazes por fumigação (≥80%). Além disso, o OE de frutos de salsa, o *trans*-anetole e o cuminaldeído apresentaram efeitos inibitórios agudos no consumo de folhas de milho tratadas e no crescimento das larvas (FDI e GI> 70%), revelando um impacto negativo no metabolismo deste lepidóptero muito significativo. O OE dos frutos da salsa foi de grande eficácia ovicida, larvicida, fago-repelente, e inibidor do crescimento larvar. Os constituintes miristicina e apiole, ambos purificados do OE de frutos de salsa por CC, mostraram eficácia similar em alguns ensaios. Além disso, distinguimos o phenylpropanoid *trans*-anetole (constituinte do OE de funcho) pela sua forte atividade inseticida, nematicida e moluscicidas, sendo mais ativo que o seu isómero (estragole).

O presente estudo demonstra que os OEs das quatro espécies de Apiaceae, e alguns dos compostos testados, apresentam propriedades anti-inseto e moluscicidas moderadas a elevadas. Os OEs e compostos apresentaram desempenhos distintos, dependendo do tipo de ensaio, do período de exposição, do organismo testado e/ ou do seu estágio de desenvolvimento. Em geral, os órgãos reprodutivos destas plantas (infrutescências, eventualmente frutos maduros) foram ótimas fontes de OEs contendo percentagens superiores de compostos biologicamente ativos (exceto o funcho amargo). No seu todo, os resultados demonstram que as quatro espécies de plantas são promissoras fontes de biopesticidas botânicos no contexto do controlo bioquímico de insetos fitófagos e vetores transmissores de doenças em humanos e animais.

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Abbreviations, Acronyms and Symbols

AChE	Acetylcholinesterase
Ach	Acetylcholine
AD	Approximated digestibility
AI	Antifeedant index
Ag-I	*Anethum graveolens* infrutescences essential oil
Ag-L	*Anethum graveolens* leaves and stems essential oil
cAMP	Cyclic adenosine monophosphate
amu	Atomic mass unit
ANOVA	Analysis of variance
atm	Atmospheric pressure unit
ATP	Adenosine triphosphate
AUCPH	Area under the curve of cumulative percentage hatching
AUCPM	Area under the curve of cumulative percentage mortality
aza	Azadirachtin
BCA	Biological control agents
BPSG	Biopesticide steering group
Bt	*Bacillus thuringiensis*
CAGR	Compound annual growth rate
CC	Column chromatography
Cc-F	*Cuminum cyminum* fruit essential oil
CL	Confidence limits
Co.	Company
corp.	Corporation
$CuSO_4$	Copper sulphate
cv.	Cultivar
DC_{50}	Concentration causing 50% of feeding deterrence
df	Degree(s) of freedom
DG-SANTE	Directorate-general for health and food safety
DMADP	Dimethyl allyl diphosphate
DNA	Deoxyribonucleic acid
DXP	1-deoxy-*D*-xylulose
EC	European commission
ED	Effective dose
ECD	Efficiency of conversion of digested food
ECI	Efficiency of conversion of ingested food
EI	Electron ionization
EIL	Economic injury level
EO(s)	Essential oil(s)
ETL	Economic threshold level
EU	European union
eV	Electron volt (as a non SI unit of energy)
F	F-value
FAO	Food and agriculture organization of the USA
FDA	Food and drug administration of the USA
FDI	Feeding Deterrence Index
FDP	Farnesyl diphosphate
FID	Flame ionization detector
Fv-I	*Foeniculum vulgare* var. *vulgare* infrutescences essential oil

Fv-L	*Foeniculum vulgare* var. *vulgare* leaves and stems essential oil
FRAC	Fungicide resistance action committee
g	Gram(s)
GC	Gas chromatography
GDP	Geranyl diphosphate
GGDP	Geranylgeranyl diphosphate
GI	Growth inhibition
GPCRs	G-protein coupled receptors
GRAS	Generally recognized as safe
H	Heterogeneity factor
HD	Hydrodistillation
HRAC	Herbicide resistance action committee
IC	Inhibitory concentration
ID	Inner diameter
IDP	Isopentenyl diphosphate
IHMT	Instituto de Higiene e Medicina Tropical
IGR	Insect growth regulator
IPM	Integrated pest management
IR	Infrared
IRAC	Insecticide resistance action committee
ISO	International organization for standardization
ITD	Ion trap detector
IVM	Integrated vector management
J_2	Second stage juvenile nematodes
KI	Kovats retention index
K_{ow}	Octanol-water partition coefficient
L_3	Third instar
LC	Lethal concentration
LD	Lethal dose
log P	Logarithm of the octanol-water partition coefficient
LSD test	Fisher's Least Significant Difference (post-hoc test)
LT	Lethal time
MAP	Medicinal and aromatic plants
MEP	2-C-Methyl-*D*-erythritol-4-phosphate
MC	Metabolic cost
MH	Monoterpenes hydrocarbons
MIC	Minimum inhibitory concentration
MRL	Maximum residue limits
MVA	Mevalonate
N	North
MDP	methylenedioxyphenyl compounds
mm Hg	Millimeters mercury (a non SI pressure unit)
MOA	Modes of action
MS	Mass spectrometry or mass spectra
Mw	Molecular weight
n	Number of observations/ replications
no.	Number
NMR	Nuclear magnetic resonance spectroscopy
OE(s)	Óleo(s) essencial(ais)
OECD	Organization for economic co-operation and development
OCM	Oxygen-containing monoterpenes

O/W	Oil in water
p	P-value
PAL	Phenylalanine ammonia lyase
Pc-F	*Petroselinum crispum* fruits essential oil
Pc-I	*Petroselinum crispum* infrutescences essential oil
PD	Phenylpropene-derivatives
PEP	Phosphoenolpyruvate
PGR	Plant growth regulator
pH	Potential hydrogen
PPP	Plant protection products
ppm	Part per million
PVOC	Plants' volatile organic compounds
r	Coefficient of correlation
r^2	Coefficient of determination
RCR	Relative consumption rate
R&D	Research and development
Rf	Retention factor
RGR	Relative growth rate
RH	Relative humidity
RNA	Ribonucleic acid
RMR	Relative metabolic rate
RyR	Ryanodine receptor
SD	Standard deviation
SEM	Mean Standard error
SH	Sesquiterpenes hydrocarbons
SI	International system of units
SPSS	Statistical package for the social sciences
spp.	Species of a genus
subsp.	Subspecies
TAL	Tyrosine ammonia lyase
TLC	Thin layer chromatography
Tyr R	Tyramine receptor
Tween®	Registered trademark for polysorbate
USA	United States of America
USEPA	Environmental Protection Agency of USA
UV	Ultra-violet
var.	Variety
V	Volume
Vo	Void volume
v/w	Volume per weight
w/v	Weight per volume
w/w	Weight per weight
WHO	World Health Organization
α	Significance level
λ	Wavelength
χ^2	Pearson's chi-square

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Sousa R.M.O.F., Rosa J. S., Oliveira L., Cunha A., Fernandes-Ferreira M., 2015. Activities of Apiaceae essential oils and volatile compounds on hatchability, development, reproduction and nutrition of *Pseudaletia unipuncta* (Lepidoptera: Noctuidae). *Ind Crop Prod* **63**: 226–237.

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Sousa R.M., Rosa J. S., Cunha A., Fernandes-Ferreira M., 2014. Activity of *Anethum graveolens*, *Foeniculum vulgare, Petroselinum crispum* and *Cuminum cyminum* essential oils and volatile compounds against the freshwater snail *Radix peregra* (Lymnaeae). *Planta Med*, P2B49.

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Sousa R.M.O.F., Novo M.T. & Fernandes-Ferreira M., 2012. Larvicidal activity of Apiaceae essential oils against *Anopheles atroparvus* Van Thiel (Diptera, Culicidae) a former European malaria vector. 43rd International Symposium of Essential Oil (ISEO2012), Faculty of Sciences, University of Lisbon, Portugal.

Forewords

This PhD thesis is organized in 7 individual chapters and a section of appendices, each one comprising independent bibliography.

In the first chapter we present a review of the state of the art, positioning the developed work in the context of plant-based biopesticides. The **chapters II** to **VI** correspond to contents that are accessible, for its most part, in scientific articles (references of publications are provided in the first page of each chapter). To conclude, the **chapter VII** contains an integrated discussion of the work and some final considerations.

In order to avoid the repetition of chemical characterization of the plant products used in several biological assessments, we found convenient to organize in a single chapter (chapter **II**) all data concerning plant material and the performed phytochemical study. The aforementioned section is addressing to the nature, biogenesis, occurrence, and function of essential oils (EOs) and assembles the results related to chemical characterization of four Apiaceae EOs, which is in line with the **phase 1** of this work. Subsequent chapters (**III, IV, V** and **VI)** are addressing to the biological activity of several EOs (fulfilling **phase 2**). Each chapter's arrangement (**II** to **VI)** includes introduction, methodology, results, discussion (or eventually results and discussion) and bibliography.

Moreover, supplementary material consisting of some preliminary results that were not published were inserted in the appendix D.

CHAPTER I

The potential of plant-based biopesticides for the management of pests and disease vectors:

Apiaceae as a source of botanical pesticides

1. Introductory notes

The purpose of this introductory chapter is to bring some focus on current and future challenges concerning the management of pests and disease vectors and their biorational control through the use of natural products of plant origin.

Emphasis will be given to essential oils (EOs) as plant added-value products, discussing the opportunities and constrains to the use of EO-based pesticides. In addition, we examine the state of knowledge concerning secondary metabolites produced by plants of the Apiaceae family, highlighting the pesticidal activities described for EOs against insects, molluscs, nematodes, pathogens, *etc.*

But this chapter would not be complete without taking a look toward the future and the feasibility of some solutions and so we will discuss the use of biopesticides in a more global perspective, giving insights about social, economical and regulatory aspects.

The current chapter is structured in an attempt to give a comprehensive outlook of several subjects, following an interdisciplinary approach. While covering each major core subject, key concepts and fundaments will be provided and informations concerning each investigated plant species will be delivered. To conclude, we explain the goals of the current work, and the reasons that motivated this research.

Keywords: Apiaceae, biocontrol, bioeconomy, biopesticides, diseases vectors, eco-friendly, environmental issues, essential oils, medicinal and aromatic plants (MAP), mode of action, pests, pest management, phytochemical, semiochemicals, toxicity.

2. Toward a biorational control of pests and disease vectors

Any organism that interferes with the convenience or well-being of humans or other species favored by humans is generally regarded as a '**pest**' (Cooper, 1991). Although this notion of pest is often entomologically biased, it also encompasses many nonarthropod animals (invertebrate and vertebrate), pathogens and weeds (Kogan, 1998).

The concept of pest is more frequently used in the agrosystem context being related to plant and stored products damage and losses. Nearly 67 000 different crop pest species, causing about a 40% reduction in the world's crop production, were described (Oerke et al., 2012). Overall, weeds are responsible for the highest potential loss (34%), with animal pests and pathogens being less important (18 and 16%, respectively). Frequently, minor crop losses are economically acceptable but damages caused by pests might become an economic burden if yield losses attain 5% or more (Paranjape et al., 2014). In less developed countries, where the access to **Plant Protection Products** (PPP) is limited, annual crop losses ranging from 30 to 50% are commonly reported, threatening the food supply (Dubey et al., 2011). Moreover, the deterioration of food commodities during storage is another relevant dimension of the problem. Insects, rodents and fungi might destroy stored foodstock, being the latter organisms a serious threat to public health due to their mycotoxin secretions (Dubey et al., 2010). In addition, a variety of insects, snails or rodents have a key role in the transmission of **vector-borne diseases**[1] as vectors of pathogens (namely microorganisms and parasites). Besides their direct impact on human health, inflicting a heavy burden on human populations particularly in developing countries in tropical and subtropical zones, vector-borne diseases are also a significant obstacle to socioeconomic development (WHO, 2012). For instance, mosquitoes act as vectors for most of the life threatening diseases (i.e. malaria, yellow fever, dengue fever, chikungunya ferver, filariasis, encephalitis, West Nile Virus infection, *etc*.) which are prevalent in more than 100 countries across the world, infecting over 700 million people every year (Ghosh et al., 2012). Therefore, vector control strategies are an important component of the prevention and management of these human diseases (WHO, 2012), being equally crucial for livestock (Aktar et al., 2009).

During the first half of the 20th century, 'pest control' was understood as the set of actions taken to avoid, attenuate, or delay the impact of pests on crops or domestic animals (Kogan, 1998). Natural pesticides (either extracts or main compounds from natural sources) were widely used with moderate success until the 1940s (Casida, 1980). However, these were progressively replaced during the following decades by a number of more potent and stable synthetic analogs (namely pyrethroids) and new chemicals displaying long lasting effects (Casida, 1980). Protection specialists began to focus on testing these chemicals, to the detriment of studying pest biology and non biocidal methods of control (Chandler et al., 2011). This advent of organosynthetic pesticides was the major step forward into the green revolution operated in different industrialized countries between the

[1] 'Vector-borne disease' is the collective term for transmitted infectious diseases. Vector-borne disease patterns are affected by climate change, environmental degradation and urbanization (WHO, 2012).

1940s and 1960s (Hüter, 2011; Dubey et al., 2010; Glare, 2015). For over 50 years, pest management has relied heavily on the use of synthetic chemical pesticides and PPP have been a first choice policy to prevent crop damage, and eventually the worst scenario of a total loss (Hüter, 2011). Similarily, interventions for vector control have relied on the use of a limited choice of insecticides (WHO, 2012).

Generally, pesticides have been used in three sectors viz. agriculture, public health and household use. Major companies of the agrochemical industry have developed a multitude of chemicals showing biocidal or protectant activity against insects, mites, nematodes, rodents, weeds, pathogens, *etc*. Such plethora of chemical formulations may be classified according to distinct criteria[2]. Although, this massive use of synthetic chemicals has undoubtedly resulted in a dramatic increase of crops yields and food production (Chandler et al., 2011) and a substantially reduction in the transmission of several vector-borne diseases (Malaria was eliminated from a number of countries) (WHO, 2012), that practice rapidly revealed to be unsustainable or hazardous. Evidences of widespread contamination, high negative impact on environment and toxicological effects of synthetic pesticides on non-target organisms, including beneficial insects and humans, are well documented (Casida, 1980; Smith, 1987; WHO, 1990; Aktar et al., 2009; van der Sluijs et al., 2015). Pests and vectors management relying on broad-spectrum synthetic chemicals have instigated the development of novel and more effective molecules and new generations of pesticides (Casida, 1980; Isman, 2002), though giving rise to a pernicious and acceletated cycle of product/ pest resistance development. Such major drawbacks (to be explored in the section 2.3 and table I.1) have also stirred an increasing interest in safer and environmentally benign strategies, namely the resurgence of non-chemical means of pest control and the development of a wide range of eco-chemical and biorational products of different origins, labelled as **biorational pesticides** or **biopesticides** (Glare et al., 2012).

At a time when environment and health are primary concerns and new legislations are leading to the withdrawal or reduced accessibility to many synthetic chemical pesticides, there is an urgent needed for alternative pest management strategies that meet the overall demands (Glare, 2015; Chandler et al., 2011). It became imperative to development environmentally benign pest control methods in compliance with the implemented regulatory actions (Arango & Salazar, 2011; Chandler et al., 2011). Over the next 20 years, crop production will have to increase significantly to meet the needs of a rising human population in food, fibers, energy, *etc.* As it happened to be true 50 years ago, the size of arable land is limited and cannot be expanded without further inroads into vital natural habitats like the rain forests (Hüter, 2011). Unquestionably, the most sustainable way to increase yields and food availability is to combine sustainable methods in integrated schemes known as **Integrated Crop Management** (ICM) (Hüter, 2011) and **Integrated Pest Management** (IPM) (Chandler et al., 2011).

[2] Supplementary information is provided in Figure A.1 of the appendix A.

In the following sections, we identify the different classes of biopesticides and examine more particularly plant-based products/chemicals, discussing their integration in pest/vectors management programs.

2.1. Biopesticides and the integrated management of pests and vectors

According to Chandler and co-workers (2011), biopesticides are a particular group of crop protection tools used in IPM that encompass a very wide range of living and non-living entities (Fig. I.1), markedly variable in their composition, mode of action and fate in the environment *etc.* This designation has been applied with a narrow focus on manufactured preparations based on natural or biotechnologically produced organisms, though a broader definition that also comprises botanical compounds and **semiochemicals**[3] is currently accepted (Copping & Menn, 2000; Arango & Salazar, 2011; Chandler et al., 2011; Glare et al., 2012; Glare, 2015). More recently, beneficial microbes (endophytes) have been suggested as an additional class of biopesticides (Glare et al., 2012; Glare, 2015) and alternative classification was established with basis on their **modes of action** (MOA) (Villaverde et al., 2014). Since the above-mentioned groups of biopesticides can display various activities/functions (Fig. I.1) they are suitable for several purposes.

In the *sensu amplo*, a biopesticide is a formulation based on the activity of an agent of natural origin that has pesticidal action. However, due to the wide range of research fields and the variety of pests that can be involved, the definition has been recurrently used in a more restricted sense (Glare, 2015). According to Copping and Menn (2000) and the US Environmental Protection Agency (USEPA)[4], biopesticides can be categorized in three or four classes considering the type of pest they address and the source or production method (Fig. I.1). Yet, this definition given by USEPA has not been followed by in the entire world community. Other organizations and bodies, specifically, the International Biocontrol Manufacturer's Association and the International Organization for Biological Control (IOBC, 2008), have been promoting the use the term **biological control agents** (BCAs).

Nowadays, biopesticides are being used across the world, more commonly in plant protection, and recognized as safe pest management tools by contrast to the conventional synthetic pesticides. Pest management agents have become an important component of environmentally friendly pest control programs and a suitable alternative to synthetic pesticides (Glare et al., 2012). Biopesticides have a range of attractive properties that make them eligible components of IPM (Table I.1). In specific contexts, the use of living systems (insects, bacteria, fungi, nematodes and microsporidia), or their toxins, may become quite successful approaches when integrated with other control measures (Kerry, 1997). By 2013, the USEPA[4] reported approximately 400 registered active

[3] Term that designate naturally occurring substances (or mixtures) comprising both information-conveying chemicals and toxin (Koul, 2000) that are used by individuals to establish intraspecific and interspecific communication.

[4] USEPA, Office of Pesticide Programs.Online: http://www.epa.gov/pesticides/about/types.htm [April 27, 2015].

ingredients and 1250 biopesticides products in the USA. Furthermore, global information with reference to commercialized biopesticides may be found in *The manual of biocontrol agents*, edited by Copping (2011) and/or in several online databases (Box 1).

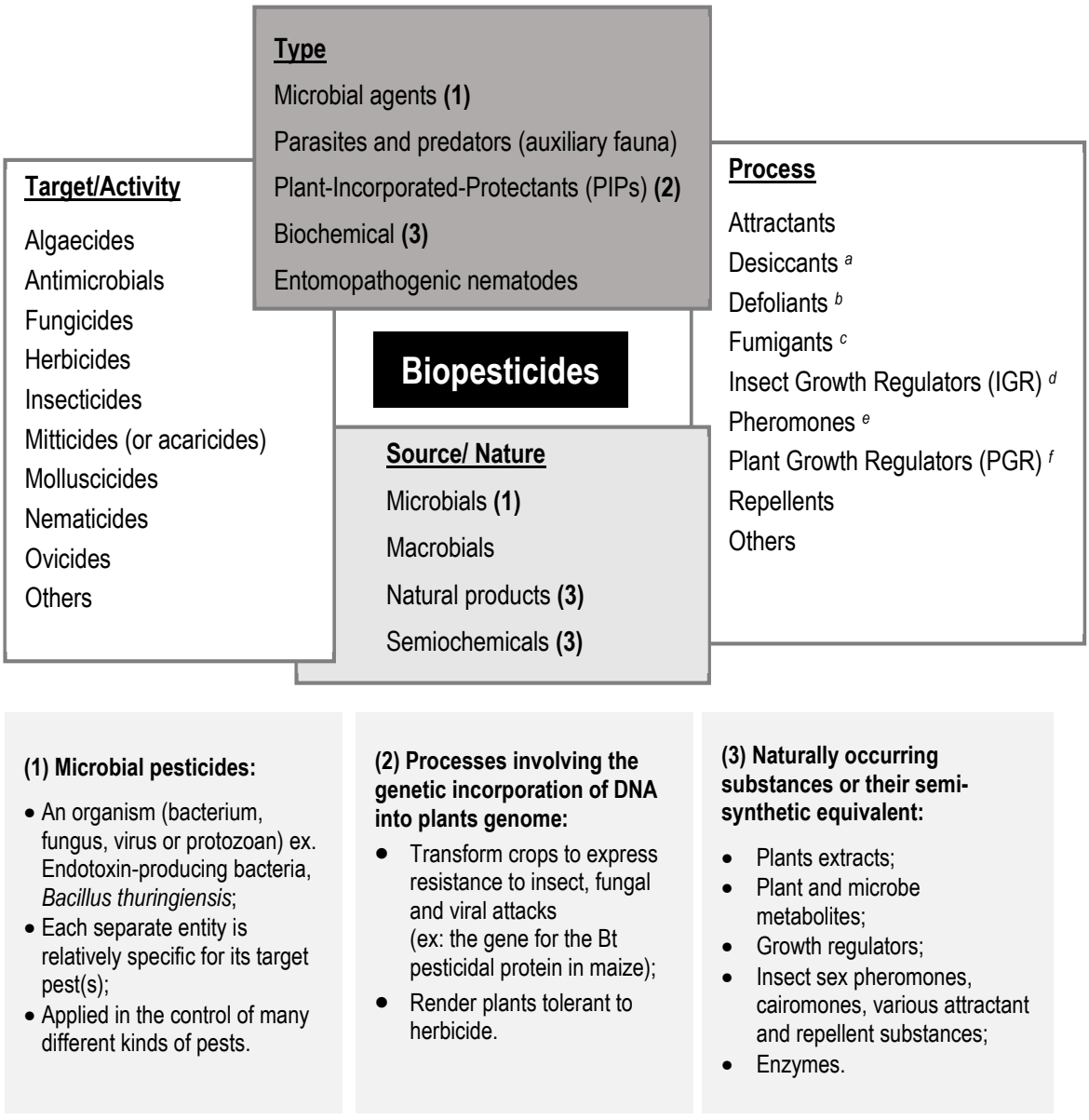


Figure I.1. Classifications and description of biopesticides.
[Sources: Copping & Menn (2000); Glare (2015); the US Environmental Protection Agency]

a Promote drying of living tissues, such as unwanted plant tops. **b** Cause leaves or other foliage to drop from a plant, usually to facilitate harvest. **c** Produce gas or vapor intended to destroy pests in buildings or soil. **d** Disrupt the molting, maturity from pupal stage to adult, or other life processes of insects. **e** Biochemicals used to disrupt the mating behavior of insects. **f** Substances that alter the expected growth, flowering, or reproduction rate of plants (excluding fertilizers or other plant nutrients).

Box 1. Databases concerning biopesticides and organisms

The Biopesticides Database (BPDB) provides around 450 records of biocontrol agents ranging from naturally derived substances to insect predators:
http://sitem.herts.ac.uk/aeru/bpdb/

A list of active substances authorized for use in the EU may be accessed online at the EU Pesticides Database: http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=homepage&language=EN

A list of BCAs widely used and commercialized in the European and Mediterranean Plant Protection Organization (EPPO) can be found at:
http://archives.eppo.int/EPPOStandards/biocontrol_web/bio_list.htm

Informations concerning biopesticide active ingredient provided by the Office of Pesticides Programs of the USEPA, are available at:http://iaspub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:46:0::NO:::

For thousands of years, agricultural practices relied essentially on crop rotation or mixed crop planting to optimize natural pest control (such as predation, parasitism, and competition). Therefore, the concept of 'natural pesticides' arose early along with the development of agriculture (Dayan et al., 2009). During the 1st half of the 20th century, 'pest control' was understood as the set of actions taken to avoid, attenuate, or delay the impact of pests on crops or domestic animals. However, it was not until 1972 that the term 'integrated pest management' and its initials IPM were accepted by the scientific community, and incorporated into the English literature (Kogan, 1998). Despite the existence of some ambiguity about the meaning of the IPM (dozens of different definitions have been suggested) there is substantial agreement about the essence of the IPM concept (Kogan, 1998). The following broader definition adopted by the FAO panel of experts (1967) has served as a template for others:

> '*Integrated Pest Control is a pest management system that, in the context of the associated environment and the population dynamics of the pest species, utilizes all suitable techniques and methods in as compatible a manner as possible and maintains the pest population at levels below those causing economic injury*.'

Theoretically, IPM is a system of holistic approach to limit pest damage to tolerable levels through a combination of environmentally benign tactics, techniques and technologies in agriculture (Bird, 1987). The objective of IPM approaches is not to eradicate pest populations but rather to maintain them below the levels that causes economic damage (ETL and EIL) by combining different practices together and by following an operational framework (Fig. I.2). IPM has been one of the major key drivers of the biopesticide industry.

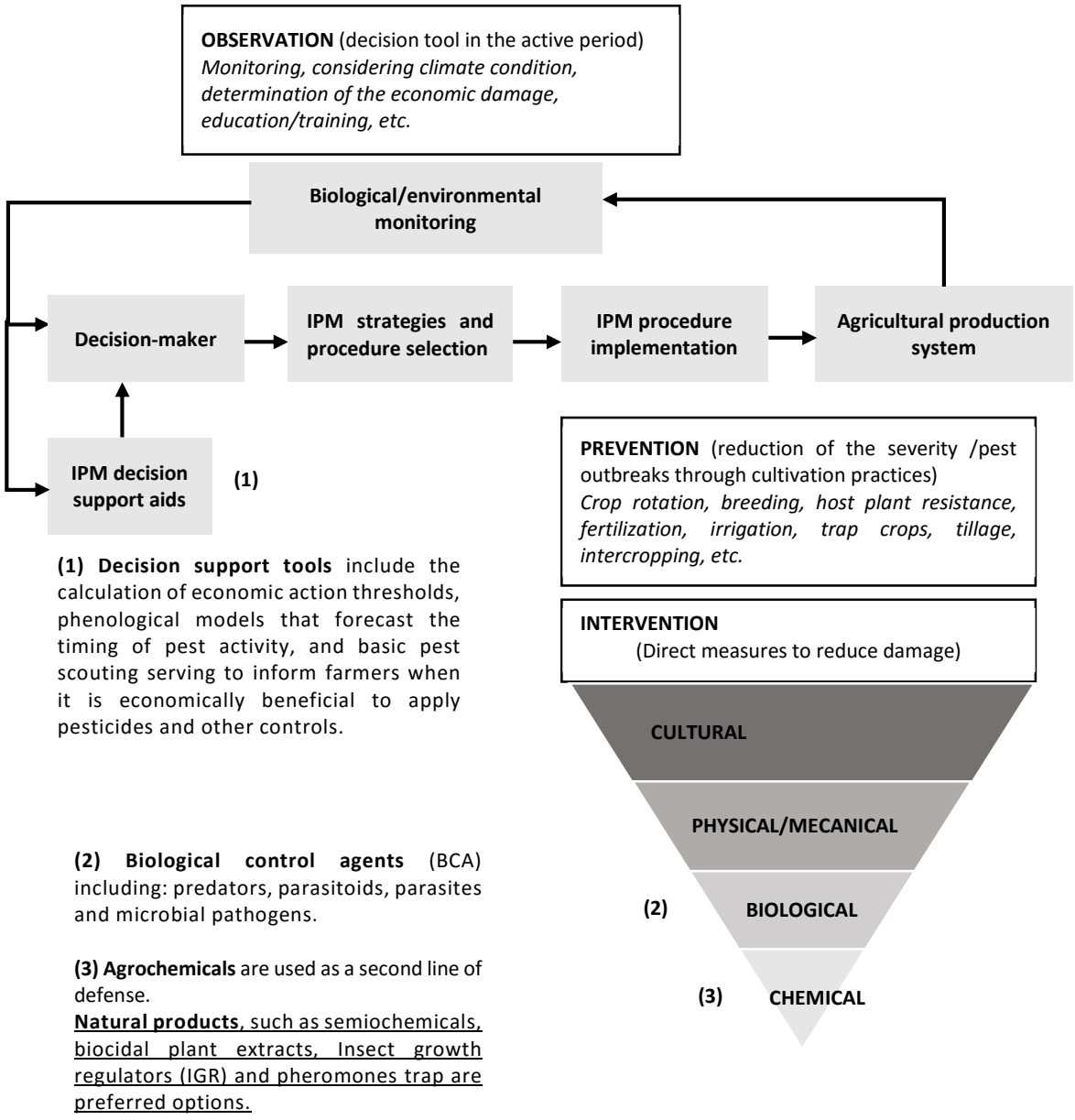


Figure I.2. Components and the operational framework defined for IPM. [Sources: Bird (1987); Chandler et al. (2011)]

The implementation of IPM is coupled to the concept of IPM/ecology integration level. By ensuring the higest level of IPM implementation, considering multiple tactical options, may be more successful if a total systematic is employed (Chandler et al., 2011). In this ideal case, the agro ecosystem's structure and dynamics (crop/pest interactions) are recognized and the farm environment is made resistant to the development of crop pests (Lewis et al., 1997; Kogan, 1998).

It is important to highlight that IPM is not limited to production systems (agriculture, horticulture and forestry). In a broadest sense, it may be implemented in several contexts including urban landscapes (gardens, squares, *etc*.) and buildings (schools, childcare facilities, households) [5]. The development of IPM in agriculture lead to appearance of an equivalent concept applicable to health protection systems, the **Integrated Vector Management** (IVM). Similarily to IPM, IVM is a rational decision-making process to optimize the use of resources for vector control (WHO 2008, 2012). The major attributes and key elements constituting IVM schemes are depicted in figure I.3. In 2004, the WHO adopted IVM globally for the control of all vector-borne diseases. In this context, multi-sectorial approaches with specific operational and timeline frameworks, have been implemented to prevent the transmission of vector-borne diseases such as malaria, dengue, Japanese encephalitis, leishmaniasis, schistosomiasis and Chagas disease transmitted by vectors to human, livestock and/or pets (WHO, 2004). The IVM seeks to improve the efficacy, cost-effectiveness, ecological soundness and sustainability of disease-vector control (www.who.int/neglected_diseases/vector_ecology/ivm_concept/en/; WHO, 2012).

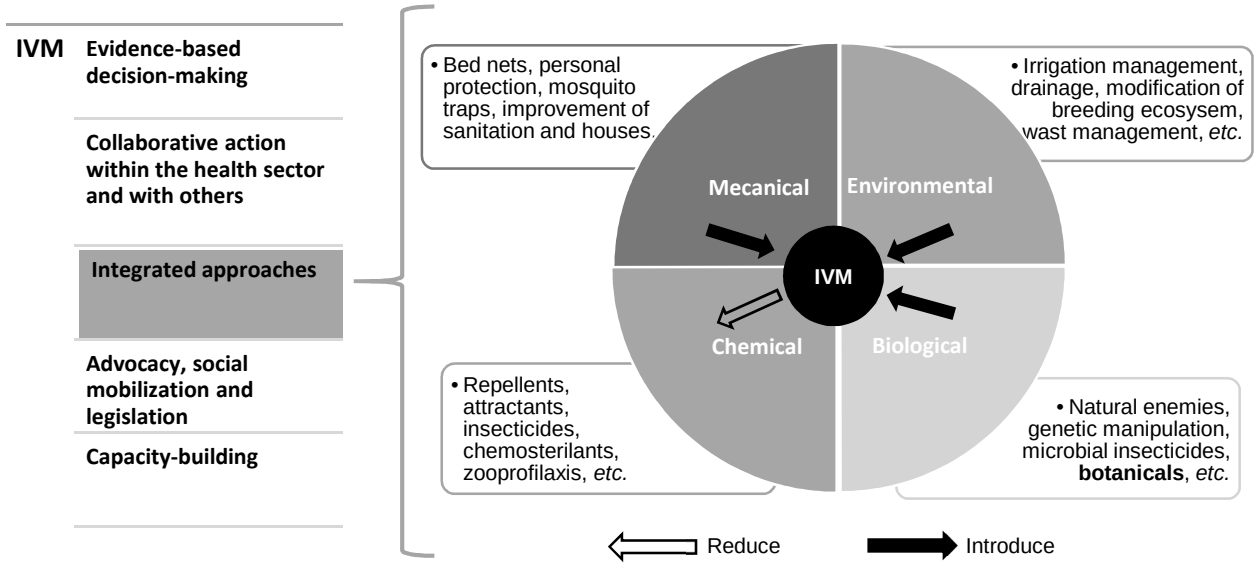


Figure I.3. Key elements of Integrated Vector Management frameworks and possible methods of intervention for vectors control. [Sources: Beier et al. (2008); WHO (2008); WHO (2012); Weber et al. (2013)]

The IVM encourages integrative approaches by engaging communities and promoting the systematic application of a range of selected interventions in synergy with each other (epidemological surveillance, advising, information sharing, collaboration with the heath sector, experts consulting, operational research; combination of environmental/physical, biological and chemical practices) (Beier et al., 2008; WHO, 2012).

[5] US National Pesticide Information Center, http://npic.orst.edu/pest/agipm.html

2.2. Regulatory issues regarding biopesticides

Given that environment safety is considered to be of paramount importance, current regulations are setting much lower Maximum Residue Limits (MRLs) for synthetic pesticides and explicitly encouraging the use of nonsynthetic pesticide-based control options or low-risk active substances (directive no. 2009/128/EC, *Framework Directive on the Sustainable Use of Pesticides*) (European Union, 2009a). The development of more and more stringent rules has been a progressive process that have accompanied the successive replacement of old pesticide by newly synthetized chemicals (Ehlers, 2009).

Although most jurisdictions recognize that biopesticides are fundamentally different from synthetic pesticides (Glare et al., 2012) they have a poor knowledge about biological control. Nevertheless, it is important to be aware that biopesticides are still pesticides (Villaverde et al., 2014) and many of these have biocidal properties. In effect, along the lines of the EU registration procedure, biopesticides are grouped together (Czaja et al., 2015) and regulated either as PPP or as biocides. Up to now, its placing on the market fall under the same regulations as their synthetic counterparts (Villaverde et al., 2014; Czaja et al., 2015). These regulations, aiming to protect human and environment safety, also have the purpose to ensure the reliability and quality of biopesticides (Chandler et al., 2011).

In the EU, any category of pesticide is currently being regulated by the:

EC regulation No. 1107/2009, **Commission Regulation (EU) No. 283/2013** and **284/2013**- concerning the placing of PPP on the market (European Union, 2009b, 2013a, 2013b);

EC regulation No. 528/2012 Biocidal Products Regulation (European Union, 2012)- concerning the placing on the market of biocidal products for protection of human, animals or goods against noxious species; aimed to harmonise the market at Union level and simplifie the approval of active substances and authorisation of biocidal products (The European Chemicals Agency website: http://echa.europa.eu/regulations/biocidal-products-regulation/understanding-bpr).

The directive no. 2009/128/EC (*Framework Directive on the Sustainable Use of Pesticides*) and the regulation (EC) no. 1107/2009 (European Union, 2009a and 2009b) set out goals for all EU countries concerning the implemention of IPM principles [6], which became mandatory after 2014. In line with the above-mentioned regulation, substances should only be included in PPP if it is established that they present a clear benefit for plant production and they are not expected to have any harmful effect on human or animal health [7] or any unacceptable effect on the environment.

[6] Supplementary information on IPM principles is delivered in Table A.1 of the appendix A.
[7] Impact on human health: the critical effect is judged of particular significance, such as developmental neurotoxic or immunotoxic effects.

Furthermore, beside the definition of a cut-off-criteria as regards low risk substances [8], this package of legislative measures also introduces an important aspect concerning the safeguard of honeybees (*Apis mellifera*) from pesticides exposure. The same regulation (EC, regulation no. 1107/2009) also asserts that Member States of the EU should regularly examine plant protection products containing active substances and give incentives for the placing on the market of low risk PPP (European Union, 2009b).

Despite the guidance notes provided by the Directorate-General for Health and Food Safety (DG SANTE) and by the Organization for Economic Co-operation and Development-Biopesticide Steering Group (OECD-BPSG) towards the harmonization of biopesticide, no specific regulatory system has been established yet. Like other PPP, biopesticides should only be authorized if they pose minimal or zero risk. That is applicable to semiochemicals and IGR, which are classed as low risk substances owing to their high level of selectivity and non-lethal effects on specific targets (OECD, 2003; Tripathi et al., 2009; Chandler et al., 2011). Regarding the regulation and authorization of plant-based products, steps are being taken to encourage their use (Tripathi et al., 2009). Several guidelines have been considered with the aim at facilitating the procedure of assessment of bioactive agentes and their registration. The Guidance document SANCO/11188/2013 lists the riteria that products have to meet to be classified as low risk substances. Products showing no toxicological concerns, or for which consumers exposure linked to PPP is negligible as compared to the daily exposure in the food chain and/or natural background, are suitable candidates to be included in a list of active substances with low toxicological risk (i.e. Annex IV of regulation no. 396/2005 of the European Parliament and the Council) (European Union, 2005). Specifically, it is the case for several flavoring and aromatic compounds (for instance, eugenol, L-carvone, D-limonene that are the main component of clove, mint and orange EO, respectively), for which consumer exposure through usual food consumption or personal care products is significant but customary (SANCO, 2013).

The decision whether or not to authorize a biopesticide product is made based on experts' judgement, from the analysis of the registration data portfolio (information about mode of action, toxicological and eco-toxicological evaluations, host range testing, *etc.*). Such process can be very expensive and time-comsuming (aprox. 4 years) and may prevent small and medium-sized enterprises from developing biological control products and take place into the biopesticide market (Ehlers, 2009). Only larger companies have the resources to support these costs and the means to conduct a compulsory research, produce a dossier and enter into the interative discussions that are part of the registration procedure (Bales et al., 2008). Due to these several obstacles inherent to the regulatory phases (fragmented or over-cautious registration and authorization process) and the reduced market size, the application of biological control in several European countries has been

[8] Appropriate risk assessment is made based on community or internationally agreed test guidelines. The regulation (EC) no. 1107/2009 defines low-risk substances as active substances that do not show any of the following properties: persistence in soil (half-life > 60 days), bioacumulation (factor >100), carcinogenic, mutagenic, toxic to reproduction, sensitising effect, very toxic or toxic, corrosive, endocrine disruptors, neurotoxic or immunotoxic.

less expedite and successful when compared with other parts of the world (Bale et al., 2008). For instance, the registration process in the USA can be completed in about 1/3 of the time required in EU even counting with some delays. This process is probably discouraging a number of non-EU leading biocontrol companies from putting efforts in the European market (New Ag International Magazine: Report on ABIM, 2013). Besides, in the particular case of the BCAs, the existence of conflicting interests of some stakeholders with industry have been identified as a decisive factor restraining their development in Europe (Ehlers, 2009). In order to address some of these issues, the EU project Registration of Biological Control Agents Action (REBECA) (terminated in January 2008) has brought forward recommendations for a Europe-wide regulatory framework (Ehlers, 2009).

As shown in the previous section, biologically active agents classified as biopesticides are extremely varied. This leads to serious complications in the approval and the registration processes. In many cases, data requirements for the registration should be less stringent. Hence, an application of common criteria to assess their safety to the consumer and environment is very difficult and, in well-described circumstances, they could be assessed on a case-by-case basis (SANCO, 2013; Czaja et al., 2015).

Presently, different countries have different rules and regulations. As consequence problems related to registration, use, import, and export do occur. A common policy regarding the use of biopesticides that could be accepted globally is now desirable (Mishra et al., 2015).

2.3. Conventional pesticides *versus* biopesticides, and their market

Synthetic pesticides and biopesticides exhibit many favorable and unfavorable aspects (Table I.1). Before taking a closer look, it should be noted that both types of pesticides are somewhat compatible and that the development of pest resistance, generally linked to the application of synthetic chemicals, might occur with products of natural origin when control is based on a single biomolecule.

The consequences of the heavy, over-reliant use of conventional synthetic chemicals, namely pest resistance, bioaccumulation through food chain, environmental pollution, loss of biodiversity and risks for human health, are well described (Bales, 2008). Currently, chemical pesticides still dominate the pest, weed and disease control scenery despite the increasing antipathy of the public and pressure of regulators (Nollet & Rathore, 2015). The global comsumption pattern of pesticides has been mostly allocated to insecticides (44%), herbicides (30%) and fungicides (21%) (Aktar et al., 2009). Over the last years, the discovering, developing and registering of new synthetic pesticides became less profitable, in part due to the high developing cost, which also include risk assessment, and the lower investment return (Bales et al., 2008).

Although very hazardous to manufacture, formulate and apply due to their acute toxicity and high persistence (Casida, 1980), chemical insecticides of the chlorinated hydrocarbons,

methylcarbamates and organophosphate classes[9] were relatively inexpensive and effective to control most insect pests of crops, livestock and man (Nollet & Rathore, 2015). Several chlorinated hydrocarbons have been restricted or banned because of unacceptable persistence, effects on wildlife, and evidence of possible carcinogenic activity (Casida, 1980). Even the neonicotinoids, the last generations of synthetic insecticides claimed to successfully address the toxicological and environmental shortcomings of these predecessors (Isman, 2008), have been largely questioned. A range of concerns have emerged as to their impacts on the ecosystems and wildlife, notably their side effects on honeybees and their link to the collony collapse disorder (Lu et al., 2014; van der Sluijs et al., 2015; van Lexmond et al., 2015). The principal classes of pesticides including those belonging to the S-triazines, a class of non-selective pre-emergent herbicides, have received most attention owing to their persistent effects (Singh et al., 2005).

Biopesticides offer effective management options and a range of solutions in several areas (Nollet & Rathore, 2015), and their use in crop protection can lead to many positive outcomes (Table I.1). Thus, these are considered versatile alternatives and key components of IPM. In addition, these could reveal some benefits if integrated with traditional plant protection products to achieve a more sustainable agricultural production (Villaverde et al., 2014). The most noteworthy and appreciated advantages of their incorporation into crop production and pest management programs are related with their biodegradability (decreased pesticide residues in food and thereby reduced risk to the consumer), and their low toxicity to non-target organisms (namely mammals and beneficials organisms, including honeybees) (Marrone, 2014; Czaja et al. 2015). The majority of biopesticides may be counted among products of a minimum risk level, which is generally lower than that of most synthetic pesticides (Czaja et al. 2015). Even so, the several positive properties of biopesticides do not acquit any product from a thorough assessment of its toxicological risk before any approval for use in pest control, since not all naturally occurring substances are safe for humans and for the environment (Duke et al., 2010; Czaja et al. 2015).

Nevertheless, their use also carries some limitations. The biological control is more inconsistent under field conditions, and eventually less effective than the control normally achieved with synthetic chemicals. Biopesticides have to be competitive on price in addition to efficacy and consistency, making development and commercialization more difficult. Besides, many natural products have inconsistent quality and their supply in industrial quantities is quite difficult. Moreover, protection of intelectual property during the development of a biopesticide is often necessary to attract investors, but overemphasis can restrict knowledge sharing and result in high legal costs (Glare et al., 2012).

[9] Supplementary information on the principal classe of pesticides is provided in Box 1 of the appendix A.

Table I.1. Summary of the negative and positive aspects concerning the use of synthetic pesticides and biopesticides. [a]

	Shortcomings	**Advantages**
Synthetic pesticides	High level of residues in food; Accumulation and biomagnification; Development of heritable resistance; Secondary pest problems; Variable levels of toxicity to non-target organisms and mammals; Hazardous to users if inadequate handling and application; Caused serious health problems to workers; High developmental and registering costs.	Generally prompt action; Broad-spectrum activity; Effective as stand-alone treatments; Well-studied and identified mode of action; Familiarity of the market; Affordability.
Biopesticides	Might display slow acting effects; Less suitable for use as stand-alone treatments; Require growers and user acceptance and awareness; Rapid degradation, susceptibility to unfavorable environmental conditions; Specificity to one pest species; Niche market products with low profit potential; Products registration is a black box; Barrier to biopesticide commercialization; The market for biopesticides remains relatively small, while the cost of R&D is high; Sale price needed to recover costs may be prohibitive.	Biodegradable, low half-life/persistence in the environment; Low mammals' toxicity/minimal hazard; Not necessarily lethal to targets; High level of selectivity; Low risk of resistance; Short intervals for crops harvesting Crops quality with little or no residues; Low developmental costs; Higer benefit per unit of money invested; New european union legislation promoting/ regulating the use of biopesticide.

[a] Sources: Copping & Menn, 2000; Bale et al., 2008; Chandler et al., 2011; Glare et al., 2012; Glare, 2015; Nollet & Rathore, 2015.

Biopesticides are currently been developed by a range of organizations, from public institutions to small or medium-sized companies (Marrone, 2014). The four major players of the global biopesticide market are AgraQuest, Inc. (USA), Marrone Bio Innovations Inc. (USA), Certis USA LLC (USA) and Koppert Biological Systems (The Netherlands) (*Markets&Markets*, 2014). Partnerships and collaborations are the key strategies adopted by these market players to ensure their growth in the market (*Markets&Markets*, 2014). With the increasing demand for biopesticides, major agrochemical companies (e.g. BASF, Syngenta, FMC corp., Novo-Nordisc and Dupont) are readily including biopesticides in their crop protection offerings (GIA, 2011; *Markets&Markets*, 2015). Larger companies have also been involved in biopesticides production and distribution through acquisitions of small biopesticide companies or licensing agreements (Nollet & Rathore, 2015; Glare et al., 2012; Marrone, 2014; *Markets&Markets*, 2015). With some exceptions, the bulk of these major companies are acquiring products rather than investing in R&D projects (GIA, 2011). In any case,

this increasing interest by multinationals brings significant new marketing channels to biopesticides (Glare et al., 2012).

In the course of the last twenty years, this paradigm shift towards biopesticides has caused a significant increase of biopesticides' penetration on the global pesticide market (Table I.2) (Hall & Menn, 1999; Copping & Menn, 2000; Marrone, 2014). The market competitiveness of biopesticides has been increasing and studies indicate that the unprecedented growth witnessed since 2009 is likely to continue up to 2020 (Table I.2) (*CPL Business Consultants*, 2013a; 2013b). Thus, it is expected that by 2020 biopesticides will generate a worldwide value of $6.6 thousand millons (*Markets&Markets*, 2015).

North America has dominated the global biopesticide market (accounting for around 40% of the worldwide demand in 2011), being followed by Europe, Asia-Pacific and Latin America regions (*Research and Markets*, 2012). In Europe, biopesticides sales in 2014 represented 5.0% of the agrochemical market, which was estimated to worth approximately $890 million at end-user level (*CPL Business Consultants*, 2013b). Spain, Italy and France have been identified as the largest individual European biopesticide markets (*Research and Markets,* 2012).

Table I.2. Evolution of the global pesticides market size and forecasts.

Year	**Global market** ($ billions)		**CAGR of the biopesticide market** (%)	**References**
	Synthetic pesticides	**Biopesticides**		
1995	29.0	0.4	10-15	Hall & Menn, 1999
2005	26.0	0.7	9.9 (5-years growth)	Tanwar et al., 2012
2008	–	1.0	–	CPL, 2010
2009	37.9	1.6	15.6	Lehr, 2010; Nollet & Rathore, 2015
2010	–	1.1	9.9	GIA, 2008
2011	37.5	1.3	–	Lehr, 2012
2012	46.1	–	–	Lehr, 2012
2013	54.8	–	–	Lehr, 2014
2014	56.0	2-3	> 15	Marrone, 2014; Nollet & Rathore, 2015
2015	–	2.8	15.7	Nollet & Rathore, 2015
2017	65.3	–	15.8 (5-years growth)	Lehr, 2012; Glare, 2015
2019	–	4.37	16.0 (5-years growth)	Markets&Markets, 2014
2020	–	6.6	18.8 (5-years growth)	Markets&Markets, 2015

CAGR: Compound Annual Growth Rate

Trends analysis of each of the submarkets, in terms of active ingredients, has shown that microbial agent-based biopesticides dominated the global market (Koul, 2011; Glare et al., 2012), whereas in Europe, BCAs and botanicals have been the most popular market segments (35 and 18.5% share; 18.5 and 20% of compound annual growth rate from 2010 to 2011, respectively). Approximately half the European biopesticide market was attributed to invertebrate biocontrol agents and pollinators (*CPL Business Consultants*, 2013b).

As previously foreseen by Kogan (1998), natural enemies such as entomopathogenic nematodes are still underused. In 2011, this particular class of biopesticides represented only 1.5% of the market share in Europe (*CPL Business Consultants*, 2013b). Although biopesticides show a great promise, they have not come up to the desired level so as to displace the dominance of chemicals (hindering regulatory, economic and societal issues) (Mishra et al., 2015). Nevertheless, there has been some notable progress in the small niche market. Globally there is a growing acceptance of botanicals to be used for the control of harmful species. Even though, their commercialization has generally proceeded much more slowly in regions where the regulatory standards are more rigorous (Isman, 2014).

2.4. Prospects for biopesticides

At the beginning of the twenty-first century, the opportunities and need for effective biological control are greater than ever (Bales et al., 2008). Governments are likely to continue imposing strict safety criteria on conventional chemical pesticides, and this will result in fewer products on the market (Copping & Menn, 2000). However, pest resistance will continue to be a problem claiming for suitable solutions (Bales et al., 2008). Hence, the development of biopesticides follows a strong trend and forecasts indicate a continued growth of the global biopesticides market (Copping & Menn, 2000; Glare et al., 2012; Nollet & Rathore, 2015) (Table I.2). Although the future of biopesticides may continue to be limited mainly to niche markets, there is great potential for a long-term development (Copping & Menn, 2000; Marrone, 2014). It is projected that such evolution of the biopesticides market will outperform the growth of synthetic pesticides in the near future (Glare et al., 2012). Likewise, a significant growth is expected for the biopesticides market in the European region, as a result of the implementation of the EU's Sustainable Use Directive of 2009 (*CPL business consultants*, 2013b). Effectively, its growth until 2020 is projected to proceed at the highest rate *(Markets&Markets*, 2015).

Even though changes in political and social attitudes towards safer, more environmentally compatible pest control alternatives have increased opportunities for biopesticides, they have not reached their potential yet (Glare et al., 2012). Considerable efforts to enhance biopesticides shelf life, efficacy, biological spectrum, reliability, and cost have been undertaken (Copping & Menn, 2000), nevertheless, there are major challenges to overcome regarding to:

- **Continued investment in expertise for R&D and implementation of biopesticides –** Industry R&D is necessary to support the development and registration of more biopesticides (Bale et al., 2008; Glare et al., 2012).

- **Adding value and assuring the performance of biopesticides –** Concerted efforts to solve limitations (raw material availability, variations of the bioefficacy, *etc*), namely by standardization of extraction methods and improvement of shelf life (Mendki et al., 2001).

- **Registration and legislative changes –** Manufacturers are still reluctant to commit to such alternatives due to difficulties with approval and registration (Villaverde et al., 2014). Concerted efforts to modify regulations will be crucial to boost the development of new biopesticides.

- **Societal Changes –** The lack of awareness, knowledge, and confidence of farmers, advisory personnel and other comsumers is one of the chief reasons for the lagging of these eco-friendly pest control alternatives (Mishra et al., 2015). Spending time creating societal awareness about the benefits of sustainable and environmentally friendly pest management is a fundamental step for a change of mindsets (Bale et al., 2008).

Currently, there is an established small market share for **pheromones** [10]. These will continue to be of significance as pest monitoring systems in special crops (Copping & Menn, 2000). By its turn, the use of BCAs, which was viewed as an environmentally friendly method of pest control, is being subjected to a more critical evaluation. Many countries have established harmonized regulation for the import and introduction of non-indigenous species (Bale et al., 2008). Moreover, alternative strategies for plant protection emerging for the 21st century also comprise the introduction of transgenic varieties of plants resistant to pesticides/pests (Mendki et al., 2001). However, in some areas of the world including Europe, there is some uncertainty over the public acceptance to genetically modified pest-resistant crops (Bale et al., 2008), probably attributable to the fact that their effects on health is not yet well clarified. Likewise, the use of microbes and their gene products introduces additional implication and toxicological issues. Infectious and immunological effects, concomitant to human exposure to these types of biopesticides in food or the environment, are plausible and require further evaluation (Sudakin, 2012).

Presently, botanical pesticides play a modest role in crop protection and several factors appear to limit their success (Ntalli & Menkissoglu-Spiroudi, 2011). Their relatively slow action, variable efficacy, lack of persistence and inconsistent availability have been claimed as main reasons for their limited market penetration (Isman, 2008). Besides, marked regulatory barriers and the challenge of protecting intellectual property based on natural products have also hindered the commercial development of botanicals (Isman, 2008). Nevertheless, there is a considerable market

[10] Pheromones are biochemicals used in IPM for the detection and monitoring of insects' populations (by attracting insects in mass trapping devices) or as a component of management strategies (by disrupting the mating behavior).

opportunity for organic food production, urban pest control and in public health (Isman, 2014). Moreover, botanicals and semi-refined plant preparations are likely to be of greater value in developing countries where useful plant species are often locally abundant, accessible and inexpensive (Isman, 2014). Owing to their affordability and the familarization of communities to plant extracts, the WHO (2012) also considers the use of botanicals as one of the applicable strategies for vectors control. There is no doubt that botanical pesticides will play a significant role in the future for pest control in both industrialized and developing countries (Isman, 2008; Dubey et al., 2011; Pavela, 2011; Isman, 2014).

2.5. Plant-based products as eco-friendly pesticides – Cases of success

Plants produce a large diversity of secondary metabolites of great importance for their adaptation to the environment. Generally, these metabolites occur as a response to chemical, biotic or physical stress (namely, infection, wounding or herbivory) (Bennett & Wallsgrove, 1994). Such diversity has been attributed to biochemical coevolutionary pressures between plants and their hosts (Speed et al., 2015). These complex mixtures of semiochemicals play not only a major role in plants' defense, but they also serve as chemical signals in beneficial biotic interactions (namely, reproductive advantage as attractants of pollinators and seed dispersers) (Croteau et al., 2000), in competition strategies, or even in carnivory (Rischer et al., 2002).

Plants semiochemicals (pheromones and **allelochemicals**[11]) have a significant influence over insects' behavior by acting as pollinator attractants or as repellent of phytophagous and parasites. Moreover, many plant metabolites also interfere with insects' reproduction, either by stimulating or inhibiting the oviposition (Regnault-Roger, 1997). In general, most plant defensive chemicals act by discouraging insect herbivory (feeding deterrent activity/ antifeedant activity)[12], or by impairing larval growth, rather than by killing the insects (Bennett & Wallsgrove, 1994; Regnault-Roger, 1997; Isman, 2002). Nonetheless, plant secondary metabolites may not exclusively work for the plant's profit, and insect-plant chemical interactions in nature are usually very subtle. Interestingly, many specialist herbivores (in this regard the Lepidoptera have been particularly studied) and pathogens have managed to circumvent the deterrent or toxic effects of secondary metabolites by adapting them to their own defense purposes and/or making use of these phytochemicals as nutrients (Rimpler, 1991; Bennett & Wallsgrove, 1994; Croteau et al., 2000).

Since ancient times, natural products from plants have been used as medicines and pesticides in both the Western and the Eastern worlds (Dubey et al., 2010). Still nowadays, the rural

[11] The term allelochemical is etymologically derived from Greek, *allelon*: 'on another'. The International Allelopathy Society have defined allelopathy as: any process involving secondary metabolites (alleochemicals) produced by plants, microorganisms, viruses and fungi that influence the growth and development of agricultural and biological systems.

[12] Antifeedant and feeding deterrent are used synonymously. According to Isman (2002), antifeedants are behavior-modifying substances that deters feeding through a direct action on peripheral sensilla in insects.

people and tribes living in some remote areas primarily depend upon the folk medicine and their ethnobotanical knowledge (Singh et al., 2005). All over the world, the recognition of the biological properties of a myriad of natural products has motivated the search for new drugs, antibiotics, insecticides, and herbicides (Croteau et al., 2000).

The scientific literature documenting the bioactivity of plant derivatives to pests is vast and reflects the renewed interest in the field of plant-based pesticides. These studies have uncovered an array of bioactive phytochemicals in several families such as Agavaceae, Asteracae, Lamiaceae, Meliaceae, Piperaceae, Rutaceae, *etc*. (Shaalan et al., 2005; Boulogne et al., 2012). Such bioactive compounds can display a wide spectrum of pesticidal activity, varying in type and degree (molluscicidal, nematicidal, piscicidal, insecticidal, larvicidal, growth inhibition, repellency, feeding-deterrence, chemosterilant, *etc.*) (Singh et al., 2005). According to an exhaustive literature survey of 1965 references (from 1923 to 2010), about 656 plant species worldwide (distributed into 110 families) were identified as having a significant insecticidal activity (Boulogne et al., 2012). Specifically, in the field of botanical insecticides the growth in research has been explosive (from 61 papers in 1980 to 1207 in 2012). Much of this progress is attributed to publications dedicated to neem (*Azadirachta indica*) activities, followed by those publications regarding EOs (Isman & Grieneisen, 2013). Researches concerning botanical pesticides have been thoroughly reviewed (Duke, 1990; Duke et al., 2000; Akhtar & Isman, 2004; Dayan et al., 2009; Koul, 2008; Srivastava et al., 2009; Dubey et al., 2010; Ntalli & Menkissoglu-Spiroudi, 2011; Boulogne et al., 2012; Gosh et al., 2012; Isman, 2014). Among the several books that contributed to the state of the art, the following ones are particularly recommendable: Koul & Dhaliwal (2000), Rai & Carpinella (2006), Dubey (2011), Gross et al. (2014a) and Singh (2014).

In the category of plant-derived biopesticides, many botanicals[13] (either as crude extracts and/or naturally occurring chemicals) show a long history of uses around the world (Ntalli & Menkissoglu-Spiroudi, 2011). Diverse bioactive allelochemicals belonging to different classes of compounds (viz. phenolics, flavonoids, quinones, tannins, terpenoids, alkaloids, saponins, sterols, *etc*.), have shown effects towards several type of noxious species (Table I.3). According to Boulogne et al. (2012), terpenoids, alkaloids, and phenolic compounds are the most regularly cited classes of compounds displaying insecticidal activity (37%, 30%, and 20% of the phytochemicals described in 1965 references, respectively). Surprisingly, only a few plant species have led to major commercial pesticidal products akin to the synthetic pesticides (Sola et al., 2014). The most well-known vegetal sources of bioactive products and their properties are summarized in table I.3 and the molecular structure of some of these plant metabolites are depicted in figure I.4.

Formulations based on pyrethrum (from *Chrysanthemum* spp., *Pyrethrum* spp., and *Tanacetum* spp.), neem, rotenone (from *Derris* spp. and *Lonchocarpus* spp.), sabadilla (*Schoenocaulon officinale*) and nicotine (from *Nicotiana* spp.) are examples of botanical insecticides

[13] The specification for plant-based products is more complex than for conventional chemicals. The term 'botanicals' is a broad concept used to covers a wide variety of different types of plant substances, while 'plant extracts' are defined as complex mixtures presenting a wide range of chemical compounds and biological activities (OECD, 2012).

that have a long history of uses and are being traded globally (Mendki et al., 2001; Sola et al., 2014). Botanical pesticides such as the powder extract of *Ryania speciosa* (containing the botanical insecticide ryanodine) were widely used until the 1940s, but were partially substituted by their synthetic analogues or by more profitable synthetic pesticides when these entered into the market (Dubey et al., 2011). In fact, many important synthetic PPP found their origin in natural products but were chemically modified to provide synthetic derivatives (e.g. the insecticides pyrethroids, the juvenile hormone mimics, the herbicide cinmethylin, *etc.*) (Duke, 1990).

Currently, the neem tree, also know as the 'wonder tree', is regard as the most important source of biopesticides (Dubey et al., 2011). The others aforementioned plant-products (excluding nicotine) are still authorized biochemicals, along with EOs from several aromatic plants, and their use in pest management is approved for organic farming, together with micro, macro-organisms (parasites and predators) and their subtances (European Union, 2008) [14]. This type of biopesticides may play a much greater role in future in pre- and postharvesting protection, especially in developing countries (Isman, 2006).

Presently, different plant products have been formulated for large-scale application and several dozens commercially produced botanical insecticides are being used as alternatives to synthetic pesticides in eco-friendly pest management (Dubey et al., 2010). Botanical insecticides and antifeedant are among the most significant reduced-risk botanical pesticides. These have been incorporated into several PPP and household products for the protection of men and domestic animals (Isman, 2000a), been a major case of success comparatively to other plant-based pesticides. Despite this fact, the current production of botanical insecticides is often insufficient to cover the rising demand for plant protection products that are safe for both the environment and health (Pavela et al., 2013). The same can be established in what concerns insect antifeedants. As a matter of fact, among more than 800 compounds reported for their feeding deterrent action, only those extracted from neem have shown consistent commercial success occupying a large share of the market (Isman, 2000b). Nowadays, about 30 neem-based formulations are being manufactured (especially in China and India) and traded internationally as pesticidal products, effective against a number of insect species as well as mites, nematodes and pathogenes (Shaalan et al., 2005; Sola et al., 2014).

Alternatively, suitable commercial options regarding herbicides, nematicides, and molluscicides of botanical origin are still very limited. Botanical pesticides can be also successfully applied in vectors management programs (Shaalan et al., 2005). For instance, saponins and saponin-containing plants such as endod are of great value as molluscicides in the control of the schistosomiasis-transmitting snail *Biomphalaria glabrata* (Marston et al., 1993). The so-called endod or African soapberry *(Phytolacca dodecandra*) have been traditionally used and cultivated by African populations as a potent botanical molluscicide, which can be extracted from the berries and incorporated into soaps.

[14] Plant protection products authorized for use in in organic farming are shown in table A.2 of the appendix A.

In several contexts, standardized plant extracts find a greater relevance (Singh & Singh, 2001; Dubey et al., 2011). The formulation of technical mixtures of active phytochemicals (blending) is a suitable alternative that could be advantageous from the pest resistence point of view. Their application should reduce the rate of evolution of conventional resistance compared with the selection pressure exerted by single pure bioactive molecules (Arnason et al., 1993) and they could be essential to prevent desensitization too (Isman, 2000b). In fact, it has been shown that following a prolonged exposure, caterpillars can show distinct feeding responses towards the plant extracts and pure allelochemicals (Akhtar & Isman, 2004), or even become cross-habituated (Isman, 2002). The use of chemically complex mixtures, rather than individual compounds, seems to be an effective approach of combating these problems (Akhtar & Isman, 2012). In this perspective, there are several studies concerning the enhanced effect of mixtures and possible interactions between constituents.

At this stage, it does seem that the commercial development of new botanical products is limited (Ntally & Menkissoglu-Spiroudi, 2011), probably because there are no specific data requirements for botanicals (OECD, 2012) and those applied for chemicals are not always appropriate. Nevertheless, the establishment of specific sets of data requirements may be challenging owing to the wide variety of plant-based products (Fontier, 2012). Moreover, there are further barriers to the exploitation of plant-based pesticides (Isman, 2000a, 2000b; OECD, 2012), namely:

(1) limited natural ressources;
(2) insufficient quality assurance, in consequence of ecotypical differences and phytochemical variation;
(3) co-occurrence of toxins in natural mixtures.

Consequently, the development, formulation and standardisation of the known pesticidal plant products is an underpinning factor for their commercialization (Sola et al., 2014). A biomarker approach in which the key compounds of the bioactive plant extract are determined might be helpful to assure the uniformity of crude extract and enhance their efficacy (OECD, 2012). Moreover, an optimized production could be reached through the selection of species/varieties showing the most interesting chemical profiles (classical method), or through a biotechnological approach in conditions allowing to optimize the capacity of plant tissues/ cells to produce specific metabolites (Duke, 1990).

Besides all these valid concerns to meet quality and safety requirements for users, consumers, and the environment, there are others considerations regarding economical and ethical issues. Any collection of wild plants must be properly managed to preserve the availability and biodiversity of the natural source. Evidently, it would be preferable to select plant species that can be cultivated or, ultimately, wild species with a rapid turnover (Regnault-Roger et al., 2012). Moreover, the production costs, prices at the end-user level and the required application rates should be considered (Regnault-Roger et al., 2012).

Table I.3. Plant sources and chemical classes of some of the most relevant phytochemicals showing activity against noxious species.

Plant source (Family and genus/species)	**Chemical Class**	**Bioactive compound(s)**	**Activity**	**References**
Acoraceae				
Acorus calamus, A. gramineus	Phenylpropanoid	*α*- and *β*-Asarone	Chemosterilant, antifeedant, nematicidal, antifungal	Saxena et al., 1977; Koul et al., 1990; Park et al., 2003; Sugimoto et al., 1995; Lee et al., 2004
Annonaceae				
Annona spp., *Asimina triloba*	Acetogenins (annonins and annonacins)	Asimicin Squamocin, cohibinsin	Insecticidal, miticidal, nematicidal Larvicidal	Mikolajczak et al., 1989 Omena et al., 2007
Annona squamosa	Flavonoids	Flavonol glycosides including myricetin-3-*O*-galactoside	Insecticidal, antimicrobial	Kotkar et al., 2001
	Acetogenins	-	Molluscicidal	Santos & Sant´Ana, 2001; Singh & Singh, 2001
Apiaceae				
Daucus carota	Phenylpropanoid	*α*-Asarone	Insect chemosterilant, nematicidal, antimicrobial	Momin & Nair, 2002a
Ligusticum hultenii, Lomatium californicum	Phenylpropanoid	Apiole *Z*-ligustilide	Antifungal, phytotoxic, termiticidal Antifungal, phytotoxic	Meepagala et al., 2005; Meepagala et al., 2006 Meepagala et al., 2005
Pastinaca sativa	Furanocoumarin	Xanthotoxin	Antifeedant	Akhtar & Isman, 2004
Pimpinella anisum, Foeniculum vulgare	Phenylpropanoid	*trans*-Anethole	Antifungal, insecticidal, antifeedant	Shukla & Tripathi, 1987; Hummelbrunner & Isman, 2001
Asteraceae				
Artemisia spp.	Sesquiterpene lactone	Artemisinin	Anthelmintic (antischistosomal)	Xiao, 2005
Artemisia iwayomogi, A. douglasiana	Sesquiterpene ketone	Vulgarone B	Molluscicidal, antifungal, termiticidal	Meepagala et al., 2004; Meepagala et al., 2003; Meepagala et al., 2006
Chrysanthemum spp., *Pyrethrum* spp., *Tanacetum* spp.	Pyretrins (Terpenoids)	Pyrethrin I, pyrethrin II, Cinerin I, cinerin II	Insecticidal, molluscicidal	Casida, 1980; Kumar et al., 2005
Tagetes spp.	Thiophene Terpenoid	α-Terthienyl (E)-Ocimenone	Nematicidal, larvicidal	Vasudevan et al., 1997; Dharmagadda et al., 2005; Lim, 2013

Table I.3. (*Continued*)

Amaranthaceae				
Chenopodium ambrosioides	Monoterpenoid	Ascaridole	Insecticidal	Tapondjou et al., 2002; Chu et al., 2011
Euphorbiaceae				
Euphorbia spp.	Tetracyclic triterpenes	…	Larvicidal, molluscicidal	Rahuman et al., 2008; Singh et al., 2004
	Tigliane-type diterpenes	4-Desoxy-phorbol	Piscicidal	Tiwari & Singh, 2004
Jatropha curcas	Tigliane-type diterpenes	Phorbol esters	Molluscicidal, antimicrobial	Devappa et al., 2012
Lamiaceae				
Thymus spp., *Satureia* spp., *Origanum* spp, *Mentha* spp., *Rosmarinus officinalis*	Monoterpenoids	Thymol, carvacrol, pulegone, menthone α-Terpineol, 1,8-cineole	Insecticidal, antifeedant, antimicrobial, nematicidal, phytotoxic	Akhtar & Isman, 2004; Hummelbrunner & Isman, 2001; Isman & Machial, 2006; Isman et al., 2008
Ocimum spp.	Phenylpropanoids	Eugenol	Insecticidal, antifungal	Obeng-Ofori & Reichmuth, 1997; Faria et al., 2006
Nepeta cataria	Iridoids	*Z,E*- and *E,Z*-nepetalactone	Insect attractant and repellent	Schultz et al., 2004
Leguminosae				
Derris spp., *Lonchocarpus* spp., *Tephrosia* spp.	Flavonoid (Rotenoid type)	Rotenone, amorpholone	Insecticidal, piscicidal	Kole et al., 1992
	Pyrrolidine alkaloid	DMDP [a]	Nematicidal, antifeedant	Birch et al., 1993; Blaney et al., 2011
Derris spp., *Tephrosia* spp.	Furanoflavonol	Karanjin, deguelin	Acaricidal, insecticidal	
Melanthiaceae				
Schoenocaulon officinale, Veratrum sabadilla	Alkaloid	Cevadine, veratrin	Insecticidal	Soloway, 1976
Meliaceae				
Aglaia duperreana, A. odorata, A. spectabilis, A. elaeagnoidea	Rocaglamides derivatives (= flavaglines)	Rocaglamide, methylrocaglate, C-3' hydroxylmethylrocaglate	Insecticidal	Chaidir et al., 1999; Nugroho et al., 1999; Schneider et al., 2000; Greger et al., 2001

Table I.3. (*Continued*)

Azadirachta indica, Melia azedarach, M. volkensii	Tetranortriterpenoid (Limonoid type)	Azadirachtin, nimbin, salannin, toosendanin	Antifeedant, insecticidal, IGR, molluscicidal, nematicidal	Isman et al., 1990; Singh et al., 1996; Akhtar & Isman, 2004; Javed et al., 2008; Senthil Nathan et al., 2005
Toona ciliata	Tetranortriterpenoid	Cedrelone	Antifungal, antifeedant	Gopalakrishnan et al., 2000; Koul & Isman, 1992
	Coumarin	Siderin	Antimicrobial, phototoxic	Chowdhury et al., 2003; Veiga et al., 2007
Myrtaceae				
Eucalyptus spp.	Monoterpene	1,8-cineole	Larvicidal, herbicidal	Sheeren, 2006; Singh et al., 2007
Leptospermum scoparium	Triketone	Leptospermone	Phytotoxic	Dayan et al., 2007
Phytolaccaceae				
Phytolacca dodecandra	Triterpenoid saponins	Oleanoglycotoxin-A	Molluscicidal	Parkhurst et al., 1973
		Lemnatoxin		Lemma, 1972; Parkhurst et al., 1974
Poaceae				
Sorghum spp.	Lipid benzoquinone(Quinoid)	Sorgoleone	Phytotoxic	González et al., 1997; Uddin et al., 2014
	Cyanogenic glucoside	Dhurrin	Phytotoxic	Nicollier et al., 1983; Forney & Foi, 1985
Polygonaceae				
Polygonum hydropiper, Drymis winteri	Sesquiterpenoid (Driman type)	Polygodial	Antifeedant	Asakawa et al., 1988; Zapata et al., 2009
Rutaceae				
Citrus spp.	Monoterpenoids	Limonene, citronellal	Insecticidal, antifungal	Singh et al., 2010
Sapindaceae				
Sapindus mukorossi	Triterpenoid saponin	*b*	Molluscicidal, larvicidal, Antifeedant, antifungal	Huang et al., 2003 Saha et al., 2009, Saha et al., 2010
Simaroubaceae				
Quassia amara, Picrasma exelsa	Tetranortriterpenoid (Quassinoid type)	Quassin	Antifeedant, nematicidal, herbicidal	Almeida et al., 2007
Salicaceae				
Ryania speciosa	Diterpenoid (Ryanoid type)	Ryanodine	Insecticidal	Rogers et al., 1948, Jefferies et al., 1992

[a] 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine

[b] 3-O-[O-acetyl-β-D-xylopyranosyl-β-D-arabinopyranosyl-β-D-rhamnopyranosyl] hederagenin-28-O[β-D-glucopyranosyl-β-D-glucopyranosyl-β-D-rhamnopyranosyl] ester

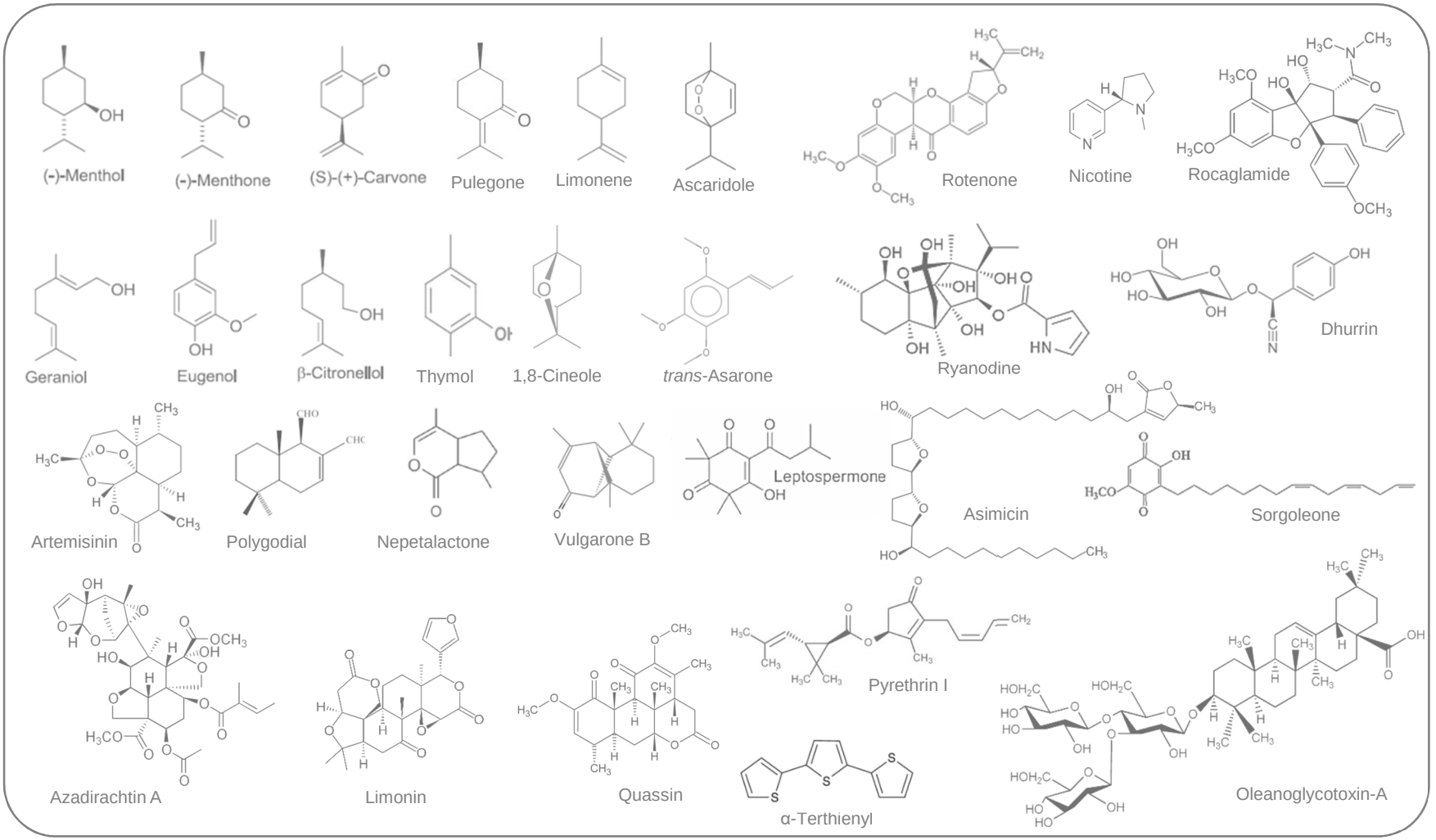


Figure I.4. Molecular structure of major phytochemicals showing activities against arthropods, molluscs, nematodes, microbes and/or weeds.

3. Plants' EOs – from chemical fundamentals and biological aspects to applications in pest management

3.1. EOs nature and occurrence

EOs are concentrated, hydrophobic liquids (at room temperature) containing volatile compounds produced by plants. These are liquid at room temperature and their constituents can easily change to a gaseous state when slightly heated without undergoing decomposition (Koul et al., 2008). As defined by the ISO technical committee, EO is the product obtained from a natural raw material of plant origin, by hydro-, steam distillation, mechanical processes as pressing (specifically for the epicarp of citrus fruits), or by dry distillation, after separation of the aqueous phase by physical processes (ISO 9235, 2013) [15]. Moreover, the distillation process permits to easily separated EOs from other products coexisting in plants storage organs, such as gums, waxes and resins (Baser and Demirci, 2007). EOs are highly soluble in organic solvents and poorly to slightly soluble in water depending on the physical-chemical properties of their constituents[16]. For an EO carries the distinctive scent, or essence, of a plant species/tissues (Olle & Bender, 2010), it is also known as the volatile or ethereal oil, or simply as the 'oil of' the plant from which it was extracted (Başer & Demirci, 2007) [17]. Nevertheless, EOs differ completely both in chemical and in physical properties from fatty oils, hence these should not be confused with fixed oils or fatty oils (Başer & Demirci, 2007).

EOs can be found in variable amounts in different plant organs (e.g., flowers, leaves, stems, fruits, seeds, bark roots and/or rhizomes) depending on the species (Tisserand & Young, 2013). There are approximately 3000 known EOs, out of which 300 are found commercially important for the industry (Bakkali et al., 2008). On account of their varied properties, EOs and plant volatiles constituents show a myriad of applications, particularly as pharmaceutics, agrochemicals, anti-microbial agents, flavoring agents, cosmetics and fragrances (Bakkali et al., 2008; Schwab et al., 2008; Regnault-Roger et al., 2012).

EO-producing plants are distinct in having specialized structures adapted to secreting and/or accumulating conspicuous quantities of volatile compounds (Loomis & Croteau, 1973). These secretory structures support the storage of metabolic products at a high concentration, thereby granting that these bioactive compounds might display their defensive and/or attractive role in specific organs under minimized risk of autotoxicity (Figueiredo et al., 2008). EO producing-plants

[15] The essential oil can undergo physical treatments (e.g. filtration, decantation, centrifugation) on the condition that these will not cause any significant change in its composition.

[16] While hydrocarbons have low solubility (< 35 ppm), oxygenated monoterpenes exhibited a solubility of one or two orders of magnitude higher. Alcohols are somewhat more soluble (183-1360 ppm) than comparable ketones in the monocyclic skeletons (between 155-6990 ppm) (Weidenhamer et al., 1993).

[17] According to the technical committee of essential oils, the terminology "essential oil of ..." should be adopt instead of "oil of …" (ISO/TC 54, 2010).

belong to many different botanical families, and specific types of secretory structures as well as their location have been associated to some plant families (Figueiredo et al., 2008).

In the Apiaceae family the accumulation of EOs is delimited to specialized structures located along plants vegetative and reproductive organs, known as secretory duct (or oil ducts) and vittae, respectively (Franz & Novak, 2009). These secretory structures form a highly interconnected network of tubular intercellular spaces extending throughout plants phloem (Senalik & Simon, 1986; Bosabalidis, 1996). Investigations on the localization of biologically active secondary products in Apiaceae have revealed that these inner secretory structures might be the place of synthesis and/or storage of different classes of compounds, including terpenoids (Senalik & Simon, 1986), lipids (Atia et al., 2009), phenolics, namely flavonoids (Reinold & Hahlbrock, 1997; Atia et al., 2009) furanocoumarins (Zobel & March, 1993; Reinold & Hahlbrock, 1997) and phenylpropene (Gersbach & Reddy, 2002; Gross et al., 2006), and in some rare cases alkaloids (Corsi & Biasci, 1998).

3.2. EOs constituents and their biosynthesis

From the chemical perspective, EOs are complex natural heterogeneous mixtures composed of various **terpenoids** of low molecular weight, as well as **non-terpenoid** volatile compounds (Başer & Demirci, 2007). Simultaneously, EOs are phytochemically diverse (i.e. containing many biosynthetically different compounds) and redundant (i.e. containing many analogs of one class) (Regnault-Roger et al., 2012). Though occasionally single constituents may predominate, EOs are generally constituted by a plethora of volatile constituents present in variable ratios, giving unique combinations of a characteristic odor and flavor. These odoriferous components exhibit heterogeneous backbones structures (straight or branched-chain, monocyclic, bicyclic or tricyclic, and aromatic or heteroaromatic) and a diversity of chemical functional groups (hydroxyl, carbonyl, carboxyl, ester, lactone, amine, epoxy, thiol) (Başer & Demirci, 2007; Schwab et al., 2008). Along with compounds belonging to the class of terpenoids, predominantly **monoterpenoids** and **sesquiterpenoids**, (rarely diterpenes), other volatile secondary metabolites of distinct biosynthetic origin may be detected in EOs mixtures, namely aromatic and aliphatic constituents (Weisshaar & Jenkins, 1998; Sangwan et al., 2001).

Terpenoids (also called isoprenoids) represent the largest class of natural chemicals. A wealth of terpenoids is produced by vascular plants, encompassing low molecular weight terpenoids and also more complex terpenoids, yet never found in EOs, such as sterols, carotenoids and ubiquinones. Despite their remarkable diversity, terpenoids are biogenetically derived of the same universal precursors, the isoprene unit isopentenyl diphosphate (**IDP**) and its isomer dimethylallyl diphosphate (**DMADP**), both constituted by a five-carbon skeleton (Croteau et al., 2000; Vickers et al., 2014) (Fig. I.5). Chain elongation consisting of repetitive additions of isoprene units, mostly through head-to-tail rearrangements, is carried out with the aid of prenyl transferases to originate different prenyl diphosphate homologs (Croteau et al., 2000). The conversion of the various prenyl diphosphates DMADP, GDP, FDP and GGDP to hemiterpenes (C5), monoterpenes (C10),

sesquiterpenes (C15) or diterpenes (C20), respectively, is catalyzed by enzymes belonging to the large family of the terpene synthases. Triterpenes (C30) and tetraterpenes (C40) are derived from the head-to-head condensation of two molecules of FDP or GGDP, respectively (Schwab et al., 2008).

In higher plants, both IDP and DMADP are synthesized via two separated pathways (Fig. I.5): the classic acetate/mevalonate pathway (**MVA**) operating in the cytosol, and a mevalonate independent route localized in the plastids: the 1-deoxy-*D*-xylulose/2-C-methyl-*D*-erythritol-4-phosphate pathway (**MEP**) [also known as non-mevalonate, DXP or Rohmer pathway] (Lichtenthaler, 1999; Rohmer, 2003; Eisenreich et al., 2004). The monoterpenes (C10), diterpenes (C20), tetraterpenes (C40), and certain prenylated quinones are produced largely, if not exclusively, in the plastids, whereas the sesquiterpenes (C15), triterpenes (C30), and polyterpenes are produced in the cytosol (Croteau et al., 2000). Although the synthesis of the terpenoids fundamental precursors (IDP/DMADP) is markedly different in both routes, there is no absolute compartmental separation of the two pathways (Hampel et al., 2005; Bartram et al., 2006). A metabolic crosstalk between the cytosolic MVA and the plastidial DXP/MEP pathways, with the probable unidirectional translocation of specific isoprenoid intermediates from the plastids to the cytoplasm, is currently accepted (Bick & lange, 2003). Therefore, the production of sesquiterpenes or sterols would not be entirely dependent on mevalonate as the exclusive pool of IDP. Nevertheless, the regulation of these dual pathways is difficult to assess (Croteau et al., 2000). Moreover, the allocation of IDP/DMADP as a resource for sesquiterpene or sterol biosynthesis (either MVA or plastidial-derived) appears to be quite variable in the plant kingdom (Hampel et al., 2005). This differential contribution of both pathways to the biosynthesis of the different groups of terpenoids is conceivably induced by stress factors (Bartram et al., 2006).

The presence of phenylpropanoids and other aromatic derivatives contribute to the aroma and scent of many plant species (Schwab et al., 2008), adding significant aromatic traits to many EOs (Sangwan et al., 2001). Volatile phenylpropenes are less frequent than the terpenes and their occurrence is restricted to some botanical families (Bakkali et al., 2008). In fact, some of these phenylpropanoids compounds, which encompass aldehydes (cinnamaldehyde), alcohols (cinnamic alcohol), phenols (chavicol, eugenol) and methylene dioxy compounds (anethole, elemicine, estragole, methyl eugenole) are typically found in EOs of Apiaceae, Lamiaceae, Myrtaceae and Rutaceae (Bakkali et al., 2008; Schwab et al., 2008). These volatile phenylpropenoids have a common biosynthetic origin as they are derived from the **shikimic acid pathway**, the general phenylpropanoid pathway through which lignin, flavonoids and other phenylalanine-derived compounds are produced (Croteau et al., 2000) (Fig. I.6). The biosynthesis of the several classes of phenylpropanoid is based on the few intermediates of the shikimate pathway as the core unit (Vogt, 2010).

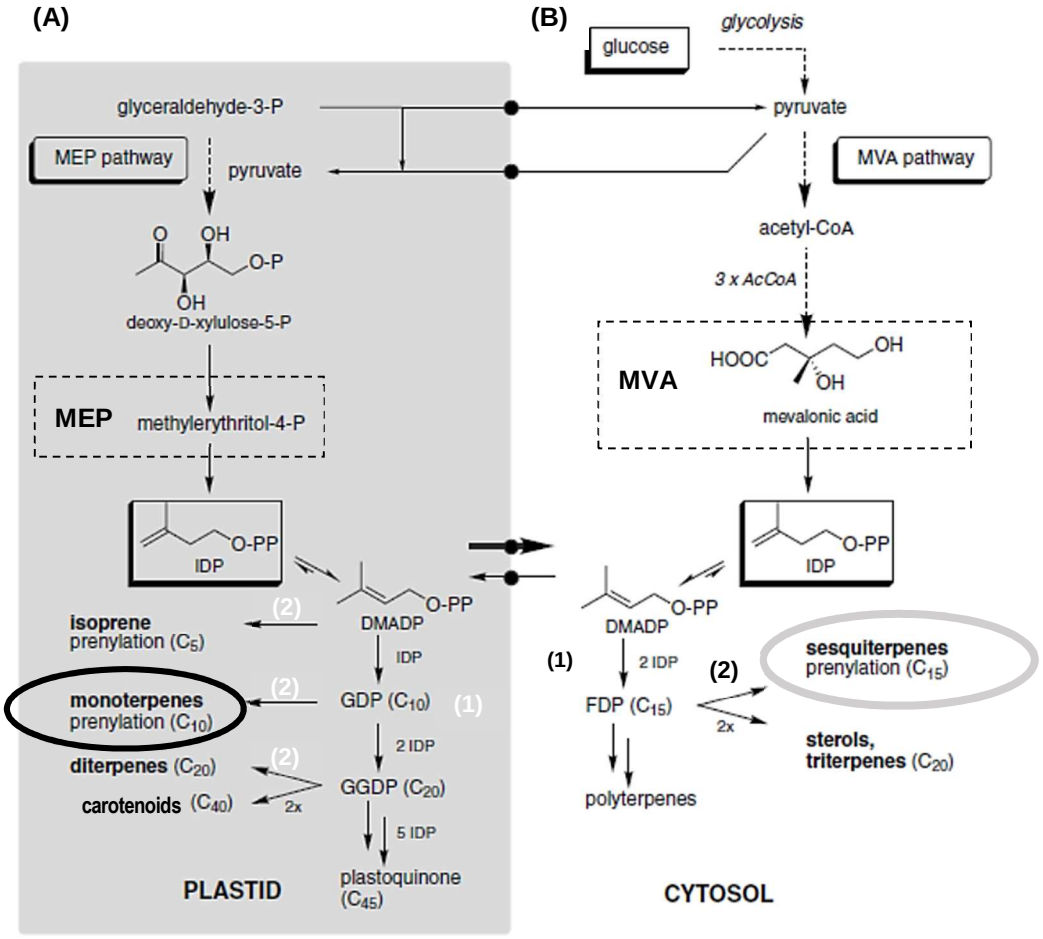


Figure I.5. Schematic map representing the two independent biosynthetic routes leading to the formation of terpenoids in higher plants, the biosynthesis of the different terpenoid classes is located in different subcellular compartments: **(A)** The 1-deoxy-*D*-xylulose/2-C-methyl-*D*-erythritol-4-phosphate route (DXP/MEV) operates in the plastids and **(B)** the acetate/mevalonate pathway (MVA) is active in the cytosol. [From Bartram et al. (2006) with some modifications]. The possible crosstalk between pathways is marked with arrows.

(1) IDP is utilized in a sequence of elongation reactions to produce a series of prenyl diphosphate homologs (GDP, FDP, GGDP), which serve as the immediate precursors of terpenoids. Linear geranyl diphosphate (GDP, C10), farnesyl diphosphate (FDP, C15) and geranylgeranyl diphosphate (GGDP, C20), are produced by the activities of three prenyl transferases. **(2)** Terpene synthases are the primary enzymes responsible for catalyzing the formation of hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15) or diterpenes (C20) from the acyclic substrates DMADP, GDP, FDP or GGDP, respectively.

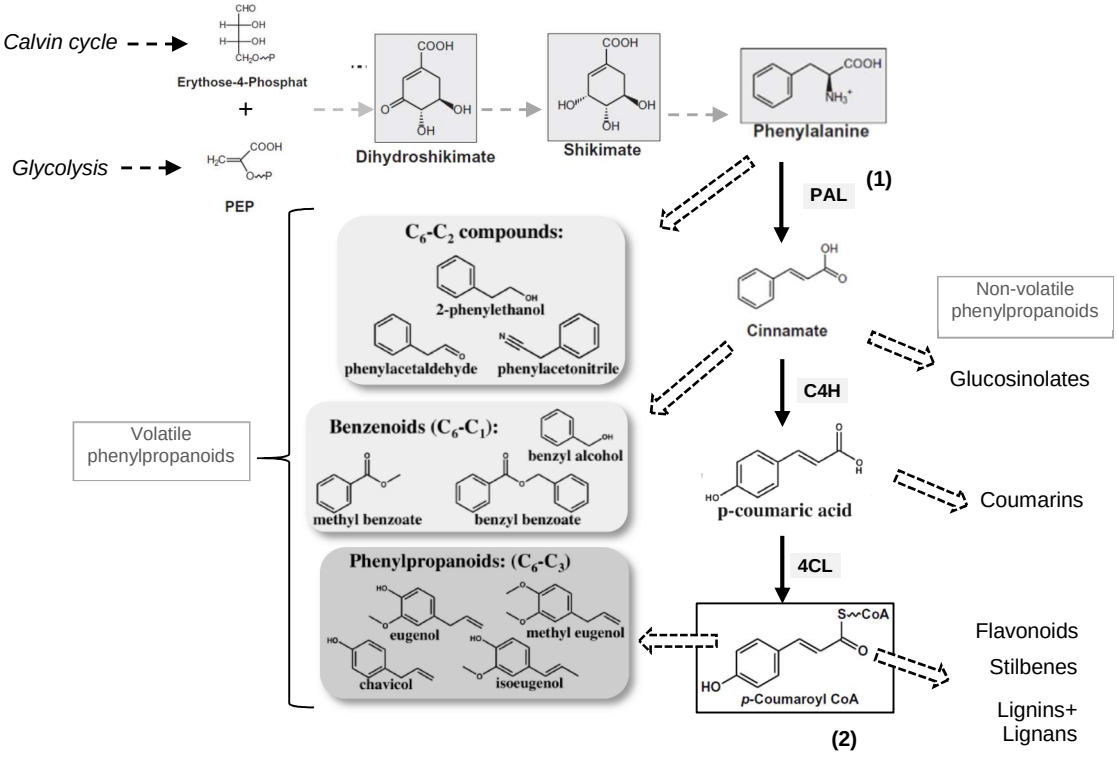


Figure I.6. Scheme depicting the general phenylpropanoid metabolism that generate a vast array of secondary metabolites, including volatile phenylpropenes of the benzenoid and phenylpropene classes found in plant essential oils [Modified from Vogt (2010) and Peled-Zehavi et al. (2015)].

(1) Phenylalanine, which is synthesized in the plastid through the shikimate and aromatic amino acids (AAA) biosynthetic pathways, is the main precursor for both volatile and non-volatile metabolites (Peled-Zehavi et al., 2015). The enzyme PAL directs carbon from aromatic amino acids to the synthesis of phenylpropanoid metabolites by converting phenylalanine to cinnamic acid. Tyrosine may also be used as an alternatively precursor being converted to *p*-coumaric acid by TAL (Croteau et al., 2000). **(2)** *p*-Coumaroyl CoA is an important branchpoint within the central phenylpropanoid biosynthesis in plants, leading to production of the monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols, and several (sub)classes of plant phenolics (Croteau et al., 2000; Vogt, 2010).

PEP, phosphoenolpyruvate; PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase, 4CL, 4-coumaroyl-CoA ligase. Omitted intermediary steps are represented by dashed arrows.

3.3. EO production and chemical variation

The production of secondary metabolites in plants, viz. the volatile components occurring in EOs, can be affected in a number of ways, from their formation to their final isolation (Figueiredo et al., 2008). Amongst many factor recognized to influence EO occurrence those related to plants inheritance (i.e. species, variety, chemotype, *etc*.) and physiology have the strongest impact on the yield and chemical profile. The occurrence of intraindividual variation of EOs between plant parts

and during ontogenesis of tissues is therefore well known (Franz & Novak, 2009). Moreover, biotic and abiotic factors have a strong modulating effects on plants response to environment. Some of the aspects influencing/stimulating the emission of plant volatile organic compounds (PVOC) are depicted in the figure I.7.

Furthermore, EO accumulation and quality can be optimized during the cultivation cycle of EO-producing plants throughout several approaches/ techniques. For instance, the mycorrhization of dill and fennel plants with *Glomus macrocarpum* and *G. fasciculatum* was found beneficial to plant growth, improving the yield and the quality of fruit EOs in both plant species (Kapoor et al., 2002; Kapoor et al., 2004, respectively). On the other hand, plant cultures may respond differently to seeding rate, row spacing, nitrogen and phosphate fertilization, with divergent effects on EOs yield and composition (Kapoor et al., 2002; Kapoor et al., 2004; Petropoulos et al., 2008).

The factors influencing content and composition of EOs extracted from umbelliferous crops were reviewed by Olle and Bender (2010). The authors discussed not only the determining factors associated to the plant species (part used, variety, landraces developmental phase), but also the impact of the cultivation conditions such as the latitude, climate, fertilization, salinity, irrigation, growth retardants, diurnal and annual rhythms, harvesting time and storage (Olle & Bender, 2010 and ref. therein). Varga and co-workers (2012) have concluded that seed morphology has a significant influence on the EO quality and that the composition changes during the ripening process in several Apiaceae species. The same authors also called the attention to the fact that EO accumulation and chemical variations are not stable or family-specific (Varga et al., 2012).

3.4. Biological/ecological functions of EOs and plants' volatiles

From an ecological perspective it is believed that PVOC can fulfill several functions acting as direct repellents or toxicants for herbivores and pathogens protecting valuable reproductive parts of plants (Schwab et al., 2008). Terpenoids dominate most plant volatile blends (Degenhardt, 2008) mediating communications between plants or their interactions with other organisms (parasites, herbivores, pollinators) (Berenbaum, 1990; Pichersky & Gershenzon, 2002; Dudareva et al., 2006; Degenhardt, 2008) (Fig. I.7). Volatile compounds, including those constituting EOs, seem to display and adaptive role by helping the plant to deal with strong environmental stress (Pichersky & Gershenzon, 2002; Pichersky et al., 2006). As a consequence, the overall production of volatiles and EOs generally increases when plants meets extreme conditions (Olle & Bender, 2010).

Moreover, a given compound may be implicated in general defense or stress responses in plants by displaying several roles. Added to a direct action against pest and pathogen, some of these substances have been demonstrated to serve as indirect plant defenses through multi-trophic interactions, namely by attracting predators and parasitoids that prey upon or parasitize the herbivores (Schwab et al., 2008). Since many monoterpenes are phytotoxic in concentrations under 100 ppm, unsaturated aqueous solutions of slightly soluble terpenoids (namely oxygenated

compounds) occurring naturally in plant tissues and soil solutions, may act as potent biological inhibitors (Weidenhamer et al., 1993).

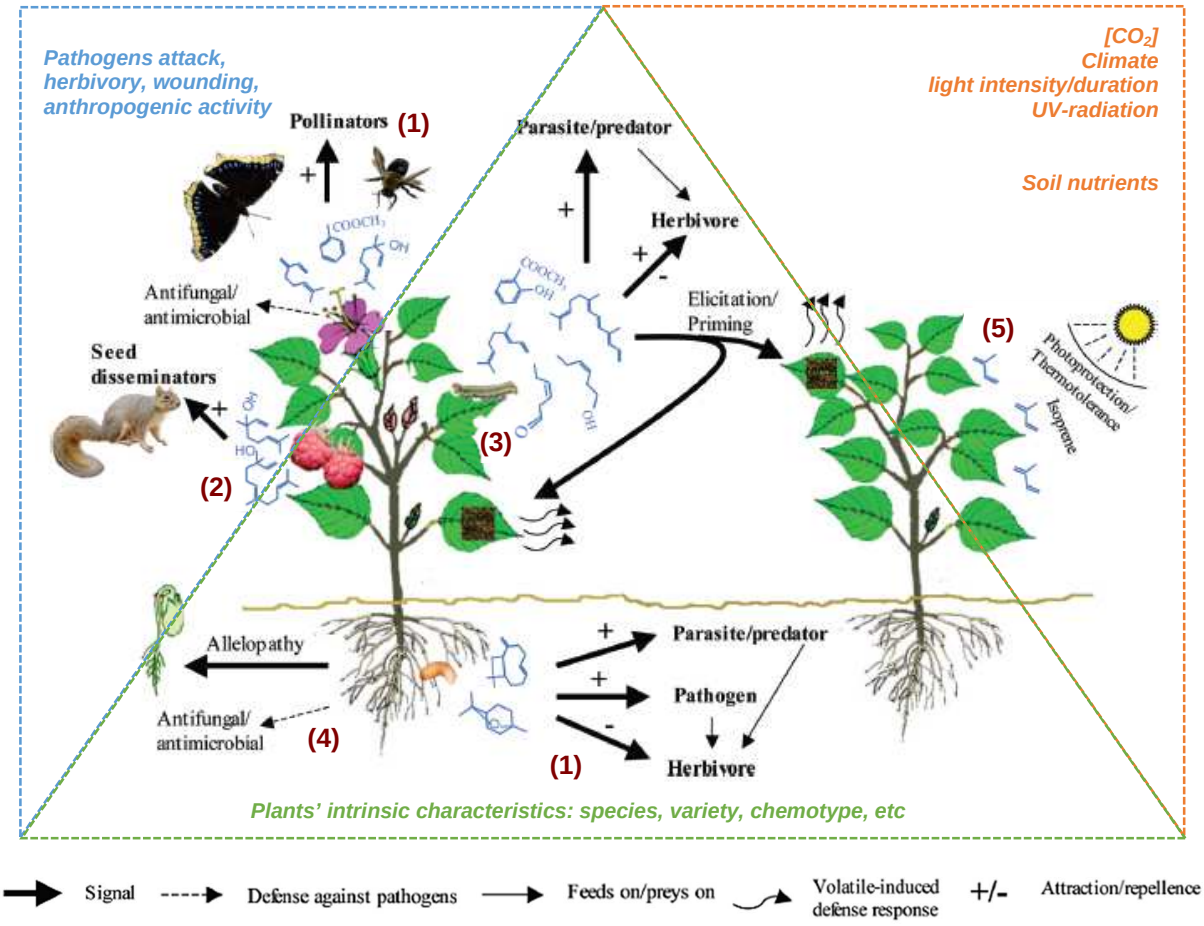


Figure I.7. Volatile-mediated interactions of plants with the surrounding environment [Modified from Dudareva et al. (2006)] and biotic abiotic factors influencing plants' metabolism and the chemical profiles of essential oils. [Sources: Sangwan et al. (2001); Dudareva et al. (2006); Degenhardt (2008); Franz & Novak (2009) and references therein]

(1) Plant-animal interactions include the attraction of pollinators and seed disseminators by floral and fruit volatiles, attraction/repellence of herbivores, and attraction of natural enemies of attacking herbivores both in atmosphere and rhizosphere (Dudareva et al., 2006). **(2)** The function of fruit volatiles as a signal of ripeness and as an attractant for seed-dispersing organisms is supported by the fact that some substances are specifically formed by ripe fruits but are absent in vegetative tissues and non-ripe fruit. **(3)** Unlike ripe fruits, flowers, and vegetative tissues often produce and release many of the volatiles only after their cells are disrupted (Schwab et al., 2008). **(4)** Similar to aboveground volatile compounds, roots emit an array of chemically and structurally diverse volatiles that can contribute to a belowground defense system and increase plants ecological competitiveness; by acting against patogens, herbivores or competiting plant species, or as a second line defense mechanism by attracting enemies of root-feeding herbivores (Dudareva et al., 2006). **(5)** In addition, leaf volatiles confer photoprotection and thermotolerance (Dudareva et al., 2006) by quenching reactive oxygen species generated from plant metabolism when these are exposed to high UV-radiation and temperature, respectively.

Phenylpropene-derivatives such as *trans*-anethole, eugenol and isoeugenol may display simultaneous advantages to plants, both as defense allelochemicals against animals and pathogens and as floral attractants of pollinators (Pichersky & Gershenzon, 2002).

The extraordinary variation/combination of emitted/accumulated plant-volatiles observed in many species has given evidences of their role in general defense mechanisms and stress responses (Pichersky & Gershenzon, 2002; Pichersky et al., 2006). Nevertheless, by providing such adaptive trait, the chemical variation also constitutes an additional challenge for producer, suppliers and regulation entities when regarding to the production of standardized products under natural conditions (Regnault-Roger, 2012).

3.5. Activity of EOs and their constituents

3.5.1. Modes of Action (MOA)

EOs exhibit a wide range of biological activities towards different types of organisms. These are also known to affect several targets at the same time, yielding a diversity of effects (Fig. I.8).

An important common characteristic of the EOs and their components is their hydrophobicity. The polarity of monoterpenes (expressed as the K_{ow}, octanol–water partition coefficient) plays an essential role in determining the speed by which compounds penetrate through the tegument. Therefore, the distribution and the ability of a xenobiotic to reaches the action site is strongly influenced by this physical parameter (Lucia et al., 2013). It is recognized that the lipophilic character of fragrance compounds and their affinity towards lipid-rich tissues, is mainly responsible for the broad spectrum of biological activities (Lahlou, 2004). In general, EOs and their components may trigger several events at the cellular level. Effects been described in the literature with reference to the general activity of EOs in cells generally comprise interaction with several cellular components, oxidative stress, membranes permeabilization and impairment of homeostasis, bioenergetic failure and cellular death (Table I.4).

Several experiments addressing the insecticidal activity of EOs, or their constituents, have suggested a neurotoxic action (Kostjukovsky et al., 2002; Shaaya & Rafaeli, 2007) given the symptoms frequently observed (Fig. I.8). The onset of toxic signs is usually rapid being similar to those produced by organophosphates and carbamates, both well-known classes of acetylcholinesterase (AChE) inhibitors (Isman, 1999; Shaaya & Rafaeli, 2007) [18]. Correspondingly,

[18] Supplementary information concerning pesticides MOA and their molecular target sites are provided in the Box 1 and figure A.2 of the appendix A.

many plant-products including EOs constituents show significant *in vitro* AChE [19] inhibitory activity in the nervous tissue of molluscs, insects and nematodes (Shukla et al., 2006; López & Pascual-Villalobos, 2010; Yeom et al., 2012; Kang et al., 2013). The inhibitory action of components identified in Apiaceae EOs on the AChE activity of *Sitophilus oryzae* and *Aedes albopictus* were recently tested (Kim et al., 2013; Seo et al., 2015). Both α- and β-pinene revealed high inhibition rates against the two insect species, followed by other compounds like limonene carvacrol or thymol. Nevertheless, not all compounds with strong toxicity consequentially show dose-related effects (Kostyukovsky et al., 2002) or a marked inhibition activity on AChE (Lucia et al., 2013). Sometimes the inhibitory effects reported are relatively weak and AChE inhibition is only obtained when EOs/compounds are administered at such higher level that could not substantiate the toxic effects observed *in vivo*. Such differences observed indicate that some chemicals should have others/different target sites (Shaaya & Rafaeli, 2007).

With respect to others EOs' MOA, the octopaminergic system primarily found in invertebrates, including insects (Gross et al., 2014b), has been avowed as a probable and more biorational target for arthropods control. Several reports indicate an interaction of monoterpene and EOs constituents with octopamine[20] and tyramine receptors (OctbR and TyrRI of the subclasses 2 and 3, respectively), both referred as G-protein coupled receptors (GPCRs) (Enan, 2005a; Verlinden et al., 2010; Gross et al., 2014b). For instance, eugenol mimicked octopamine in increasing intracellular calcium levels in cloned cells from the brain of *Periplaneta americana* and *Drosophila melanogaster* (Enan, 2005b). The lack of the octopamine class of receptors in vertebrates probably accounts for the biorational mode of action of EOs and their selectivity/ suitability as insecticides and acaricides (Price & Berry, 2006; Verlinden et al., 2010).

In spite of the evidence for a cholinergic and octopaminergic MOA for certain monoterpenoids and phenylpropene derivatives (Bischof & Enan 2004; Kostyukovsky et al., 2002), some effects on the hormone and pheromone system and cytochrome P450 monooxygenase of insects have also been described (García et al., 2005). Thus, the exact molecular target of EOs or their constituents have not been fully elucidated neither the mechanisms been clearly established. At this point, it is reasonable to conjecture that EOs and compounds activity would be occasioned by multiple mechanisms that affect multiple targets, on account of the chemical complexity, thereby disrupting more effectively cellular activity and biological processes (Regnault-Roger et al., 2012), rather than by a specific cellular target or a unique MOA.

[19] The acetylcholinesterase (AChE, EC3.1.1.7) hydrolyses acetylcholine (ACh) a neurotransmitter that when released from synaptic vesicles briefly depolarises the postsynaptic cell membrane and modulate the transmission of the nerve impulse (Siegfried & Scott, 1990). Therefore, compounds that inhibit AChE might significantly increase the levels of this neurotransmitter in the synaptic gap, resulting in the continuous stimulation of the cholinergic fibers at the central and pheripheral system and ultimately leading to paralysis and death (Shaaya & Rafaeli, 2007).

[20] Octopamine is present in the nervous system of invertebrates and display several physiological roles (as neurotransmitter, neurohormone, and neuromodulator) been essential for mediated-transynaptic signaling (Verlinden et al., 2010; Gross et al., 2014b). In arthropods, all energy-demanding activities (e.g. heartbeat, jumping, flying, ventilation, light emission, *etc*) are modulated by the dopaminergic system and depend on octopamine. As a consequence, the interruption of its function may result in a total breakdown of insects' nervous system (Dubey et al., 2011).

Likewise, as it is true for most insecticides, the MOA of a molluscicide should be a multi-component process affecting more than one system (Fig. I.8), culminating in the death of the organism (McCullough et al., 1980; WHO, 1983). Price and Berry (2008) have demonstrated that millimolar concentrations of EOs compounds have a blocking effect on action potentials of *Planorbis corneus* buccal ganglion neurons. Authors have attributed the reversible knockdown effects observed and the impaired response to some neurotransmitters (octopamine, dopamine and acetylcholine) to nonspecific mecanisms, other than a direct targeting of octopamine GPCRs (Price & Berry, 2008). Moreover, Janahmadi and collaborators (2008) examined the capacity of anise EO to induce neuronal hyperexcitability in the snail *Helix aspersa* suggesting an inhibitory action over voltage and/or Ca^{2+}dependent K^+ channels.

With reference to EOs activities against plants, a relatively large number of highly phytotoxic phytochemicals is derived from the terpenoid pathway. For instance, the monotepenes 1,8-cineole and its analogue (1,4-cineole) display growth inhibitory action, although apparently through different MOA, including the inhibition of all stages of mitosis and inhibition of mitochondrial respiration (Duke & Oliva, 2004). The sesquiterpene lactone artemisinin from *Artemisia* spp. EOs, which showed herbicidal potential has been considered as a structural lead for the development of natural product based herbicides. This compound behaves primarily as a strong energy transfer inhibitor of isolated thylakoid membranes (Bharati et al., 2012). In addition, it is known that phenolic compounds occurring in EOs exert their phytotoxicity on cell membranes, resulting in nonspecific permeability changes that alter ion fluxes and hydraulic conductivity of roots, followed by a cascade of physiological effects involving changes in ion balance, stomatal function, rates of photosynthesis and respiration and/or plants' water uptake (Einhellig, 2004). In addition, phenolic compounds might also interfere with phytohormones, enzymes and carbon flows, causing deviations in metabolites biosynthesis (Einhellig, 2004). Nevertheless, there is little correspondence with the known molecular target sites of commercial herbicides [20] and those of natural phytotoxins (Duke & Oliva, 2004).

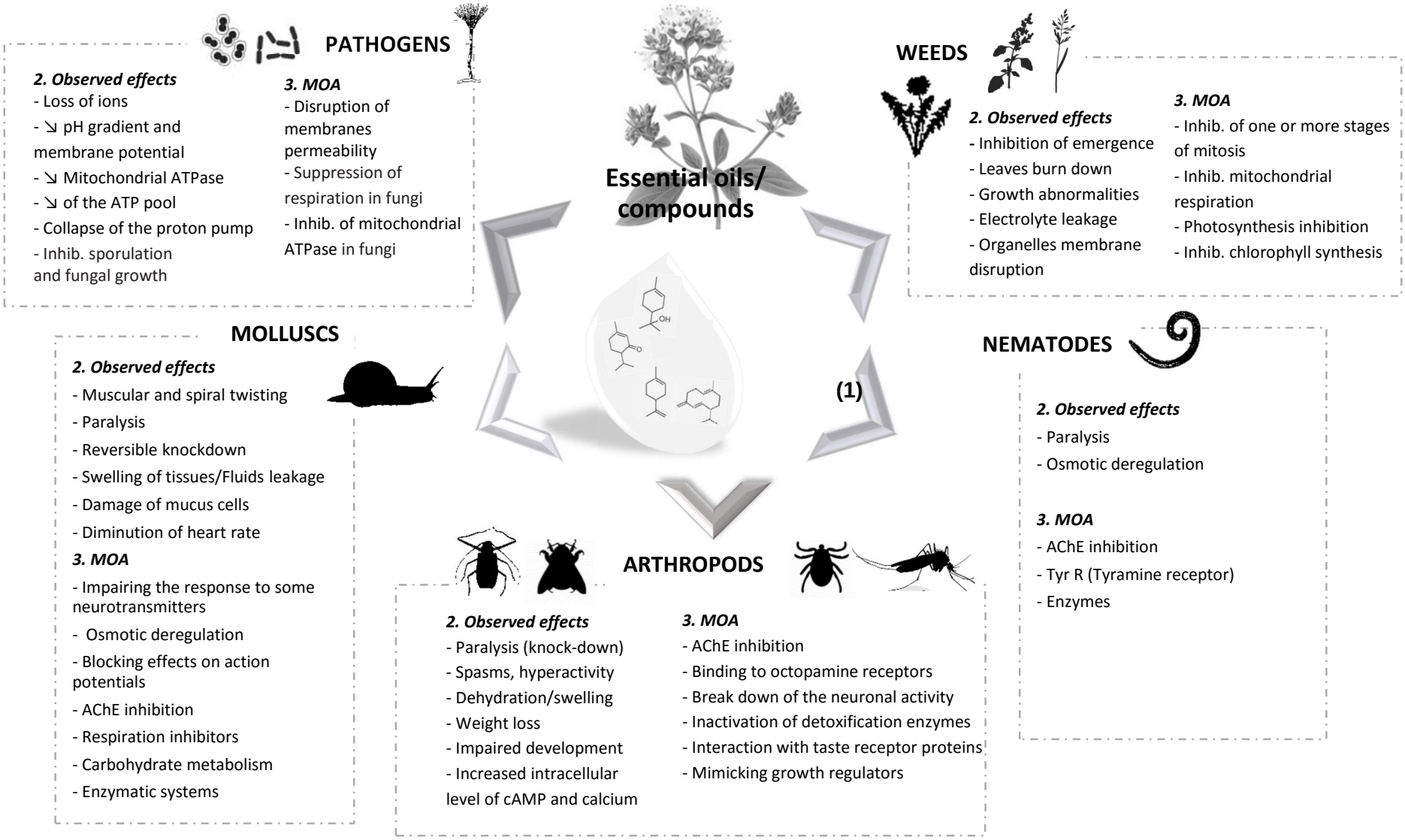

Essential oils/ compounds
(1)
PATHOGENS
2. Observed effects
- Loss of ions
- ↘ pH gradient and membrane potential
- ↘ Mitochondrial ATPase
- ↘ of the ATP pool
- Collapse of the proton pump
- Inhib. sporulation and fungal growth
3. MOA
- Disruption of membranes permeability
- Suppression of respiration in fungi
- Inhib. of mitochondrial ATPase in fungi
WEEDS
2. Observed effects
- Inhibition of emergence
- Leaves burn down
- Growth abnormalities
- Electrolyte leakage
- Organelles membrane disruption
3. MOA
- Inhib. of one or more stages of mitosis
- Inhib. mitochondrial respiration
- Photosynthesis inhibition
- Inhib. chlorophyll synthesis
MOLLUSCS
2. Observed effects
- Muscular and spiral twisting
- Paralysis
- Reversible knockdown
- Swelling of tissues/Fluids leakage
- Damage of mucus cells
- Diminution of heart rate
3. MOA
- Impairing the response to some neurotransmitters
- Osmotic deregulation
- Blocking effects on action potentials
- AChE inhibition
- Respiration inhibitors
- Carbohydrate metabolism
- Enzymatic systems
NEMATODES
2. Observed effects
- Paralysis
- Osmotic deregulation
3. MOA
- AChE inhibition
- Tyr R (Tyramine receptor)
- Enzymes
ARTHROPODS
2. Observed effects
- Paralysis (knock-down)
- Spasms, hyperactivity
- Dehydration/swelling
- Weight loss
- Impaired development
- Increased intracellular level of cAMP and calcium
3. MOA
- AChE inhibition
- Binding to octopamine receptors
- Break down of the neuronal activity
- Inactivation of detoxification enzymes
- Interaction with taste receptor proteins
- Mimicking growth regulators

Figure I.8 (of the previous page). Systematic overview of EOs constituents' activities on several type of organisms based on the literature.

(1) The ***routes of exposure*** is one important aspect of the biological activity. EOs entrance into organisms may happen after a direct contact with the body (absorption thru the outer layer tissues and/or digestive system), or, if applicable, via the respiratory tract through vapor phase (fumigation) before reaching tissues/cells and molecular targets (Regnault-Roger, 1997; Shaaya & Rafaeli, 2007). Different routes can give rise to variable symptoms and level of effectiveness. Even though, the exposure to EOs may occur simultaneously via two or more routes, affecting more than one system.

Table I.4. General cytotoxicity induced by essential oils or their constituents and reported specific events taking place at the cellular level. [a]

EOs	Damage the cellular membrane and organelles; Mitochondrial dysfunction-induced reactive oxygen species accumulation; Decreased ATPase and dehydrogenase activities; Causes nuclear and cytoplasmic mutagenicity.
Most monoterpenes	Cause reduction in the number of intact mitochondria and golgi bodies; Impair respiration and photosynthesis.
Hydrocarbons	Act as solvents of the cellular membrane compromising its integrity and increasing the movement of other molecules across the cell.
Phenolics	Produce very reactive phenoxyl radicals when oxidized by contact with reactive oxygen species.
Phenols, aldehydes and alcohols	Act as prooxidants on proteins and DNA.
Furocoumarins [b]	Act as photoactive molecules; Produce radical reactions and oxygen singlet; Crosslink both strands of DNA by binding to pyrimidine bases, thereby inhibiting DNA replication and transcription.

[a] [Sources: Lahlou, 2004; Bakkali et al., 2008; Tripathi et al., 2009]; [b] Some EOs are known to contain residual or low content of coumarins.

3.5.2. Structure-activity relationships

Several attemps to relate the activity of EOs to compounds chemical structure have been made. In some cases, an indication of an EO's toxicity can be obtained from the analysis of the chemical structure of its constituents. The basic carbon skeleton, the type of functional group, the degrees of saturation, volatility, and type of derivatization all influence insecticidal activity of the monoterpenoids (Rice & Coats, 1994). Moreover, the stereochemistry of isomeric forms seems to play an important modulating role in a compound's potency (Seo et al., 2009; Santos et al., 2011). Nevertheless, compounds holding the same moieties may act idiosyncratically or display quite different degrees of toxicity. Hence, broader assumptions based on chemical functional group should be made with precaution (Tisserand & Young, 2013).

Studies on insecticidal activities of phytochemical compounds have revealed that one important factor is related to the number of hydroxyl functions. Enan (2005a; 2005b) have identified some structural characterisitic that are required for EOs compounds such as eugenol to interact with octopaminergic receptors of the fruit fly. Both the presence and location of a hydroxyl group and a spacing group, e.g. a methoxy group, appears equally important features of phenylpropanoids' activities. However, according to Santos and collaborators (2011) the presence of hydroxyls in the cyclic structure of some monoterpenes resulted in a decrease of the larvicidal potency on *A. aegypti,* while lipophilic groups, conjugated and exo double bonds appear to increase the potency. Furthermore, Park and co-workers (2008) found that compounds with functional groups, namely phenol, alcohol, aldehyde, and ketone, were more active than hydrocarbons when evaluated by the fumigation assay. Other findings have highlighted the contribution of the allyl side chain existing in molecules such as dillapiole and eugenol, which, according to some authors, greatly contribute for the respective synergistic and larvicidal activities observed, when tested against different mosquito larvae (Belzile et al., 2000; Barbosa et al., 2012). Moreover, according to some authors, variations in the functionalities attached to either the aryl ring or the alkyl side chain of phenylpropane derivatives have significant influence on compounds' efficiency against the Japanese termite, *Reticulitermes speratus* (Seo et al., 2009).

In an attempt to relate the anti-nematode activity to the chemical functional group of several EOs compounds, Choi and co-workers (2007) showed that monoterpene hydrocarbons and ketones had weak or no activity, whereas phenols, aldehydes and primary alcohols were the most active followed by secondary and tertiary alcohols. Echeverrigaray and co-workers (2010) found that compounds with hydroxyl and carbonyl groups exhibited higher nematicidal activity than other terpenoids against *M. incognita* hatching.

On the other hand, when compared to oxygenated compounds, in particular phenylpropanoids and alcohols or phenolic terpenes, hydrocarbons showed a less significant antimicrobial activity (Bassolé & Juliani, 2012). The following hierarchy for antifungal activity, depending on the type of chemical functions, was suggested by some authors: phenols > cinnamic aldehydes alcohols > aldehydes ≥ ketones > ethers > hydrocarbons (Kalemba & Kunicka, 2003).

In what concerns the allelopathic activity, it has been found that among monoterpenoids with varied chemical groups ketones and alcohols displayed superior inhibitory effects over lettuce germination and seedlings growth, being followed by compounds with an aldehyde and phenol groups (Vokou et al., 2003).

3.5.3. Interactions of EOs compounds

Apart from the above highlighted aspects, it is known that the biological activity of a substance may be influenced by the presence of other molecules; hence, the toxicity being commonly the result of their interactions and a combined effect of both bioactive and non active compounds towards the biological system. Such interactions become tremendously difficult to predict for complex mixtures

with dozens of components (Tisserand & Young, 2013). In this context, the study of artificial blends has been, to a certain extent, a helpful approach for the understanding of compounds interactions. When two or more substances are employed simultaneously three possible outcomes migh be expected depending on the nature of their interaction (Tisserand & Young, 2013):

- **Additivity,** when the biological activity of a AB mixture = A + B (sum of constituent's activities when applied separately)
- **Synergism or potentiation**, when the biological activity of a AB mixture > A + B
- **Antagonism**, when the the biological activity of a AB mixture < A + B

These concepts of synergism, antagonism and/or additivity modulated by two EOs compounds once blended have been verified under *in vivo* and *in vitro* assays for a number of organisms and different ways of exposure (Hummelbrunner & Isman, 2001; Vokou et al., 2003; Pavela, 2010; Ntali et al., 2011; Koul et al., 2013; Pavela, 2014a), molecular targets and enzymes (Kumrungsee et al., 2014). So far, most of these studies have give evidences that the constituents of a binary mixture may interact additively or synergistically (for example thymol combined with others compounds), thereby increasing the overall effect of a mixture as an effective antifeedant, insecticide, growth regulator, *etc*. (Hummelbrunner & Isman, 2001; Akhtar & Isman**,** 2012). Nevertheless, significant antagonistic effects may also occure, like those observed by Kumrungsee and co-workers (2014) for binary mixtures combining 1,8-cineole in different proportions with thymol or linalool (tested against diamondback moth, *Plutella xylostella*), or those reported by Ntalli and co-workers (2011), regarding the nematicidal activity of *R*-(–)-carvone blended with five different compounds on *Meloidogyne incognita*. Furthermore, most studies concerning antimicrobial activity have attributed additive and synergism effects to phenolic and alcohol compounds. Moreover, compounds with similar structures generally exhibited additive rather than synergistic effect (Bassolé & Juliani, 2012). EOs terpenes are known synergizing agents that act as solvents being able to potentiate the effects of other toxins by favouring their passage through membranes (Rattan, 2010).

Owing to the great number of constituents, it is expectable that natural mixtures, such as EOs, would implicate an extremely complex pattern of interactions. The overall activity being the result of each compound's contribution and concentration, ratios and the underlying biological mechanisms (Ntali et al., 2011). Artificial blends replicating the EO composition with missing single components were further tested to corroborate the role of individual constituents and their contribution toward the whole EO activity (Yeom et al., 2012). Findings from these previous studies have sustained the notion that **1) minor compounds play a role in the overall property of EOs** and that, **2)** in general, **'the whole being not the sum of its parts'**. In fact, it was confirmed that 'inactive constituents' (more correctly not active individually) may potentiate the 'active' constituents and that their presence is necessary to achieve full toxicity (Ntalli et al., 2011), most likely by exerting any influence on bioavailability, resorption rate, reaction of the active compounds with molecular target, *etc*. For example, some EOs constituents typically found in Apiaceae and Rutaceae species, namely methylenedioxyphenyl (MDP) phytochemicals (i.e. dillapiole, apiole and myristicin) are highly

effective insecticide synergists when combined with a variety of insecticides (Lichtenstein et al., 1974) owing to their specific inhibitory action over cytochrome P450 monooxygenases (mixed function oxidases) (Neal, 1989; Regnault-Roger et al., 2012), one component of the enzymatic system responsible for the detoxification and metabolism of deleterious secondary plant metabolites in insects (Feyereisen, 1999). For instance, myristicin is able to increase the antifeedant activity of xanthotoxin toward the lepidopteran *Helicoverpa zea* (Berenbaum & Neal, 1985; Neal, 1989) and the navel orangeworm, *Amyelois transitella* (Niu et al., 2012), also enhancing the phototoxicity of the former compounds in the presence of UV light (Neal, 1989). Moreover, the mutual synergy between three phenylpropanoid derivatives found in *Pimpinella isaurica* EO [4-(2-propenyl)-phenylangelate (PPA), 4-(1-propenyl)-phenyltiglate (PPT), 4-methoxy-2-(1-propenyl)-phenylangelate (MPPA)] was found remarkable by comparison with their minor aphidicidal activity when tested separately (Sampson et al., 2005). The research centered on finding synergistic combinations of compounds may trigger the development of complex and unique (tailor-made) control agents performing at much lower doses, enabling also to reduce the cost and phytotoxicity constrain of some EOs constituents (Hummelbrunner & Isman, 2001).

In spite of some ongoing progresses about the identification of MOA and synergistic effects of some monoterpenes and phenylpropanoids, there is still a limited knowlegde concerning the impact of other EOs constituents, namely sesquiterpenoids, and the occurrence of multiples interactions in more complex mixtures. Such understanding is of upmost importance for attesting the efficiency of EO-based pesticides. Besides, it could be a key factor from the viewpoint of normalizing botanical pesticides (Pavela, 2010). Both issues (overcoming optimization and standardization) have been perceived as restrictive factors to commercialization of plant-based pesticides in general.

3.6. EOs applications in pest management- Opportunities and constraints

Nowadays, EOs are gaining high popularity and extending their application opportunity, mostly on account of the growing consumer awareness concerning its health benefits and as a result of the generalized trend to ‘green consumerism’ (Dubey et al., 2011). In 2014, the EO market was estimated at US $5.51 billions, corresponding to a total world production of 165,000 tons (Grand View Research, 2015). With the rising demand for this type of plant-derived product and the diversification of their appliance, it is expected that the global EO market will grow significantly for the next years (CAGR of 8.6% from 2015 to 2022), likely reaching $11.67 billions by 2022 (Grand View Research, 2015). At the worldwide scale, orange (*Citrus sinensis*) EO is the leading product segment (about 29% share of the total volume consumption in 2014), alongside with eight other EOs (from cornmint, eucalypts, citronella, peppermint, lemon, clove leaf, lime and spearmint) (Grand View Research, 2015).

For the last three decades, these plant-derived products have received a great deal of attention from scientists, corporations and society in general. EOs have been widely employed around the world as therapeutic and biochemical controlling agents (as bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, *etc.*) (Bakkali et al., 2008). One can distinguish several attributes that make EOs and their constituents attractive candidates for use as active ingredients of biorational pesticide (Isman, 2000b; Hummelbrunner & Isman, 2001):

- Pre-existing availability and established market through worldwide commerce; with several suppliers and competitive prices (Isman et al., 2011; Isman & Grieneisen, 2014);
- Familiarization and acceptance from consumers as a conseqence of a long history of human use (traditional medicine, pharmaceutic preparations, herbal beverages, as natural flavoring agents, soaps and fragrances) (Koul et al., 2008; Rajendran & Sriranjini, 2008; Isman & Grieneisen, 2014);
- Favorable safety profiles and comparatively low mammalian toxicity (Koul et al., 2008);
- Simplicity of the extration procedure (principally through hydro- and steam distillation) (Isman & Grieneisen, 2014);
- Constituents have low molecular weight and relatively simple chemical structures (Regnault-Roger, 1997);
- Many EOs are classified with the GRAS (Generally Recognized as Safe) status [21] and acknowledged as safe for human use (USGOV, 2015);
- Several EOs and their ingredients are fully approved in the US for food and beverage consumption by the Food and Drug Administration (FDA) (Kostjukovsky et al., 2002; Dubey et al., 2011);
- Natural mixtures contain several active components which can exhibit positive effects though additive and synergistic interactions (Dubey et al., 2010);
- Broad-spectrum of efficacy (Koul et al., 2008; Isman & Miresmailli, 2010).

Owing to the GRAS status of certain EOs, plant-derived EO products, in general, are eligible for minimum-risk pesticide products (Cloyd et al., 2009; Tripathi et al., 2009). Most EOs constituents are relatively non-toxic to mammals and fish in toxicological tests (Koul et al., 2008). In the USA, active ingredients meeting the criteria for minimum risk, set by the 40 Code FR 152.25(f) guidance, have been exempted from toxicity data requirements and pesticide registration.[22] Consequently, since 1996, American companies have benefit from this window of opportunity to develop and

[21] US Government Publishing Office, Substances generally recognized as safe. Available Online [25 January 2016]: http://www.ecfr.gov/cgi-bin/text idx?SID=b7a5dbb2dc9be4c8c18afa5003962e74&mc=true&node= pt21.3.182&rgn=div5

[22] US Environmental Protection Agency, Minimum Risk Pesticides. Available Online [25 January 2016]: http://www.epa.gov/minimum-risk-pesticides/minimum-risk-pesticide-definition-and-product-confirmation.

introduced EO-based pesticides to the market in a shorter time period (Tripathi et al 2009; Dubey et al., 2011). EcoSMART Technologies, Inc., is one example of a leading company (Khater, 2011). Besides USA, other jurisdictions (namely Japan, Korea, EU) are also considering the reduction of data requirements for products of this type (Isman, 2008).

Several professional formulations containing EO are being commercialized in developed countries for urban, agriculture and consumer purposes (Isman et al., 2011). Many fragrance-based products available in the market have successfully combined several EOs and constituents showing specific pesticidal properties (Table I.5). The whole EOs or the pure active ingredients extracted from clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum* spp.), eucalypt (*Eucalyptus* spp.), geranium (*Pelargonium graveolens*), lemongrass (*Cymbopogon citratus*), citronella (*C. nardus*), oregano (*Origanum vulgare*), peppermint (*Mentha* x *piperita*), rosemary (*Rosmarinus officinalis*) and thyme (*Thymus* spp.) are among the phytochemicals which have been regularly incorporated into formulations (Table I.5). Carvacrol, citronellal, 1,8-cineole, eugenol, *d*-limonene, pulegone and thymol have been mentioned amongst the most active constituents of EOs against insects, some being active ingredients of commercially available flea shampoos, mosquito repellents, agrochemicals, *etc.* (Dubey et al., 2010). There are numerous opportunities for EO-based pesticides usage at satisfactory relative efficacy/application cost ratio, above all as regards to human and animal safety (management of structural pests, household pests, ectoparasites and mosquitoes) (Isman et al., 2011). The majority of patents have been assigned to products of veterinary and domestic uses, essentially aimed to prevent mosquito bites (Tripathi et al., 2003; Coats et al., 2011) and preserve belongings from moths, beetles, termites and ants (Koul et al., 2008; Tripathi et al., 2009). For example, insect-repellent formulations against cockroaches, mosquitoes, mites, ticks and other household insects have been elaborated by incorporating iridoids found in *Nepeta cataria* EO [i.e. *E,Z*- and *Z,E*-isomers of nepetalactone (Fig. I.4) or dihydronepetalactone] (Coats et al., 2003; Scialdone, 2006; Scialdone & Palefsky, 2014).

Besides the anti-insect formulations, EOs present several other applications in agriculture including organic farming systems (Huter, 2010). EOs and constituents have shown great antifungal activities against phytopatogenic fungi (Yoon et al., 2013). Owing to their volatile nature, EOs are considered suitable substitutes to toxic fumigants for the biorational control of stored-products damaging species (Shaaya et al., 1997; Lee et al., 2001; Park et al., 2003; Shaaya & Rafaeli, 2007; Rajendran & Sriranjini, 2008). These volatile compounds could also represent a sound solution for the control of greenhouse pests (Tunç & Şahinkaya, 1998; Aslan et al., 2004; Tripathi et al., 2009). In addition, some might be useful for the control of arthropod pests through a chemosterilant action. This is the case of *Acorus calamus* EO, which contains the volatile phenylpropane-derivatives α- and β-asarone (*trans* and *cis*-isomer of 2,4,5-trimethoxy-1-propenylbenzene); both well known for their significant antigonadal activity (Saxena et al., 1977). EOs are also promising candidates for weed management, however, there is still a long way to go until suitable formulations are available to deliver EO-based herbicides into the field. Volatile oils from pine, clove, lemongrass, citronella, thyme, lemon eucalyptus (*Eucalyptus citriodora*), cotton lavender (*Santolina chamaecyparissus*),

winter savory (*Satureja montana*), Syrian oregano (*O. syriacum*), white savory (*Micromeria fruticosa*) and manuka (*Leptospermum scoparium*) which contain the triketone leptospermone (Fig. I.4), could be used to inhibit weeds emergence (Dudai et al., 1999; Angelini et al., 2003; Dayan et al., 2009; Grosso et al., 2010). Clove EO and its major aromatic compound, eugenol, are active ingredients of traded bioherbicides used as postemergent treatments. According to specifications given by manufacturers the former products act by contact on green vegetation (burn down effect) rather than systemically (Table I.5). The phenolic compounds, carvacrol and thymol, exhibit some herbicidal potential owing to their strong effects on weed germination and seedling growth evidenced over a variety of plant species (Dudai et al., 1999). Moreover, EOs/ constituents showing physiological effects as PGR-like find additional application in crops production and tuber conservation. For instance, S-carvone (Fig. I.4), a monoterpene found in *Carum carvi* and *A. graveolens* EOs, is widely traded as a commercial suppressant of potato sprouting (Dubey et al., 2010).

In addition, their economic relevance to the food industry goes far beyond their utility as food flavoring additives. EOs have been found effective in delaying food spoilage caused by food-borne pathogens and several findings indicate that spices and herbs EOs should find a practical application in the inhibition of mycotoxin production. In fact, various EOs significantly inhibited the growth of toxigenic fungi (namely *Fusarium* and *Aspergillus* strains), thus preventing food commodities from mycotoxin contamination during their storage (Soliman & Badeaa, 2002; Zabka et al., 2009; Singh et al., 2010). Owing to their broad-spectrum activity, thyme, clove, cinnamon, bay, oregano, garlic and lemongrass EOs are the topmost interesting candidates displaying activity against food-borne pathogens and spoilage (Omidbeygi et al., 2007).

Notwithstanding the clear advantages of EOs and constituents in several contexts, their application in crop protection in open field may find severe limitations by the fact that they most likely volatilize relatively quickly, which is evidently detrimental to their optimal efficacy (Dayan et al., 2009). Thus, EOs purpose as fumigants and repellents appear more adequate to confined environments, namely warehouses and greenhouses. Furthermore, like any natural ou synthetic compound, reliable results under field conditions might be occasionally compromised. By experimenting commercially available products under greenhouse conditions, Cloyd and collaborators (2009) have verified that the effectiveness of some EO-based pesticides could vary in terms of their protection efficacy and plant safety. According to these authors, not all products were performing as expected or showed protection against every arthropod pest specified on their labels. Apart from unpredictable interspecific differences in the response of targeted organism, the changes of chemical profile of volatile emissions over time is one of the many aspects that might have a severe impact on their biological effects and the overall effectiveness (Isman & Miresmailli, 2010).

With few exceptions, the EOs themselves or their compounds exhibited little toxicity to mammals. On the basis of the acute oral toxicity in rodents (LD_{50} values), most EOs and constituents would fall into the slightly-toxic or nontoxic categories (LD_{50} superior to 1 g kg^{-1} for pure constituents and 5 g kg^{-1} for formulated products) (Koul et al., 2008; Isman et al., 2001; Tisserand & Young, 2013). Even though, as many safe products, EOs might carry some constrains, especially if handled

or employed inadequately. Some acute effects on animal and human, namely symptoms of poisoning, have been reported as a result of accidental overdose. Nevertheless, the most prevalent adverse are related to skin reactions (Regnault-Roger et al., 2012; Tisserand & Young, 2013). The photosensitization (chronic toxicity) resultant from long-term exposure to subacute dosage of coumarin-containing EOs (lemon, orange, bergamote and lime) and the contact dermatitis triggered by cinnamon, turpentine, laurel berry or tea tree are widely recognized (Tisserand & Young, 2013).

In terms of ecotoxicology, no bioaccumulation or biomagnification of EOs has been reported to date, probably because of the high volatility and biodegradability of their constituents (Regnault-Roger et al., 2012). Even so, unintended effects on soil and water organisms and on natural enemies in consequence of large-scale use have yet to be evaluated under field conditions (Koul et al., 2008). Some cases of phytotoxicity toward important crops have been mentioned in the literature (Cloyd et al., 2009).

Nonetheless, when taken as a whole, the positive attributes of EOs strongly substantiate their valuation. Plant EOs or their constituents have revealed efficacy against a range of pests, from pre- and postharvest cultures, as well as domestic and blood feeding pests (Isman & Machial, 2006). Along with limonoids extracted from Meliaceae species (Table I.3), plant-derived EOs could represent the most versatile, sustainable and acceptable approach for the control of pest and diseases vectores, therefore, justifying their title of ‘eco-friendly’ and ‘green’ pesticides.

Among a number of reviews concerning EOs anti-insect activity, we point out the interesting chapter of Shaaya & Rafaeli (2007), as well as the publication of Tripathi et al. (2009) and Regnault-Roger et al. (2012), which provide a full insight into EOs potential for insect-pest management. To whom concerns the use of EOs for the control of plant parasitic nematodes we recommend the review of Andrés and co-workers (2012). Moreover, Lahlou (2004), Isman & Machial (2006), Bakkali and co-workers (2008) and Koul and co-workers (2008) have presented indispensable overviews about the multiple biological activities ascribed to EOs.

Table I.5. Examples of commercially available pesticides containing essential oils or their constituents. [a]

Product trade name	EOs/Bioactive ingredients	Activity	Use	Manufacturer
Cinnamite and Valero	Cinnamaldehyde (30%) from cinnamon oil	Aphidicidal, miticidal and fungicidal	Crops: grapes, berry crops, citrus and nuts	Mycotech Corp.
Organic insect repellent	Rosemary, cinnamon leaf, lemongrass oils (0.5% each) and geraniol (1%)	Insect repellent	For protection from mosquito bites (skin and/or clothing)	EcoSMART Technologies Inc.
Repel	Lemon eucalyptus oil (30%)	Insect repellent	For protection from mosquito bites (skin and/or clothing)	Spectrum brands, Inc.
Eco-Exempt	Rosemary (10%) and peppermint (2%) oils	Insecticidal	Indoor, outdoor and on pets to control crawling and flying insects	EcoSMART Technologies Inc.
Essentria				Zoecon professional products
Garden insect killer	Rosemary, thyme, clove, peppermint oils (0.25% each)	Insect repellent and biocide	Garden insects and mites all insect stages	EcoSMART Technologies Inc.
Avenger Natural Bed Bug Killer	Clove and peppermint oils (no specifications about%)	Insecticidal	For adult bed bugs (indoor uses)	Cutting Edge Formulations, Inc.
EcoPCO WPX	Thyme oil (5%) [b]	Insecticidal	For outodoor and indoor uses. Against crawling and flying insects	Zoecon professional products
Apilife VAR	Thymol (74%), eucalyptus oil (16%), camphor and L-menthol (each 3.7%)	Miticidal	For use in honeybee colonies for suppression of Varroa mites	Chemicals Laif
Pestoban	*Cedrus deodara* oil and other botanicals	Molluscicidal	Snails	Indian Herbs, Research and Supply Co. Pvt. Ltd.
Organic garden	Rosemary (48%) and thyme (20%) oils	Fungicidal	Against anthracnose, botrytis, powdery mildew, *etc.*	EcoSMART Technologies Inc.
Talent	Carvone (95%) from *C. carvi*	Antimicrobial and PGR	Against food-borne pathogens and sprouting inhibitor of potato tubers	Luxan B.V.
GreenMatch EX	Lemongrass oil (50%)	Herbicidal	Weeds management	Marrone Organic Innovations, Inc.
GreenMatch O	D-Limonene (70%)	Herbicidal	Weeds management	Marrone Organic Innovations, Inc.
Avenger AG	D-Limonene	Herbicidal	Weeds management	Cutting Edge Formulations, Inc.
BioWeed control	α-Terpineol (42-47%)	Herbicidal	Weeds management	Certified Organics Australia Pty Ltd.
Weed Zap	Clove and cinnamon oils (19% each)	Herbicidal	Weeds management	JH Biotech, Inc.
Matran II	Clove oil (46%)	Herbicidal	Weeds management	EcoSMART Technologies Inc.
Organic Weed & Grass killer	Eugenol (5%)	Herbicidal	Weeds management	EcoSMART Technologies Inc.
OrganoCide	Oregano oil (25%)	Broad spectrum fungicide and bactericide	Soil treatment and growing crops protection against *Rhizoctonia*, *Pythium*, *Aspergillum*, *Botrytis*, *etc.*	BotanoCap Green microencapsulation solutions

[a] Information of products were compilled from manufacturers´ websites. [b] Also contain pyrethrins (0.5%) and 2-phenethyl propionate (3%) as insecticidal ingredients. [c] The product also contains a high content of fatty acids.

4. The Apiaceae family as a source of botanical pesticides

4.1. Taxonomy of the Apiaceae

The family Apiaceae Lindl. (1836) (=Umbelliferae Juss.) is one of the most numerous, including several vegetable and herbs of high economic and medicinal value (Olle & bender, 2010) (Table I.6).

Table I.6. Economically important species of the Apiaceae family.

Scientific name	English common name	Portuguese common name
Ammi visnaga	Toothpick weed	Âmio-bisnaga
Anethum graveolens	**Dill**	**Endro; aneto**
Angelica archangelica	Angelica	Angélica medicinal
Apium graveolens	Celery	Aipo; salsão
Carum carvi	Caraway	Alcaravia; cominho-dos-prados
Coriandrum sativum	Coriander	Coentro
Cuminum cyminum	**Cumin**	**Cominho**
Daucus carrota	Wild carrot	Cenoura
Ferula assafoetida	Asafoetida	Férula; funcho-gigante
Foeniculum vulgare	**Fennel**	**Funcho**
Levisticum officinale	Lovage	Levístico
Pastinaca sativa	Parsnip	Pastinaca; cherovia
Petroselinum crispum	**Parlsey**	**Salsa; salsinha**
Pimpinella anisum	Anise	Anis; erva-doce
Trachyspermum ammi	Ajowan; Bishop's weed	Ajowan; tomilho indiano

Highlighted in bold are the species that were studied in this work.

Drude (1898) traditionally divided the Apiaceae into 3 subfamilies (Table I.7). This division system, almost exclusively based on fruit anatomical characters was widely adopted and has been used by many authors (e.g., Pimenov & Leonov, 1993; Plunkett & Downie, 1999). Nevertheless, the floral and fruits morphological characters, traditionally viewed as stable characters, are prone to convergence (Plunkett & Downie, 1999; Downie et al., 2001). The almost exclusive use of morphological-based criteria to delimit suprageneric groups has confounded interpretations and caused considerable controversy surrounding affiliations at the tribal level (Berenbaum, 1990). As a consequence, the resolution of the family classification has been difficult. Since Pimenov and Leonov's classification (1993), several authors have proposed a few taxonomic changes within the family based on hypothesized phylogenetic relationships established from a combination of morphological and molecular data (e.g., Downie et al., 2001; Spalik & Downie, 2001; Calviño et al., 2008). Major advances towards the production of an acceptable modern classification within Apioideae, mostly based on DNA sequence data from both chloroplast and nuclear markers, was

achieved for the last two decades by Downie and co-workers (Downie & Katz-Downie, 1996; Plunkett & Downie, 1999; Downie et al., 2000; Downie et al., 2001, Downie et al., 2010).

Apiaceae species are readily identifiable flowering plants with obvious distinctive characters[23] (Pimenov & Leonov, 1993; Downie et al., 2000), but their identification to the genus and species levels is of known difficulty (Plunkett & Downie, 1999). Umbellifers exhibit a remarkable array of morphological and anatomical modifications of their fruits, many of which are adaptations for various modes of seed dispersal (Downie et al., 2000).

Table I.7. Drude's division of Apiaceae into three subfamilies (1897–1898) and fruits major morphological characters.

Hydrocotyloideae Link	Endocarp sclerified; fruit lacking free carpophore; companion canals present but vittae lacking.
Saniculoideae Burnett	Endocarp parenchymatous; exocarp bearing scales, prickles, or barbs (rarely smooth); stylopodium ring-like; oil canals or cells variable.
Apioideae Drude	Endocarp parenchymatous (fruit sometimes hardened by a woody subepidermal layer); styles emerging from the apex of the stylopodium; vittae and companion cells present.

The largest subfamily, Apioideae, consists of 1800–3000 herbaceous plants of temperate climat (250–400 genera) (Plunkett & Downie, 1999). Species of the subfamily Apioideae may be distinguished from those of the other subfamilies with basis on the following distinctive characters:

Morphological: Presence of compound umbels; more or less finely-divided leaves; two one-seeded mericarps attached to a central bifurcate carpophore; a terminal style; absence of stipule and presence of well-developed schizogenous secretory canals (Downie & Katz-Downie, 1996; Downie et al., 2010).

Biological: A relatively distinctive insect fauna and insect specialists (e.g. *Depressaria* spp. and *Papilio* spp.) (Berenbaum, 1990).

Chemical: The occurrence of some classes of compounds, namely, furanocoumarins (Fig. I. 9), phenylpropenes (Fig. I.10), methylated flavonoids, flavones, *etc*. (Berenbaum, 1981; Berenbaum, 1990).

[23] Supplementary information about morphology, reproductive characteristics and important genera of the Apiaceae family is presented in table A.3 of Apendix A.

4.2. Phytochemicals from Apiaceae and their bioactivities

Besides their popularity and high commercial relevance, umbelliferous crops are also important sources of bioactive compounds. The Apiaceae family is rich in secondary metabolites; yielding distinctive compounds such as sesquiterpenic lactones, furanocoumarins, monoterpene coumarins, polyacetylenes, volatile phenylpropenes, phtalides, *etc.* (Hadaček et al., 1994; El-Razek et al., 2001; Christensen & Brandt, 2006; Evergetis et al., 2012).

More than a few species of the carrot family have proven to have phytotherapeutical potential. Some extracts or compounds have been described as antimicrobials (Ozçelik et al., 2004), antioxidants (Momin & Nair, 2002a; Singh et al., 2006a, 2000b; Zhang et al., 2006), anti-inflammatory (Tabanca et al., 2007), vasorelaxants (Ko et al., 1991), chemopreventive agents (Zheng et al., 1992), as well as phytoestrogens (Yoshikawa et al., 2000; Baser et al., 2007). For example, a group of aliphatic C_{17} polyacetylenes identified in carrot, celery, parlsey and parsnip have revealed interesting anti-tumor (namely antileukemic), anti-inflammatory and antiplatelet aggregatory effects in mammals (Konoshima & Lee, 1986; Chistensen & Brandt, 2006; Chen et al., 2015). Furthermore, the linear furanocoumarin psoralen (Fig. I.9) has been successfully used in the treatment of skin disorders (eczema, psoriasis) by means of a combination of oral ingestion and UV-A treatment (Croteau et al., 2000).

Nevertheless, the Apiaceae family also ascribes renowned poisonous species. Some conjugated polyacetylenes (viz. cicutoxin, oenanthotoxin, virol A, virol B, virol C, *etc*.), which are produced by species of the *Cicuta* and *Oenanthe* genera (C. virosa, *C. maculata*, *C. douglasii*, *O. crocata*), have been identified amongst the strongest plant neurotoxins (Uwai et al., 2000). *Conium maculatum* (hemlock) has been known since ancient times for its acute narcotic neurotoxic effect. *C. maculatum* toxicity is due to the strong phytotoxins piperidinic alkaloids [viz. conmaculatin, γ-coniceine, (S)-(+)-coniine/(R)-(−)-coniine and N-methylconiine]. Besides its acutes effects, *C. maculatum* have been found to induces a chronic teratogenic activity on livestock and humans (López et al., 1999; Radulović et al., 2012). Nonetheless, the carrot family comprises very few alkaloid-producing species, with alkaloids showing a very erratic distribution (Berenbaum, 1990).

4.2.1. Distinctive terpenoids and non-terpenoids constituents from Apiaceae EOs

Several EOs and oleoresins from Apiaceae species are extracted and currently commercialized. Notwithstanding the larger size of the Apioideae subfamily, some taxa remain poorly known (Kapetanos et al., 2008) and many more are still to be characterized in a phytochemical sense. A modest number of species have been studied in terms of their EOs composition. In a review published more than thirty years ago, Kubeczka (1982) stated that only 10% of the known species had been investigated for their EOs. However, at that time, about 760

different constituents belonging to different chemical classes had already been identified (Kubeczka, 1982).

EO compounds encountered in Apiaceae have proved to be very diverse; at least eight distinct classes of phytochemicals, occurring in minor or larger amounts, have been found in a variety of sources and/or materials (Chizzola, 2010). An overview of such chemical diversity within Apiaceae EOs is evidenced in the Table I.8 and in the introduction of Chapter II. In addition, a broader study comprising more than 150 species (about 50 genera of the Apiaceae growing wild in Europe and the Mediterranean region, including Asia Minor) can be found in Chizzola´s review (2010). As can be seen from table I.8, the predominance of single compounds exceeding 30% of the overall EO are very frequent. Outstandingly, numerous Apiaceae EOs are almost fully constituted by a combination of non-terpenoids compounds, namely those extracted from fruits of *P. sativa*, *F. vulgare* and *Heracleum* sp. (Kurkcuoglu et al., 2006; Pitasawat et al. 2007; Radjabian et al., 2014, respectively), the aerial part of *F. foetida* and *F. latisecta* (Kanani et al., 2011), or roots of *P. crispum* and *E. planum* (Petropoulos et al., 2004; Thiem et al., 2011, respectively). Besides, EOs qualitative and quantitative profiles may be quite variable and distinct between organs of the same plant species. Moreover, EO composition may be especially contrasting when comparing roots and aerial parts (Table I.8) [for example in *Lomatium* spp. (Asuming et al., 2005), *C. cyminum* (Bettaieb et al., 2010), *D. carota* subsp. *maxima* (Chizzola, 2010), *D. muricatus* (Bendiabdellah et al., 2012), *E. planum* (Thiem et al., 2011) and *T. villosa* (Drew et al., 2012)].

Table I.8. Major constituents of Apiaceae essential oils, including those from the studied species (*).

Species	Part	**Main components** (%) (> 5%, by decreasing order of abundance)	Reference(s)
Amni visnaga	–	linalool (32), isoamyl 2-methyl butyrate (36), isopentyl isovalerate (15)	Khadhri et al., 2011
*Anethum graveolens**	Hrb	α-Phellandrene (57), dill ether (21), limonene (11)	Vera & Chan-Ming, 1998
	Fw	(R)-Limonene (36), α-phellandrene, dill ether (15)	Góra et al., 2002
	Fr	Limonene (42), α-phellandrene (23), (S)-carvone (22)	
	Ap	α-Phellandrene (59), β-phellandrene (7), methyl eugenol (29)	Evergetis et al., 2013
	Ap	α-Phellandrene (32), apiole (15), dill ether (13), limonene (12), geraniol (11)	Rana & Blazquez, 2014
Angelica archangelica	Fr	α-Pinene (14-41), β-phellandrene (0-55)	Sigurdsson et al., 2005
	Ro	α-Pinene (21), δ-3-carene (17), limonene (16), α-phellandrene (9)	Fraternale et al., 2014
Anthriscus cerefolium	Lf	Estragole (83),1-allyl-2,4-dimethoxybenzene (15)	Baser et al., 1998
A. sylvestris	Ro	β-Phellandrene (45), Z-β-ocimene (17), α-pinene (5)	Bos et al., 2002
	Lf	β-Phellandrene (39), β-myrcene (17), sabinene (6), trans-sabinyl acetate (8), Z-β-ocimene (5)	Bos et al., 2002
Apium graveolens	Fr	D-Limonene (60), selinene (10), phthalides (3)	Pitasawat et al., 2007
A. graveolens var. *dulce*	Lf	(Z)-3-butylidenephthalide (28-38), 3-butyl-4,5-dihydrophthalide (34-41), α-thujene (8-10)	Sellami et al., 2012
	Ro	(Z)-3-butylidenephthalide (31-52), 3-butyl-4,5-dihydrophthalide (12-13), α-thujene (7-12)	Sellami et al., 2012
Athamantha densa	Ap+ Fw	Unknown (20), β-bisabolene (13), unidentified alkaloid (12), β-pinene (9), unidentified alkaloid (9)	Evergetis et al., 2012
Bunium persicum	Fr	γ-Terpinene (44), cuminaldehyde (17), γ-terpinen-7-al (11), *p*-cymene (8)	Oroojalian et al., 2010
Bupleurum fruticosum	Fr	β-Pinene (39), α-pinene (35), β-phellandrene (7)	Chizzola, 2008
	Ap	β-Pinene (38), α-pinene (29), limonene (22)	Evergetis et al., 2009
Cachrys ferulacea	Ap	γ-Terpinene (27), β-phellandrene (16), α- pinene (10), *p*-cymene (7), α- terpinolene (9), E-β-ocimene (9)	Evergetis et al., 2013
Carum carvi	Fr	Carvone (33), limonene (17), γ-terpinene (17)	Pitasawat et al. 2007
	Fr	Carvone (40), limonene (14), β-pinene (7)	de Almeida et al., 2010
	Fr	(S)-(+)-carvone (56), limonene (27)	Yeom et al., 2012
C. montanum	Ap	Nothoapiole (63), dillapiole (9), α-humulene (7)	Laouer et al., 2009
C. nigrum	Fr	dillapiole (30), germacrene B (21), β-caryophyllene (8), β-selinene (7), nothoapiole (6)	Singh et al., 2006a
Chaerophyllum aromaticum	Ap	*cis*-Ocimene (24), α-terpinolene (12), *p*-cymene (7), γ-terpinene (7), β-sesquiphellandrene (7)	Evergetis et al., 2013
C. heldreichii	Ap+ Fw	Sabinene (72), β-phellandrene (11)	Evergetis et al., 2012

Ap, aerial part; Fr, fruit; Fw, Inflorescence; Herb, Hrb; Inf, infrutescence; Lf, leaf; Ro, root; Um, ripe umbel; Rz, rizhome

Table I.8. *(Continued)*

Cnidium officinale	–	cis-Butylidene phthalide (33), 3-butyl phthalide (21), cis-3-isobutylidene phthalide (10), terpinen-4-ol (8)	Choi et al., 2002
Coriandrum sativum	Fr	Linalool (88)	Msaada et al., 2007
	Fr	Linalool (68), γ-terpinene (7)	Grosso et al., 2010
Conopodium capillifolium	Ap+ Fw	α- Pinene (38), sabinene (29)	Evergetis et al., 2009
Crithmum maritimum	–	Dillapiole (15-47), γ-terpinene (17- 35), sabinene (7-22), thymol methyl ether (10-18)	Pateira et al.1999
	–	γ-Terpinene (25- 44), sabinene (17-34), thymol methyl ether (10-18)	
*Cuminum cyminum**	Fr	Cuminaldehyde (30), *p*-cymene (14), γ-terpinene (13), safranal (9), β-pinene (6)	Oroojalian et al., 2010
	Ro	Bornyl acetate (24), diepi-α-cedrene (18), β-pinene (13), hexadecanoic acid (10), camphor (6), citronellal (6)	Bettaieb et al., 2010
	Fw	γ-Terpinene (52), 1-Phenyl-1,2-ethanediol (13), cuminaldehyde (9), α-phellandrene (8), β-pinene (7)	
	Fr	γ-Terpinene (26), 1-Phenyl-1,2-ethanediol (23), cuminaldehyde (15), *p*-cymene (9)	Bettaieb et al., 2011
	Fr	*p*-Cymene (27), cuminaldehyde (17), γ-terpinene (16), β-pinene (9)	Yeom et al., 2012
Daucus carota	Fr	Carotol (67), daucene (9), (Z, Z)-α-farnesene (6)	Ozcan & Chalchat, 2007
	Fr	Geranyl acetate (53)	Radulović et al., 2011
D. carota subsp. *carota*	Hrb	α-Pinene (30), sabinense (30), terpinen-4-ol (6)	Staniszewska et al., 2005
	Fw	α-Pinene (42), sabinense (20)	
	Fr	Geranyl acetate (65), α-pinene (13)	Maxia et al., 2009
	Fr	β-bisabolene (51), E-methylisoeugenol (10), 11αH-himachal-4-en-1-β-ol (9)	
D. carota subsp. *halophilus*	Um	Elemicin (31), sabinene (29), α-pinene (12), limonene (6)	Tavares et al., 2008
D. crinitus	Ap+ Fw	Isochavicol isobutyrate (39), octyl acetate (12), isochavicol 2-methylbutyrate (7)	Lanfranchi et al., 2010
D. gingidium subsp. *gingidium*	Lf	Sabinene (27), α-pinene (11), germacrene D (7), limonene (6)	Flamini et al., 2007
	Fr	Sabinene (61), α-pinene (12)	
D.carota subsp. maximus	Lf	Shyobunone (17), preisocalamendiol (18), β-cubebene (13)	Saad et al., 1995
	Fr	E-Methylisoeugenol (37), β-bisabolene (35), β-asarone (18)	
D. guttatus subsp. *zahariadii*	Ap+ Fr	Apiole (43), β-bisabolene (34)	Radulović et al., 2011
D. muricatus	Ro	Eicosane (19), undecan-2-one (10), tridecanol (6)	Bendiabdellah et al., 2012
	Um	Limonene (22), α-pinene (22), *trans*-sabinyl acetate (12)	
D. sahariensis	Ap+ Fr	Myristicin (52), α-pinene (12), limonene (12), myrcene (7)	Flamini et al., 2013
Distichoselinum tenuifolium	Fw	Myrcene (77), limonene (6)	Tavares et al., 2010
	Um	Myrcene (85)	

Ap, aerial part; Fr, fruit; Fw, Inflorescence; Herb, Hrb; Inf, infrutescence; Lf, leaf; Ro, root; Um, ripe umbel; Rz, rizhome

Table I.8. *(Continued)*

Eleoselinum asclepium	Ap+Fw	Sabinene (35), α-pinene (27)	Evergetis et al., 2009
Eryngium planum	Ro	(Z)-Falcarinol (64), 2,3,4-trimethylbenzaldehyde (17)	Thiem et al., 2011
	Fw	cis-Chrysanthenyl acetate (43), α-pinene (11)	
Ferula assafoetida	Ap	1-Methylpropyl (1E)-prop-1-en-1-yl (33), α-pinene (11), β-pinene (6), germacrene B (6)	Kanani et al., 2011
F. diversivittata	Ap	Verbenone (69), *ar*-curcumenene (6)	Kanani et al., 2011
F. heuffelli	Ro+Rz	Elemicin (35), myristicin (21)	Pavlović et al., 2012
F. foetida	Ap	2,3,4-Trimethylthiophene (49), 2,5-diethylthiophene (28) elemicine (8)	Kanani et al., 2011
F. latisecta	Ap	1-Methylpropyl (1Z)-prop-1-en-1-yl disulfide (89)	Kanani et al., 2011
F. oopoda	Ap	α-Terpinyl acetate (79), sabinene (18)	Kanani et al., 2011
F. orientalis	Ap	Nonane (46), 2-methyloctane (19)	Kanani et al., 2011
F. ovina	Ap	α-Pinene (61-69)	Kanani et al., 2011
Foeniculum vulgare	Fr	*trans*-Anethole (70), fenchone (9)	Singh et al., 2006b
	Fr	trans-Anethole (86), estragole (5), D-limonene (4), *p*-anisaldehyde (3)	Pitasawat et al., 2007
	Fr	*trans*-Anethole (69), limonene (9), estragole (8)	Ntalli et al., 2011
F. vulgare var. *vulgare**	Fr	*trans*-Anethole (88-1), estragole (50-0), fenchone (14-1), limonene (16-6)	Bahmani et al., 2005
	Herb	*trans*-Anethole (53), limonene (35)	Stefanini et al., 2006
	Fr	*trans*-Anethole (74), fenchone (15), limonene (5)	
Heracleum anisactis	Fr	Octyl acetate (49), octyl butanoate (17), octyl 2-methylbutanoate (8), octyl 2-methylpropanoate (7)	Radjabian et al., 2014
H. gorganicum	Fr	Hexyl butanoate (33), octyl acetate (18), Hexyl 2-methylbutanoate (10), hexyl hexanoate (6), hexyl isobutanoate(6)	Radjabian et al., 2014
H. pastinacifolium	Fr	Octyl acetate (60), octyl butanoate (10), octyl 2-methylbutanoate (8), octyl 2-methylpropanoate (7)	Radjabian et al., 2014
H. persicum	Fr	Octyl acetate (21), hexyl butanoate (18), octyl 2-methylbutanoate (10), hexyl 2-methylbutanoate (9), Octyl 2-methylpropanoate (7)	Radjabian et al., 2014
H. rawianum	Fr	Octyl acetate (75), hexyl butanoate (7)	Radjabian et al., 2014
H. rechingeri	Fr	Hexyl butanoate (39), octyl acetate (14), hexyl 2-methylbutanoate (8)	Radjabian et al., 2014
H. sphondylium subsp. *ternatum*	Fr	Octanol (39), octyl butyrate (24), octyl acetate (11)	Ozek et al., 2002
H. sphondylium subsp. *pyrenaicum*	Ap+Fw	Octyl acetate (17), limonene (13), trans-β-farnesene (6), β-caryophyllene (6)	Evergetis et al., 2009

Ap, aerial part; Fr, fruit; Fw, Inflorescence; Herb, Hrb; Inf, infrutescence; Lf, leaf; Ro, root; Um, umbel.

Table I.8. *(Continued)*

Levisticum officinale	Ro	(Z)-Ligustilide (37), β-pinene (9), pentylcyclohexadiene (9), α-pinene (8)	Santos et al., 2005
	Ro	(Z)-Ligustilide (9-71), β-phellandrene (0-49), (Z)-3-butylidene phthalide (1-29), phenylacetaldehyde (0-17), pentylcyclohexadiene (0-12)	Raal et al., 2008
	Lf	α-Terpinyl acetate (56), (Z)-Ligustilide (17), β-phellandrene (11)	
	Fw	β-Pinene (18), β-phellandrene (12), spathulenol (9), γ-curcumene (8)	Mirjalili et al., 2010
	Fr	β-Phellandrene (62), δ-3-Carene (6)	
Lomatium dasycarpum subsp. *dasycarpum*	Fr	3-Methyl-2-buten-1-yl-3-methylbutanoate (36), lavandulyl-2-methyl-butyrate (15), -Methyl-2-buten-1-yl-2-methylbutanoate (10), senkyunolide (10), β-caryophyllene (6)	Asuming et al., 2005
	Ro	(Z)-Falcarinol (22), osthol (9), palmitic acid (9), neocnidilide (7), senkyunolide (6)	
L. lucidum	Fr	β-Phellandrene+limonene (22), decanal (6),α-humulene (5)	Asuming et al., 2005
	Ro	(Z)-Falcarinol (45), (E)-β-farnesene (6), α-chamigrene (6), β-acoradiene (6)	
L. macrocarpum var. *macrocarpum*	Fr	β-Pinene (54), α-pinene (25)	Asuming et al., 2005
	Ro	Osthol (68), peucenin-7-methyl ether (9)	
L. utriculatum	Fr	Sabinene (34), (Z)-ligustilide (13), terpinen-4-ol (7), β-caryophyllene (6), β-phellandrene/limonene (6)	Asuming et al., 2005
	Ro	(Z)-Falcarinol (17), (Z)-ligustilide (17), senkyunolide (7), palmitic acid (6)	
Malabaila aurea	Ap	Hexyl butyrate (36), hexanoic acid hexyl ester (36), *n*-hexanol (11)	Evergetis et al., 2013
Oenanthe pimpinelloides	Ap	γ-Terpinene (43), *O*-cymene (18), β-sesquiphellandrene (8), β-pinene (7)	Evergetis et al., 2009
Pastinaca sativa	Ro	Terpinolene (40-69), myristicin (17-40)	Kubeczka & Stahl, 1975
	–	(Z)-β-Ocimene (11), hexyl butanoate (10), (E)-β-farnesene (6)	Kapetanos et al., 2008
P. sativa subsp. *urens*	Fr	Octyl butyrate (80), octyl hexanoate (5)	Kurkcuoglu et al., 2006
P. hirsuta	–	Hexyl butanoate (55)	Kapetanos et al., 2008
*Petroselinum crispum**	Ro	Apiole (52), myristicin (36)	Petropoulos et al., 2004
	Lf	Myristicin (22), apiole (18), β-phellandrene (17), *p*-1,3,8-menthatriene (14), α-, *p*-dimethylstyrene (9)	
	Lf	Sabinene (21), 3-hexyne-2,5-diol, 2-5-dimethyl (11), p-mentha-1,5,8-triene (8), apiole (8), p-cymene (7), myristicin (6)	Knio et al., 2008
	Lf	Myristicin (31-43), β-phellandrene (22-36), *p*-1,3,8-menthatriene (6-10), β-myrcene (5-9)	Vokk et al., 2011
	Fr	Apiole (11-68), myristicin (1-38), allyltetramethoxybenzene (1-29), α-pinene (8-17), β-pinene (5-11)	Lamarti et al., 1991
P. crispum subsp. *tuberosum*	Ro	β-Pinene (31), apiole (20), β-phellandrene (17), myristicin (16)	Petropoulos et al., 2004
Peucedanum officinale	Ap+ Fw	1-Bornyl acetate (81)	Evergetis et al., 2012
P. lancifolium	Ro	*trans*-Sesquilavandulol (53), sabinene (7), β-eudesmol + α-cadinol (6)	Kubeczka et al., 1989
P. neumayeri	Fw	γ-Terpinene (32), α-pinene (21), β-phellandrene (13)	Evergetis et al., 2012

Ap, aerial part; Fr, fruit; Fw, Inflorescence; Herb, Hrb; Inf, infrutescence; Lf, leaf; Ro, root; Um, umbel.

Table I.8. *(Continued)*

Pimpinella anisum	Fr	*trans*-Anethole (77), anisalacetone (7), estragol (6)	Knio et al., 2008
	Fr	*trans*-Anethole (86)	Ntalli et al., 2011
	Fr	*trans*-Anethole (97)	de Almeida et al., 2010
P. haussknechtii	Fr	4-(2-propenyl)-phenyl 3'-methylbutyrate (57), bicyclogermacrene (9), germacrene D (8)	Sajjadi et al., 2015
P. peregrina	Ap+ Fw	α-Bergamontene (62), aristolene (20)	Evergetis et al., 2012
P. rigidula	Ap+ Fw	*trans*-Epoxypseudoisoeugenyl 2-methylbutyrate (27), β-selinene (24), *trans*-isomiristicin (8), α-zingiberene (8), myristicin (7), *trans*-pseudoisoeugenyl 2-methylbutyrate (7)	Evergetis et al., 2012
P. serbica	–	Apiole (77), (+)-(E)-β-caryophyllene (10)	Kapetanos et al., 2008
P. tragium	Ap+ Fw	Germacrone (23), unknown (20), germacrene B (19), geigerene (10)	Evergetis et al., 2012
Prango ferulaceae	Ap	β-pinene (23), δ-3-carene (16), α -pinene (13), epi-α-bisabolol (8)	Sefidkon et al., 1998
	Fr	β-pinene (33), δ-3-carene (10), α -pinene (10), limonene (9)	
	Fr	2,3,6-Trimethyl benzaldehyde (67), chrysanthenyl acetate (15)	Ercan et al., 2013
P. uloptera	Ap+Fw	α-Bisabolol (31), safrole (19), (+)-spathulenol (13)	Razavi et al., 2011
	Ap+Fr	γ-Terpinene (36), *trans*-anethole (24)	
Seseli montanum	Ap+Fw	α -Pinene (32), *β*-phellandrene (19), sabinene (17)	Evergetis et al., 2009
S. montanum subsp. *peixotoanum*	Um	α -Pinene (36), β-pinene (22), limonene (9), myrcene (7), β-elemene (6)	Gonçalves et al., 2012a
S. parnassicum	Ap+ Fw	β-Sesquiphellandrene (30), germacrene D (13), germacrene B (11), β-elemene (11), bicyclogermacrene (6)	Evergetis et al., 2012
S. tortuosum	Um	α -Pinene (25), β-pinene (24), Z-β-ocimene (13)	Gonçalves et al., 2012a
Smyrnium olusatrum	Ro	Isofuranogermacrene (21), *β*-phellandrene (20), furanodiene (17), limonene (12), Furanoeremophil-1-one (11)	Mölleken et al., 1998
	Fr	*β*-Phellandrene (27), 1β-Acetoxyfurano-4*(15)*-eudesmene (12), α-pinene (12), myrcene (11)	
	Lf	Furanoeremophil-1-one (29), curzerene (29)	Papaioannou et al., 2010
	Fw	Curzerene (38), germacrone (20), furanoeremophil-1-one (20)	
	Inf	1β-Acetoxyfuranoeudesm-4*(15)*-ene (31), curzerene (17)	
S. rotundifolium	Ap+ Fw	1β-Acetoxyfuranoeudesm-3-ene (21), 1β-Acetoxyfuranoeudesm-4*(15)*-ene (9), furanodiene (12), myrcene (11)	Evergetis et al., 2012
Thapsia garganica	Ro	δ-Cadinene (17)	Drew et al., 2012
T. minor	Ap	Geranyl acetate (82)	Gonçalves et al., 2012b
T. villosa	Fr	Geranyl acetate (79-92)	Avato et al., 1996
	Fw	Geranyl acetate (37)	Drew et al., 2012
	Ro	Guaiol (15), elemol (10)	
Trachyspermum ammi	Fr	Thymol (42), γ-terpinene (28), *p*-cymene (24)	Park et al., 2007
	Fr	Thymol (48), *p*-cymene (22), γ-terpinene (21)	Oroojalian et al., 2010
	Fr	Thymol (42), γ-Terpinene (28), *p*-cymene (24)	Yeom et al., 2012

Ap, aerial part; Fr, fruit; Fw, Inflorescence; Herb, Hrb; Inf, infrutescence; Lf, leaf; Ro, root; Um, umbel.

4.2.2. Application of Apiaceae phytochemicals for the biocontrol of pests and vectors/ hosts

The supporting evidences of Apiaceae pesticidal activities against varied type of damaging/noxious organisms are significant. Several studies have pointed out the value of Apiaceae plants and their potential application in the context of IPM and IVM (Hadaček et al., 1994; Eckenbach et al., 1999; Oroojalian et al., 2010; Boulogne et al., 2012; Evergetis et al., 2009; Evergetis et al., 2012; Yeom et al., 2012; Kim et al., 2013; Seo et al., 2015). The use of several Apiaceae species was found interesting in particular for the management of leaf-cutting ants (Boulogne et al., 2012), as well as potent emerging agents for mosquito control (Evergetis et al., 2009; Evergetis et al., 2012). The biopesticidal activities that have been investigated in Apiaceae species are summarized in table I.9.

Among the family taxa, there are several species showing noteworthy activities. For example, plants of the genus *Pimpinella* have been described as excellent sources of strongly active extracts/compounds showing insecticidal, acaricidal and antifungal properties (Reichling et al., 1991; Soliman & Badeaa, 2002; Sampson et al., 2005; Baser et al., 2007). In a broader perspective, *Carum carvi*, *Foeniculum vulgare* and *Pimpinella anisum* are probably the most extensively investigated species. Considering their spectrum of activities (Table I.9), these species might present a tangible potential of application in the biochemical-based control of species in several fields.

In general, the biopesticidal activity of Apiaceae extracts was verified toward a large range of arthropod species, with a strong focus in mosquitocidal activity (Table I.9). In addition, there is considerable evidence concerning the antimicrobial activities (Syed et al., 1986; Soliman & Badeaa, 2002; Ozçelik et al., 2004; Singh et al., 2006a and 2006b; Baser et al., 2007) of extracts from umbellifers. A few studies have also underlined the potential of this plant family toward plant parasitic nematodes (Kong et al., 2006; Ntalli et al., 2011). Nevertheless, to our knowledge, investigation contemplating EOs molluscicidal action are scarce, despite some evidences of their potential for the control of harmful molluscs (viz. slugs and snails) (Dodds et al., 1999; Kumar & Singh, 2006; Singh et al., 1997). Likewise, limited information was found on the activity of Apiaceae extracts towards weeds or other plant species (Kleiman et al., 1988; Reichling et al., 1991; Azirak & Karaman, 2008; de Almeida et al., 2010).

A number of purified compounds found in considerable amount in plants of the carrot family have been evaluated and mentioned in the literature as anti-arthropods, antinematodes, molluscicides, antifungals and/or herbicidal/antigerminative agents. These phytochemicals have largely been ascribed to the classes of terpenoids, phenylpropanoids (coumarins and phenylpropenes) (Table I.10 and Fig. I.9 and I.10) and phtalides (Fig. I.11).

Table I.9. Overview of Apiaceae activity against arthropods, nematodes, molluscs, plants and food-borne pathogens.

Plant species	Part	Bioactivity	Target organism tested	Dosage	References
Ammi visnaga	Fr [c]	Mosquitocidal (Larvae)	*Culex quinquefasciatus*	24 h-LC_{50} = 9 ppm	Pavela, 2008
	Fp [a]	Nematicidal	*Bursaphelenchus xylophilus*	-	Kim et al., 2008
Anethum graveolens	Fr [a]	Insecticidal (contact)	*Blatella germanica*	0.5 mg per adult, mortality >60% at 48 h	Yeom et al., 2012
	– [a]	Mosquitocidal (Larvae)	*Culex quinquefasciatus*	$LC_{50} \leq$ 10 ppm	Amer & Mehlhorn, 2006
	– [a]	Mosquitocidal (Larvae)	*Aedes aegypti*	$LC_{50} \approx$ 50 ppm	
	Lv [b]	Mosquitocidal (Larvae)	*A. aegypti*	24 h- LC_{50} = 27 µg mL^{-1}	Promsiri et al., 2006
	Fr [a]	Antifungal Complete inhibition of mycelial growth)	*Aspergillus flavus* *A. oryzae* *A. niger*	2 µL mL^{-1} Idem Idem	Tian et al., 2011
Angelica archangelica	Fr [a]	Insecticidal (Contact)	*Spodoptera littoralis*	24 h-LC_{50} = 96 µg per larvae	Pavela & Vrchotová, 2013
	Fr	Antifeedant	*S. littoralis*	ED_{50} = 0.54 µg cm^{-2}	
	Fr [c]	Insecticidal (Contact)	*Acyrthosiphon pisum*	24 h-LC_{50}=1.1 mg mL^{-1}	Pavela et al., 2013
Angelica dahurica	Ro [c]	Antifeedant	*Attagenus unicolor japonicus*	AI = 100%, at 1.3 mg cm^{-2}	Han et al., 2006
Apium graveolens	– [a]	Insecticidal	*Lucilia sericata*	LC_{50} = 4.6%	Khater & Khater, 2009
	Fr [a]	Mosquitocidal (adults)	*Aedes aegypti*	24 h- LC_{50} = 6.1 µg mg^{-1} of female body weight	Chaiyasit et al., 2006
	– [a]	Mosquitocidal (Larvae)	*A. aegypti*	LC_{50} = 42 ppm	Pitasawat et al., 2007
	– [a]	Mosquitocidal (Larvae)	*Anopheles dirus*	LC_{50} = 59 ppm	
	Fr [a]	Mosquitocidal (Larvae)	*Aedes albopictus*	0.1 mg mL^{-1} Mortality = 98% at 48 h	Seo et al., 2015
Bifora radians	Ap [a]	Aphicidal	*Lipaphis pseudobrassicae*	1 h-LC_{50} = 0.3 mg mL^{-1}	Sampson et al., 2005
Bunium persicum	Fr [a]	Antibacterial	*Staphylococcus aureus* *Bacillus cereus* *Listeria monocytogenes* *Escherichia coli*	MIC = 0.75 mg mL^{-1} MIC = 0.18 mg mL^{-1} MIC = 0.75 mg mL^{-1} MIC = 1.5 mg mL^{-1}	Oroojalian et al., 2010
Bupleurum exaltatum	St [c]	Mosquitocidal (Larvae)	*Culex quinquefasciatus*	24 h-LC_{50} = 120 ppm	Pavela, 2008
Bupleurum fruticosum	Ap [a]	Mosquitocidal (Larvae)	*Culex pipiens molestus*	48 h-LC_{50} = 65 ppm	Evergetis et al., 2009
Carum carvi	Fr [a]	Insecticidal	*Blatella germanica*	5 mg/filter Mortality >90% at 48 h	Yeom et al., 2012
	Fr [a]	Mosquitocidal (adults)	*Aedes aegypti*	24 h- LC_{50} = 5.5 µg mg^{-1} of female body weight	Chaiyasit et al., 2006
	– [a]	Mosquitocidal (Larvae)	*A. aegypti*	LC_{50} = 55 ppm	Pitasawat et al., 2007
	– [a]	Mosquitocidal (Larvae)	*Anopheles dirus*	LC_{50} = 72 ppm	
	– [a]	Antifungal	*Aspergillus parasiticus* *A. flavus*	MIC = 2000 ppm Idem	Soliman & Badeaa, 2002
	Ub [a]	Nematistatic	*Meloidogyne javanica*	800 µL L^{-1}, 100% after 2 days	Oka et al., 2000
	Fr [b]	Molluscicidal	*Lymnaea acuminata*	48 h-LC_{50} = 101 mg L^{-1}	Kumar & Singh, 2006
	Fr [a]	Herbicidal	*Triticum aestivum*	IC_{50} = 48 nL mL^{-1} of air	Dudai et al., 1999
	Fr [a]	Herbicidal (emergence)	*Lepidium sativum* *Raphanus sativus*	0.625 µg mL^{-1}, inhibition > 90% 0.625 µg mL^{-1}, inhibition = 100%	de Almeida et al., 2010
Carum nigra	Fr [a]	Antifungal	*Penicillium purpurogenum* *Acrophialophora fusispora*	3000 ppm, complete inhibition of mycelial growth	Singh et al., 2006a
Cnidium officinale	Rh [c]	Antifeedant	*Attagenus unicolor japonicus*	AI = 100%, at 2.6 mg cm^{-2}	Han et al., 2006
Conopodium capillifolium	Ap [a]	Mosquitocidal (Larvae)	*Culex pipiens molestus*	LC_{50} = 69 ppm at 48 h	Evergetis et al., 2009

[a] Essential oil; [b] Crude ethanolic extract; [c] Crude methanolic extract; [d] Crude methylene chloride extract; [d] Crude hexane extract.
Ap, aerial parts; Fw, flowers; Fp, flowering plant; Fr, fruit; Hrb, herb; Lf, leaf; Lx, latex; Res, resin; Rh, rhizome; Ro, root; St, stem; Ub, umbel; Wp, whole plant. AI, antifeedant index; ED_{50}, the effective dose required to achieve the desired response in 50% of the population; IC_{50}, median inhibitory concentration; LC_{50}, median lethal concentration causing 50% of death; LCIC, lowest complete inhibitory concentration; MIC, minimum inhibitory concentration.

Table I.9. *(Continued)*

Plant species	Part	Bioactivity	Target organism tested	Dosage	References
Coriandrum sativum	Fr [a]	Aphicidal	*Lipaphis pseudobrassicae*	1 h-LC_{50} = 2.9 mg mL^{-1}	Sampson et al., 2005
	– [a]	Insecticidal	*Acanthoscelides obtectus*	24 h-LC_{50} < 10 mg L^{-1} of air	Regnault-Roger et al., 1993
	Hrb [a]	Antifeedant	*Spodoptera litura*	AI = 85%, at 4 mg cm^{-2}	Elumalai et al., 2010
	Fr [a]	Nematicidal	*Bursaphelenchus xylophilus*	24 h-ED_{50} = 2.8 mg mL^{-1}	Kong et al., 2006
	Hrb [a]			24 h-ED_{50} = 0.14 mg mL^{-1}	
	Fr [a]	Herbicidal (Emergence)	*Portulaca oleracea*	ED_{50} = 5.4 µL per Petri dish	Grosso et al., 2010
			Vicia sativa	ED_{50} = 8.8 µL per Petri dish	
			Lactuca sativa	ED_{50} = 5.2 µL per Petri dish	
			Triticum durum	ED_{50} = 4.5 µL per Petri dish	
	Fr [a]	Herbicidal	*T. aestivum*	IC_{50} = 56 nL mL^{-1} of air	Dudai et al., 1999
Cuminum cyminum	Fr [a]	Insecticidal (contact)	*Blatella germanica*	1 mg per adult, mortality 100% at 48 h	Yeom et al., 2012
	– [a]	Insecticidal	*Acanthoscelides obtectus*	24 h-LC_{50} < 10 mg L^{-1} air	Regnault-Roger et al., 1993
	Hrb [a]	Insecticidal	*Spodoptera litura*	AI = 90%, at 4 mg cm^{-2}	Elumalai et al., 2010
	Fr [a]	Acaricidal	*Rhipicephalus microplus*	Mortality = 100% at 1.25%	Martinez-Velazquez et al., 2011
Daucus carota	Fr [a]	Mosquitocidal (Larvae)	*Aedes albopictus*	0.1 mg mL^{-1} Mortality = 100% at 48 h	Seo et al., 2015
Distichoselinum tenuifolium	Ub [a]	Antifungal	*Aspergillus fumigatus*	MIC = 2.5 µl mL^{-1}	Tavares et al., 2010
			A. niger	MIC = 2.5 µl mL^{-1}	
Ferula assafoetida	Lx [b]	Molluscicidal	*Lymnaea acuminata*	48 h-LC_{50} = 111 mg L^{-1}	Kumar & Singh, 2006
Foeniculum vulgare	Fr [a]	Aphicidal	*Lipaphis pseudobrassicae*	1 h-LC_{50} = 9.9 mg mL^{-1}	Sampson et al., 2005
	– [a]	Mosquitocidal (Larvae)	*Aedes aegypti*	LC_{50} = 49 ppm	Pitasawat et al., 2007
	– [a]	Mosquitocidal (Larvae)	*Anopheles dirus*	LC_{50} = 35 ppm	
	Fr [a]	Mosquitocidal (Larvae)	*Culex quinquefasciatus*	1 h-LC_{50} = 25 ppm	Rana & Rana, 2012
	Fw [a]	Mosquitocidal (Larvae)	*Culex pipiens molestus*	24 h-LC_{50} = 25 mg L^{-1}	Traboulsi et al., 2005
	Fr [a]	Insecticidal	*Sitophilus granarius*	24 h-LC_{50} = 27 µL L^{-1} air	Ebadollahi, 2011
	Fr [a]	Insecticidal	*Sitophilus oryzae*	24 h-LC_{50} = 44 µL L^{-1} air	
	Fr [a]	Nematistatic	*Meloidogyne incognita*	24 h-LC_{50} = 109 µg mL^{-1}	Ntalli et al., 2011
	Ub [a]	Nematistatic	*M. javanica*	800 µL L^{-1}, 100% after 2 days	Oka et al., 2000
	Fr [a]	Antifungal (Complete inhibition of mycelial growth)	*Aspergillus niger*	4 µL	Singh et al., 2006b
			Fusarium graminearum	6 µL	
			F. monoliforme	6 µL	
	– [a]	Antifungal	*Aspergillus parasiticus*	MIC = 2000 ppm	Soliman & Badeaa, 2002
			A. ochraceus		
			F. moniliforme		
Ligusticum hultenii	Fr [d]	Antifungal	*Colletotrichum fragariae*	-	Meepagala et al., 2005
	Fr [d]	Algicidal	*Oscillatoria perornata*	LCIC = 100 ppm	
Lomatium californicum	Fr [d]	Algicidal	*Oscillatoria perornata*	LCIC = 100 ppm	Meepagala et al., 2005
			Selenastrum capricornutum	Idem	
Oenanthe pimpinelloides	Ap [a]	Mosquitocidal (Larvae)	*Culex pipiens molestus*	LC_{50} = 40 ppm at 48 h	Evergetis et al., 2009
Ostericum sieboldii	Fp [a]	Insecticidal (contact)	*Sitophilus zeamais*	14 µg per adult	Liu et al., 2011
			Tribolium castaneum	8 µg per adult	

[a] Essential oil; [b] Crude ethanolic extract; [c] Crude methanolic extract; [d] Crude methylene chloride extract; [d] Crude hexane extract.

Ap, aerial parts; Fw, flowers; Fp, flowering plant; Fr, fruit; Hrb, herb; Lf, leaf; Lx, latex; Res, resin; Rh, rhizome; Ro, root; St, stem; Ub, umbel; Wp, whole plant. AI, antifeedant index; ED_{50}, the effective dose required to achieve the desired response in 50% of the population; IC_{50}, median inhibitory concentration; LC_{50}, median lethal concentration causing 50% of death; LCIC, lowest complete inhibitory concentration; MIC, minimum inhibitory concentration.

Table I.9. *(Continued)*

Plant species	Part	Bioactivity	Target organism tested	Dosage	References
Petroselinum crispum	Fr [a]	Acaricidal	*Dermatophagoides farinae* *D. pteronyssinus*	LD_{50} = 1.9 µg cm^2 LD_{50} = 2.3 µg cm^2	Song et al., 2011
	Hrb [a]	Mosquitocidal (Larvae)	*Aedes albopictus*	0.1 mg mL^{-1}, mortality 100% at 48 h	Seo et al., 2015
Pimpinella anisum	Fr [a]	Aphicidal	*Lipaphis pseudobrassicae*	1 h-LC_{50} = 4.6 mg mL^{-1}	Sampson et al., 2005
	Fr [a]	Mosquitocidal (Larvae)	*Culex quinquefasciatus*	24 h-LC_{50} = 27 mg mL^{-1}	Pavela, 2014b
	Fr [a]	Nematistatic	*Meloidogyne incognita*	24 h-LC_{50} = 125 mg mL^{-1}	Ntalli et al., 2011
	– [a]	Antifungal	*Aspergillus flavus* *A. parasiticus* *A. ochraceus* *Fusarium moniliforme*	MIC = 500 ppm Idem Idem Idem	Soliman & Badeaa, 2002
Pimpinella isaurica	Ap [a] (excl. Fr)	Aphicidal	*Lipaphis pseudobrassicae*	10 mg mL^{-1} Mortality > 90%, 10 min	Sampson et al., 2005
Trachyspermum ammi	Fr [a]	Insecticidal (contact)	*Blatella germanica*	0.1 mg mL^{-1} per adult mortality = 76% at 48 h	Yeom et al., 2012
	Fr [d]	Mosquitocidal (Larvae)	*Aedes aegypti*	24 h-LC_{50} = 65 ppm	Kumar et al., 2012
	Fr [a]	Mosquitocidal (Larvae)	*Aedes albopictus*	0.1 mg mL^{-1} Mortality = 100% at 48 h	Seo et al., 2015
	-	Molluscicidal	*Lymnaea acuminata*	48 h-LC_{50} = 173 mg L^{-1}	Singh et al., 1997

[a] Essential oil; [b] Crude ethanolic extract; [c] Crude methanolic extract; [d] Crude methylene chloride extract; [d] Crude hexane extract.

Ap, aerial parts; Fw, flowers; Fp, flowering plant; Fr, fruit; Hrb, herb; Lf, leaf; Lx, latex; Res, resin; Rh, rhizome; Ro, root; St, stem; Ub, umbel; Wp, whole plant. AI, antifeedant index; ED_{50}, the effective dose required to achieve the desired response in 50% of the population; IC_{50}, median inhibitory concentration; LC_{50}, median lethal concentration causing 50% of death; LCIC, lowest complete inhibitory concentration; MIC, minimum inhibitory concentration.

Table I.10. Secondary metabolites from Apiaceae species showing insecticidal and fungicidal activities.

Plant species	Phytochemicals
Ammi majus	Xanthotoxin [a], isopimpinellin [a]
A. visnaga	Xanthotoxin, isopimpinellin
Anethum graveolens	Eugenol [b], elemicin [b], myristicin [b]
Angelica acutiloba	Xanthotoxin, isoimperatorin [a]
A. archangelica	Xanthotoxin, psoralen [a], isopimpinellin
A. dahurica	Xanthotoxin, psoralen
Apium graveolens	Eugenol, thymol [c], menthone [c], myristicin, xanthotoxin, psoralen, isopimpinellin
Carum carvi	Myristicin
Coriandrum sativum	Myristicin, psoralen
Cuminum cyminum	Eugenol, caryophyllene oxide [c], anisaldehyde [b]
Daucus carota	Eugenol, elemicin, caryophyllene oxide, myristicin, isopimpinellin, psoralen, xanthotoxin
Ferula alliacea	Isopimpinellin
F. assafoetida	Isopimpinellin
Foeniculum vulgare	Myristicin, anisaldehyde, isopimpinellin, psoralen, xanthotoxin
Glehnia littoralis	Psoralen, xanthotoxin, anethole [b]
Heracleum laciniatum	Isopimpinellin
H. lanatum	Isopimpinellin
Levisticum officinale	Eugenol, myristicin, psoralen
Oenanthe aquatica	Eugenol, myristicin
O. crocata	Myristicin
O. javanica	Myristicin
Pastinaca sativa	Myristicin, isopimpinellin, psoralen, xanthotoxin
Petroselinum crispum	Elemicin, myristicin, isopimpinellin, psoralen, xanthotoxin
Pimpinella anisum	Eugenol, myristicin, anisaldehyde
Trachyspermum ammi	Thymol

[Source: Boulogne et al., 2012]; [a] Coumarin; [b] Phenylpropene-derivative; [c] Terpenoid. Highlighted in bold are the species that were studied in this work.

The phenylpropanoid family is rich in biologically active compounds beneficial for the plants themselves. For instance, the coumarins, furanocoumarins, and stilbenes are natural biocontrol agents that show protecting properties against bacterial and fungal pathogens, discourage herbivory, and inhibit seed germination, evidencing their roles in plant defense (Croteau et al., 2000).

Accordingly, several furanocoumarins found in Apiaceae species, namely xanthotoxin, isopimpinellin, psoralen and isoimperatorin provide significant pesticidal effects (Table I.10). Xanthotoxin is a highly effective feeding deterrent against the Noctuids *Trichoplusia ni* and *Spodoptera litura* (DC_{50} value = 0.5 and 0.3 µg cm^2, respectively) (Isman, 2002), exhibiting a significant insecticidal activity similar to that of isopimpinellin (Miyazawa et al., 2004). Together with other furanocoumarins, imperatorin was the main compound of *Angelica archangelica* extract being responsible for the chronic toxicity in *Spodoptera littoralis* and the significantly larvae growth inhibition (Pavela & Vrchotová, 2013). In addition, the strong molluscicidal property of umbelliferone found in extracts of *Ferula assafoetida* latex was evidenced against *Lymnaea acuminata* (48 h-LC_{50} = 1.9 mg L^{-1}) (Kumar & Singh, 2006). The antimicrobial properties of bergaptene umbelliferone and xanthotoxin (MICs = 0.015%) were also reported (Ozçelik et al., 2004). Furanocoumarins are present in relatively high dosages in edible Apiaceaesuch as celery, parsley, carrots and parsnip (Pavela & Vrchotová, 2013). The total concentrations of psoralen, bergapten, xanthotoxin inside fruits of the latter species can reach as much as 3.2 mg g^{-1} of fresh weight (Beier & Nigg, 2000), while the whole content of linear furanocoumarins in fresh parlsey leaves is approximately 28 times lower (112 µg g^{-1}) (Beier et al., 1994). A rare halogenated furocoumarin, saxalin (Fig. I.9), have been found in parsley leaves along with seven other furanocoumarins (psoralen, bergapten, xanthotoxin, isoimperatorin, isopimpinellin, oxypeucedanin, and oxypeucedanin hydrate) (Beier et al., 1994).

Phenylpropenes (C6–C3 unit, consisting of a modified benzene ring bearing a propenyl side chain) are another major group of naturally occurring phenolic compounds found in the carrot family (Fig. I.10) that have attracted the attention of researchers for their pesticidal properties. Since phenylpropanoids have a unique structure and biological activity, they may have potential for developing new agents for use in agriculture and medicine (Baser et al., 2007).

The propenylbenzene *trans*-anethole is a volatile constituent frequently present in numerous Apiaceae extracts (i.e. anise, fennel), being also the major compounds of star anise (*Illicium verum*, Schisandraceae) EO. As we will discuss latter, this compound possesses a wide range of bioactivities exhibiting a great potential as biopesticidal agent. Also, the epoxypseudoisoeugenol and pseudoisoeugenol derivatives (propenylbenzenes mainly restricted to *Pimpinella* species) have rised some interest as regard to their biopesticidal properties (Tabanca et al., 2005; Baser et al., 2007). For instance, (*E*)-pseudoisoeugenol presents a significant insecticidal action (Reichling et al., 1991). Likewise, several biological activities were attributed to allyl-phenylpropenes (Fig. I.10). The volatile phenylpropanoids apiole, dillapiole, and myristicin were toxic to the insect species *Drosophila melanogaster* and *Aedes aegypti* (Lichtenstein et al., 1974). Additionally, apiole and myristicin, both major

components of *Petroselinum sativum* EO, showed acaricidal effects against *Dermatophagoides* spp. mites (Song et al., 2011). Myristicin, which is found in several plants including Apiaceae (Fig. I.10), is also a strong synergistic agent (Lichtenstein and Casida, 1963; Lichtenstein et al., 1974; Berenbaum and Neal, 1985). Similarly, apiole and dillapiole (both with a more restricted natural occurrence) were also reported to potentiate the action of synthetic insecticides (Lichtenstein et al., 1974; Berenbaum & Neal, 1985). Furthermore, dillapiole, apiole and myristicin (from dill EO) exhibited inhibitory activity on aflatoxin G1 production by the mycotoxigenic fungus *Aspergillus parasiticus* (IC_{50}=0.15, 0.24 and 3.5 µM, respectively) (Razzaghi-Abyaneh et al., 2007). In addition, *Carum montanum* EO was reported as a source of nothoapiole, an allylbenzene with interesting antimicrobial activity (Laouer et al., 2009).

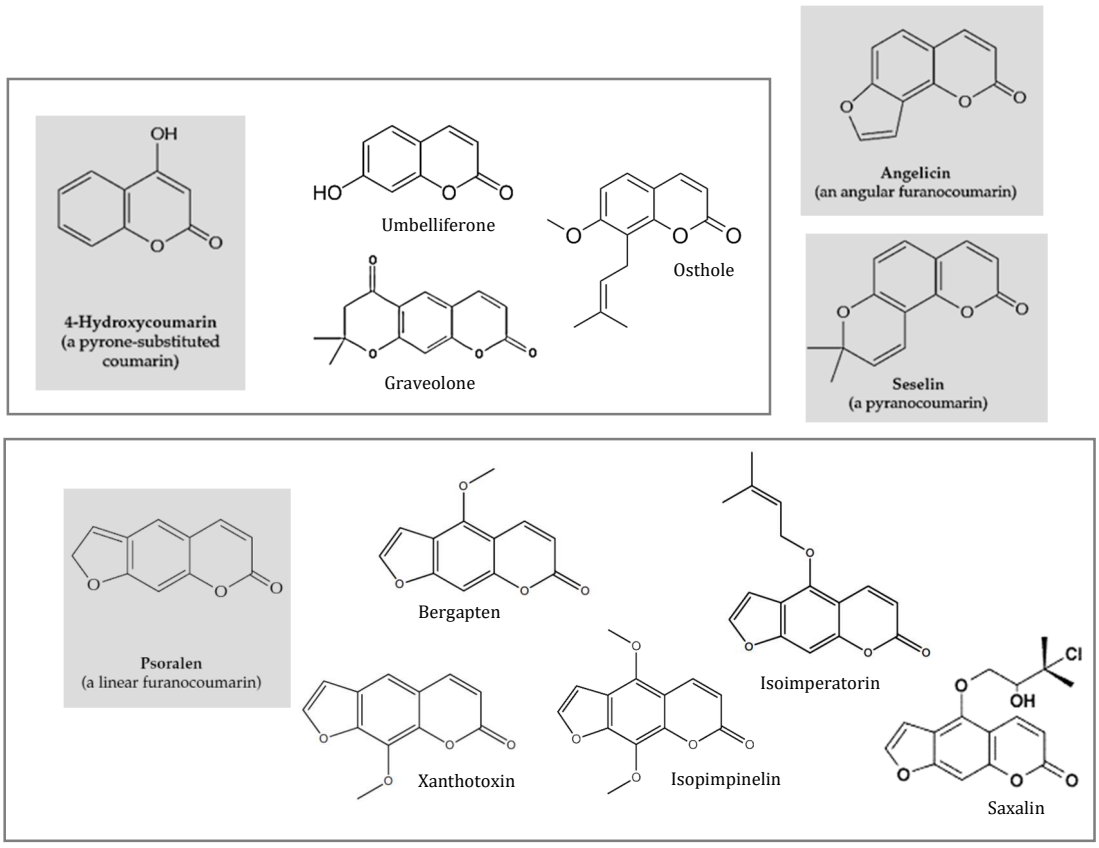


Figure I.9. Type of bioactive coumarins found in Apiaceae species showing allelopathic and/or pesticidal activity.

Coumarins are considered phytoalexins that are produced by several Apiaceae species as a constitutive as well as an inducible mechanism, conferring to plants an adaptive flexibility to their chemical defense. Linear furanocoumarins are often phototoxic to generalist insects but a metabolic adaptation to furanocoumarins is widespread among umbellifer specialists, namely caterpillars that feed on furanocoumarin-containing plants (Umbelliferae and Rutaceae) (Berenbaum, 1990). Moreover, linear furanocoumarins are potent photosensitizing toxins that may cause photophytodermitis on UV-exposed skin. Under UV light, linear furanocoumarins are able to intercalate with DNA or RNA through covalent binding with pyrimidine bases (formation of adducts), thereby inhibiting replication and transcription (Beier & Nigg, 2001).

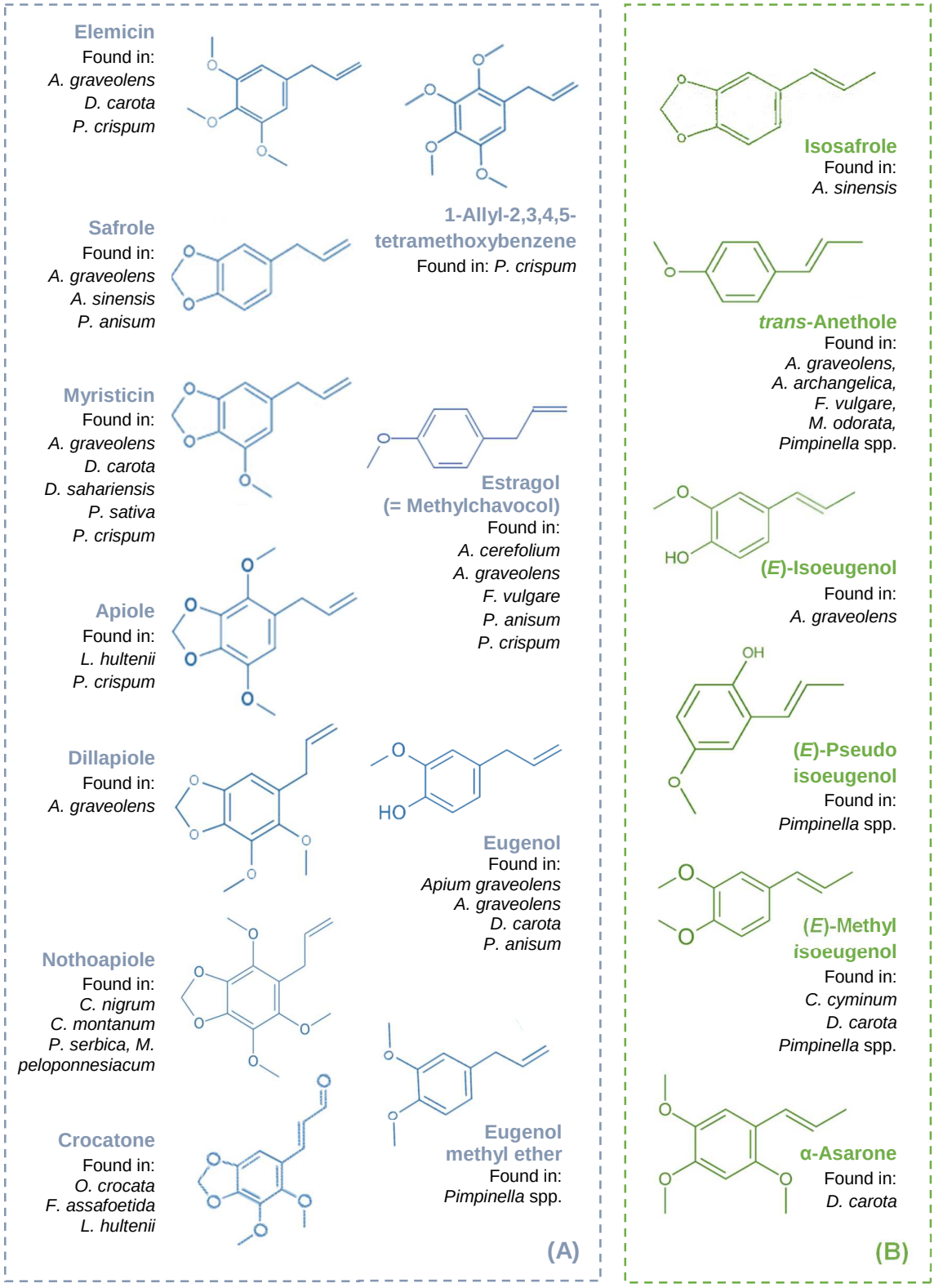


Figure I.10. Examples of volatile bioactive phenylpropene-derivatives **(A)** allylbenzenes and **(B)** propenylbenzenes, and their occurrence in Apiaceae species.

[Source: Baser et al. (2007); Dr. Duke's Phytochemical and Ethnobotanical Databases, https://phytochem.nal.usda.gov/phytochem/search, accessed May 2016].

Natural phtalides produced by Apiaceae species are another important class of referenced compounds (Fig. I.11) that exhibit one or more pesticidal activities, namely acaricidal (Kwon & Ahn, 2002), insecticidal (Momin et al., 2000; Miyazawa et al.,2004; Tsukamoto et al., 2005), antifungal (Momin et al., 2000) and herbicidal (Meepagala et al., 2005). (Z)-Butylidenephthalide extracted from *Cnidium officinale* and *Angelica acutiloba* were reported for their potent acaricidal (Kwon & Ahn, 2002) and insecticidal activities against *Drosophila melanogaster* (LD_{50}=0.84 *µ*g per adult) (Miyazawa et al., 2004). Moreover, the butylphthalides senkyunolides A, N and J isolated from *Apium graveolens* were found to possess nematicidal activity (Momin et al., 2000; Momin & Nair, 2001). The *isomers Z*- and *E*- of ligustilide are widely occurring in *Ligusticum* and *Lomatium* spp. (Meepagala et al., 2005) with *L. californicum* being one of the most importante sources of *Z*-ligustilide (97 mg g^{-1} of dry seed) (Meepagala et al., 2005). On the other hand, all parts of celery *(A. graveolens* var. *dulce*) could be considered as good sources of (*Z*)-3-butylidenephthalide and 3-butyl-4,5-dihydrophthalide (Sellami et al., 2012). Phtalides such as butylidenephthalide and 3-butylphthalide are the main component of chunkung (*Cnidium officinale*) EO (Choi et al., 2002) (Fig. I.11).

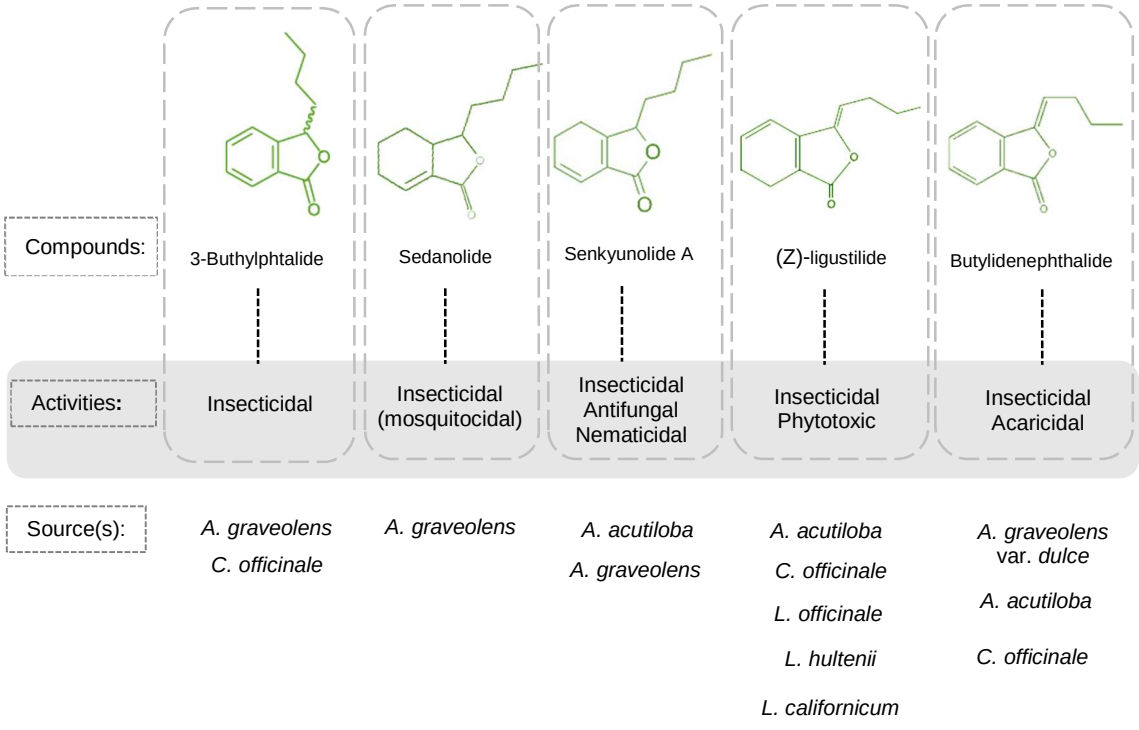


Figure I.11. Examples of naturally occurring volatile phtalides found in Apiaceae species and showing activity against pest species.

5. The research work plan

5.1. The selected plant species: *Anethum graveolens* L., *Cuminum cyminum* L., *Foeniculum vulgare* Mill., *Petroselinum crispum* (Mill.) Nym. ex A.W. Hill (Apiaceae)

The four Apiaceae species contemplated in the present study are amongst the industrial crops with greater economical importance (Table I.6). All of them are ascribed to the subfamily Apioideae (Table I.11). *Cuminum cyminum* belongs to the Scandiceae tribe (Downie et al., 2010), whereas the remaining three species, *A. graveolens, F. vulgare* and *P. crispum*, are included within the Apieae clade (Downie et al., 2001; Downie et al., 2010). In the former classification (Pimenov & Leonov, 1993) the genus *Anethum* was compiled in a separated tribe (Peucedaneae). Supplementary information concerning each plant is organized in individual fact sheets (Appendices A pages 282-285).

Table I.11. Taxonomic classification of the four studied plant species.

Taxon	Taxonomic hierarchy			
Kingdom	Plantae			
Superdivision	Spermatophyta			
Division	Magnoliophyta			
Subdivision	Spermatophytina			
Class	Magnoliopsida			
Subclass	Dipsapiidae D.C. Tank & M.J. Donoghue [a]			
Order	Apiales Nakai			
Suborder	Apiineae Plunkett & Lowry			
Family	Apiaceae Lindl.			
Subfamily	Apioideae Drude			
Clade	Apieae Takht. ex V.M. Vinogr. [b]	Daucinae Dumort. [c]	Apieae Takht. ex V.M. Vinogr. [b]	Apieae Takht. ex V.M. Vinogr. [b]
Genus	*Anethum* L.	*Cuminum* L.	*Foeniculum* Mill.	*Petroselinum* Hill
Species	***A. graveolens***	***C. cyminum***	***F. vulgare***	***P. crispum***
Species authority	Linnaeus	Linnaeus	Miller	(Miller) Nyman ex A. W. Hill
Common name	Dill[24]	Cumin[25]	Fennel[26]	Parsley[27]
Similar species	*Foeniculum vulgare*	*Carum carvi*	*A. graveolens* *Ridolfia segetum*	*Aethusa cynapium*

[*] Suprageneric composition within subfamily *Apioideae* established based on phylogenetic analyses of molecular data.
[a] Tank & Dononghue (2010); [b] Downie et al. (2000, 2001, 2010); [c] Downie et al. (2010).

[24] A variant called sowa occurs in India (Jana & Shekhawat, 2010). It is frequently referred as the Indian dill in opposition to the so-called European dill. Experts disagree on whether or not *A. sowa* can be considered as its own species, or as sub-species.
[25] Should not be confounded with *Bunium bulbocastanum* (Apiaceae) or *Nigella sativa* (Ranunculaceae), both frequently called black cumin.
[26] The monospecific genus *Foeniculum* comprises two sub-species: subsp. *vulgare* or *capillaceum* and *piperitum.* The subsp. *vulgare* is subdivided in three varieties: var. *vulgare*, var. *dulcis* and var. *azoricum*.
[27] According to some authors (Petropoulos et al., 2004), parsley is subdivided in three subspecies, while others distinguish three varieties (var. *neapolitanum*, var. *crispum* and var. *tuberosum*) (Charles, 2012).

5.2. The target-animal species: *Anopheles atroparvus* van Thiel *(*Culicidae*), Meloidogyne javanica* (Treub) Chitwood (Meloidogynidae*), Pseudaletia unipuncta* Haworth (Noctuidae) and *Radix peregra* Müller (Lymnaeidae)

The target-animal species used for the toxicological assays (Table I.12) were chosen in virtue of their biological and economical significance in the agricultural and public health contexts. Both ***Pseudaletia* (*Mythimna*) *unipuncta*** and ***Meloidogyne javanica*** have a worldwide distribution[28] and are major concerns in crops production systems. On the other hand, ***Anopheles atroparvus*** (mosquito) and ***Radix peregra*** are present in temperate zones, namely in Europe[29], and although more localized geographically, they also exhibit potential impacts as it will be discussed below.

While progressing through the larval stages (six larval instars), *P. unipuncta* can cause important economic losses in small grains, maize and grasses (http://www.cabi.org/isc/datasheet/45094). Herein, an in-depth study was undertaken with this lepidopteran **specialist** classified among the most voracious and **phytophagous** pests.

M. javanica is indicated as one of the top 10 most damaging plant-parasitic nematodes, and the 2nd most importante of the genus, after *M. incognita.* Infective second-stage juveniles of *M. javanica* penetrate into host roots causing root swelling and galls formation. Moreover, the interaction of this root-knot nematode with other pathogens may occasion a disease complex with severe symptoms (http://www.cabi.org/isc/datasheet/33246).

Classified as a key vector of malaria in the European continent, ***A. atroparvus*** (a member of the *Maculipennis* complex) have been formerly implicated in Malaria transmission during the 20th century (Jetten & Takken, 1994). Nowadays, although the disease is no longer endemic, the continuous occurrence of imported cases and the possibility of its transmission by this competent vector presently maintain the risk of future endemic infections (Capinha, 2009).

On the other hand, the freshwater snail ***Radix peregra*** has been identified an alternative intermediary host of livestock fascioliasis (and other food-borne diseases) in some European countries. The prevalence of alternative hosts, namely the snail *R. peregra,* generates an opportunity for the liverfluke parasite to survive under natural condition (Dreyfuss et al., 2000).

Even though the prevalence of both species could be considered to have a low-moderate impact at present, the predictable changes in the habitat and climate in the 21st century could generate suitable conditions for these species to proliferate strengthening the risk of transmission of

[28] ***P. unipuncta*** is a strongly migratory species (originally a neotropical species) and has been known in Europe since the 19th century. With the exception of southern France and the Azores where populations are present all year round, in Europe *P. unipuncta* occurs as a migrant establishing temporary population under favorable conditions (e.g. rising temperatures) (http://www.cabi.org/isc/datasheet/45094).

M. javanica is widely distributed in warm and tropical climates where it is often the dominant root-knot species. The geographic range includes Africa, Australia, South America, Asia, the USA and greenhouses in Europe (http://www.cabi.org/isc/datasheet/33246).

[29] A predictive map showing the probability of occurrence of ***A. atroparvus*** in Europe was provided by Sinka et al. (2010), being available at: http://www.map.ox.ac.uk/browse-resources/vector-occurrence/atroparvus/world/.

Whole Europe is inhabitated by ***R. peregra***, with a frequent distribution in Britain, France and Spain (Bargues et al., 2001).

both diseases in Europe (remark: vector density is only one of many variables). Based on these assumptions, we found pertinent to explore alternative biochemical forms of control for both species in view of their application in foreseeable scenarios.

Lastly, we considered that these 4 animal species, belonging to distinct taxa, could be of great utility for a wide range survey on the value of Apiaceae EOs as potential pest management agents. Each one of the targeted species will be presented and appraised with more details in the following chapters.

Table I.12. Taxonomic classification of the tested animal species.

Taxon	Taxonomic hierarchy			
Kingdom	Animalia			
Superphylum	Ecdysozoa			Lophozoa
Phylum	Arthropoda		Nematoda	Mollusca
Subphylum	Hexapoda		-	-
Class	Insecta		Secernentea	Gastropoda
Subclass	Pterigota		-	-
Order	Lepidoptera	Diptera	Tylenchida	Basommatophora
Superfamily	Noctuidea Latreille	-	-	Lymnaeoidea Rafinesque
Family	Noctuidae Latreille	Culicidae	Meloidogynidae	Lymnaeidae Rafinesque
Subfamily	Noctuinae Latreille	Anophelinae Grassi	-	-
Genus	*Pseudaletia* Franclemont (sin *Mythimna* Ochsenheirmer)	*Anopheles* Meigen	*Meloidogyne* Goeldi	*Radix* Montfort (sin *Lymnaea* Lamarck)
Species	*Pseudaletia unipuncta* [a]	*Anopheles atroparvus* [b]	*Meloidogyne javanica* [a]	*Radix peregra* [a]
Species authority	Haworth, 1809	van Thiel, 1927	(Treub, 1885) Chitwood, 1949	Müller, 1774

[a] Authoritative taxonomic information retrieved from the Integrated Taxonomic Information System (ITIS): http://www.itis.gov/
[b] Harbach R.E. 2013. Anopheles classification. Mosquito Taxonomic Inventory, http://mosquito-taxonomic-inventory.info/simpletaxonomy/term/6048, accessed April 2016.

5.3. Context, aims and relevance of the study

Enclosed in the scientific domain of agricultural sciences, the present work intends to add value to a selection of Apiaceae species as a source of biochemicals with biopesticidal properties, specifically EOs and volatile compounds. The research is focused exclusively on worldwide traded and well-known Apiaceae species that do possess the GRAS status (Generally Recognized as Safe) and/or being listed as a source of GRAS ingredients[30]. Conscious that natural chemical variation is a matter of great concern if extracts of a given plant species are to be used for pest management

[30] EOs from the four studied species are generally regarded as having a 'safe' regulatory status, and their use as food flavoring ingredients been approved by the FDA. The GRAS regulatory status has been accorded by the FEMA (Flavor & Extract Manufacturers association) to fennel and sweet fennel EOs (GRAS 2481 and 2483); to cumin and its EO (GRAS 2340 and 2343); to dill, dill EO and dill seeds (GRAS 2382, 2383, 2384); to parsley, parsley EO and parsley oleoresin (GRAS 2835,2836, 2837) (FEMA reference numbers found in: Hall & Oser, 1965).

(Isman & Grieneisen, 2013), we intended to address that question by considering the collection of several plant materials and the testing of more that one extract for each species.

In addition to a contribution to the current knowledge and expertise in the domain of biochemical-based biocontrol, this research project was conceived in the perspective to ascertain the biopesticidal potential of the selected four economically important crops and substantiate their use in integrated pests and vectors management, as major goals.

The current scenario of pest and vectors management is characterized by a changing regulatory framework with growing severe restraints to the use of synthetic pesticides, and an emergent awareness/ agreement of consumers to environmentally friendly solutions. In view of such scenario, the present study was planned taking into consideration the significant evolution of the worldwide biopesticides market in the recent years, and the prospects of a growing demand for new products in European for the upcoming years.

The Portuguese industrial sector in the domain of the added-value products from medicinal and aromatic plants (MAP) is deeply underdeveloped and directly related to severe scientific knowledge constraints, lack of technology, unwise entrepreneurship and short investment in this area of the bio-industry. Therefore, we consider also this research as a sound starting point for the conception of MAP fragrances-based products with high impact, finding marked relevance in both plant protection and health protection schemes.

To reach the major goals above highlighted, we outlined specific objectives organized in a two-phase research plan:

Phase 1 | Phytochemical study of fragrances produced *in vivo* by the selected Apiaceae species

(a) Chemical characterization of EOs produced by *A. graveolens*, *C. cyminum*, *F. vulgare* var. *vulgare* and *P. crispum;*
(b) Relating bio-production of EOs constituents with plant developmental stages, tissues and/or origin.

Phase 2 | Evaluation of Apiaceae EOs and volatile compounds activities against several targeted animal organisms

(c) Evaluation of insecticidal activities on *A. atroparvus* and *P. unipuncta;*
(d) Evaluation of sublethal effects on *P. unipuncta;*
(e) Evaluation of the molluscicidal activities on *R. peregra*;
(f) Evaluation of the nematicidal activity on *M. javanica*.

5.4. An overview of the research work

The following pages will provide a preview of the research performed and the topics focused of each chapter (from II to VI), in order to briefly introduce the major outcomes of each investigation.

CHAPTER II – Highlights



Anethum graveolens
Dill



Foeniculum vulgare* var. *vulgare
Bitter fennel



Petroselinum crispum
Parsley

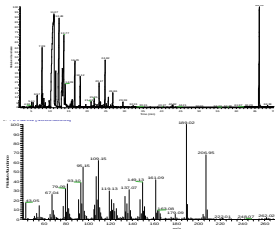


Cuminum cyminum
Cumin



Chemical analyses:

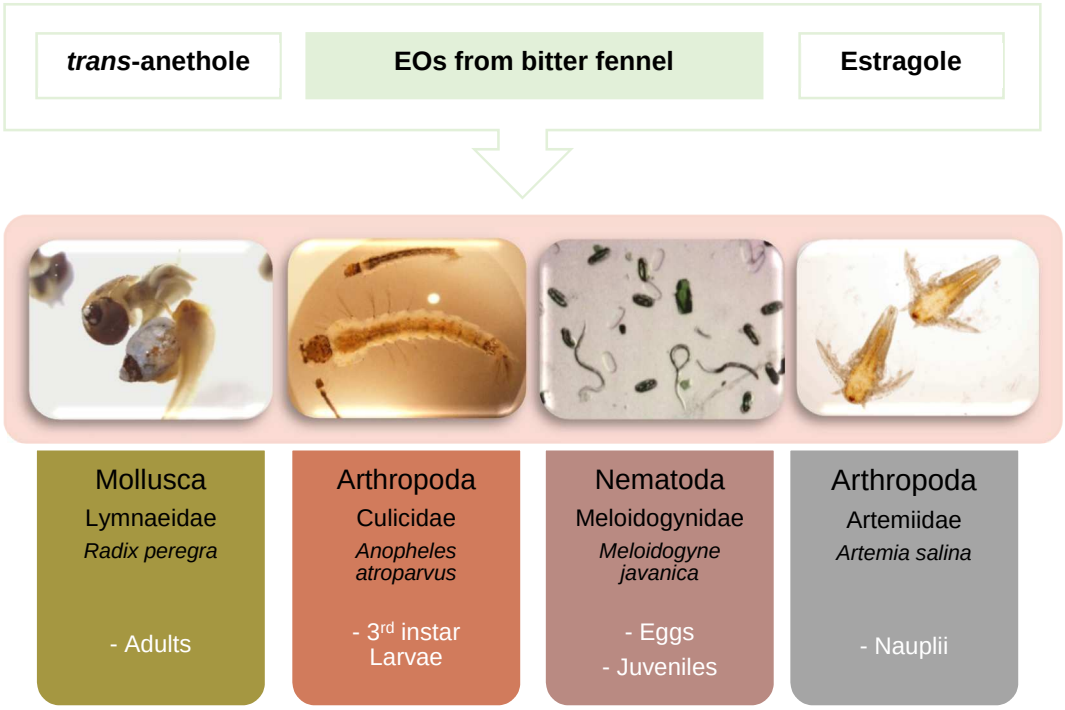
✓ Gas Chromatography (GC-FID)
✓ GC coupled to Mass Spectrometry (GC-MS)



***A. graveolens* EO**	***F. vulgare* var. *vulgare* EO**	***P. crispum* EO**	***C. cyminum* EO**
Carvone	Estragole	Myristicin	Cuminaldehyde
β-Phellandrene	Fenchone	Apiole	γ-Terpinene
α-Phellandrene	α-Phellandrene	β-Pinene	β-Pinene
Dill ether	α-Pinene	α-Pinene	*p*-cymene

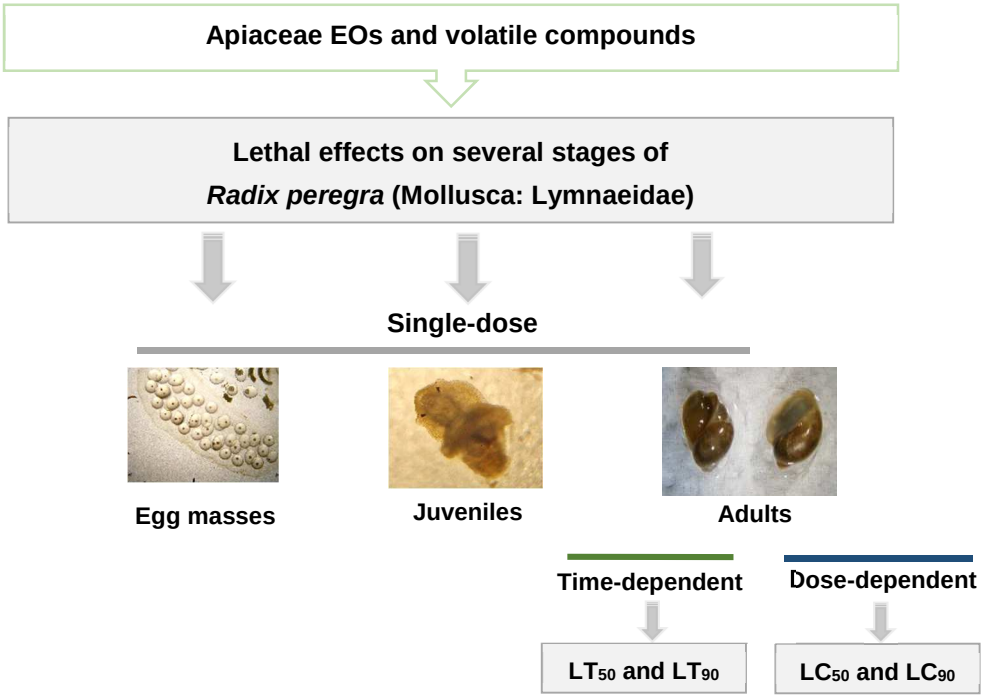
- EOs accumulated by *A. graveolens*, *F. vulgare* and *P. crispum* plants were characterized at different developmental stages and/or tissues.
- Dill and parsley plants showed intra-individual variations of their EO composition.
- An intraspecific variation of EOs composition was found among bitter fennel plants.
- Cumin EOs composition varied according to the origin of the plant material.

CHAPTER III – Highlights



- We evaluated the potential of bitter fennel as a productive natural source of biopesticides.
- Bitter fennel EOs showed satisfactory molluscicidal and mosquitocidal activities and moderate toxicity to *A. salina* (model-organism).
- Bitter fennel EO was poorly active against eggs and juveniles of the root-knot nematode.
- EOs from this perennial fennel variety could be advantageous for local pest management programs.
- *trans*-Anethole could be a good alternative for the control of freshwater snails, mosquitoes and plant-parasitic nematodes.

CHAPTER IV – Highlights



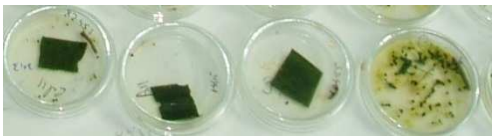
- Bitter fennel, parsley, cumin and dill EOs are highly toxic against snails.
- Estimated 48 h-LC_{50} of EOs and compounds ranged from 13.7 to 46.5 mg L^{-1}.
- Parsley fruit EO exhibited the most significant dose-effectiveness of the assayed EOs against adults.
- A short-time exposure (8 h) to bitter fennel EOs was effective for snails control.
- Apiaceae EOs and compounds have great potential as biomolluscicides.

CHAPTER V–Highlights

Apiaceae EOs and volatile compounds

Pseudaletia unipuncta (Lepidoptera: Noctuidae)

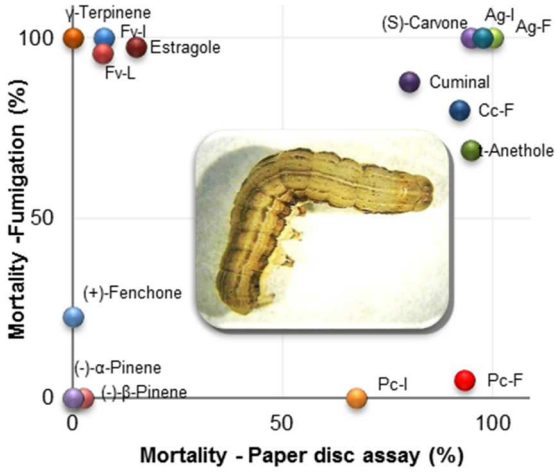
Feeding deterrence/ growth inhibition



$$FDI\ (\%) = 100\ \frac{(\text{Diet consumed in the control} - \text{Diet consumed in the treatment})}{\text{Diet consumed in the control}}$$

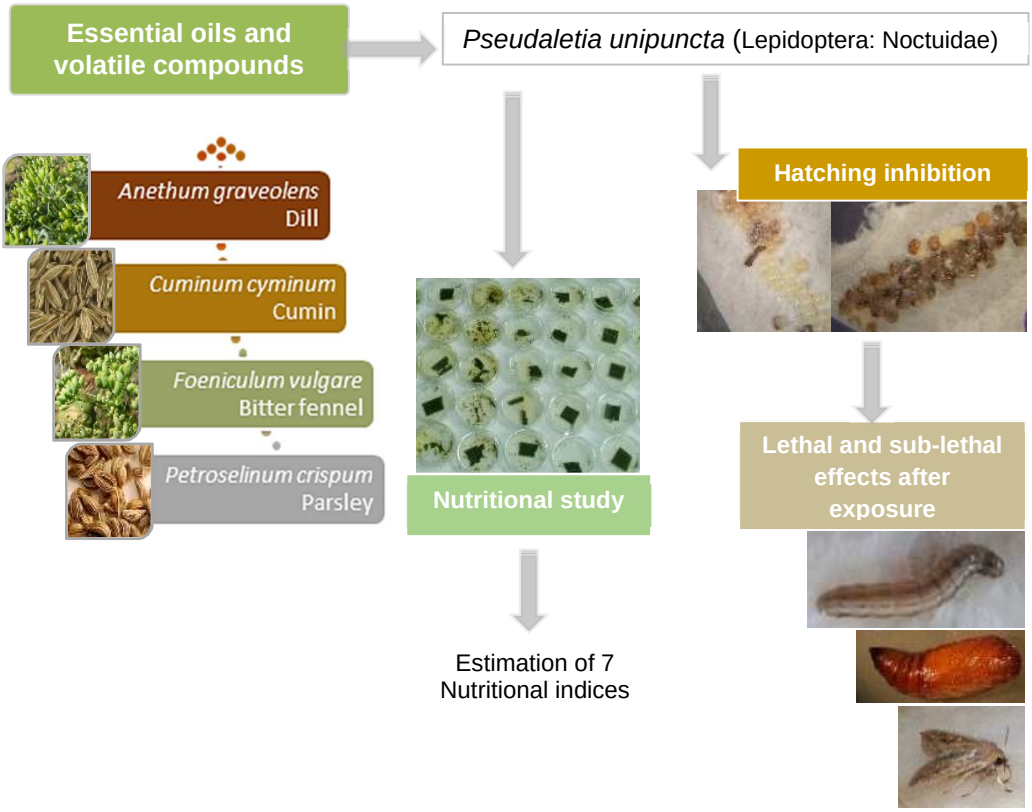
$$GI(\%) = 100\ \frac{(\text{Weight gain in the control} - \text{Weight gain in the treatment})}{\text{Weight gain in the control}}$$

Lethality



- Dill, cumin and parsley EOs were effective against *P. unipuncta* larvae.
- Anti-insect activity varied according to plant species/composition, type and exposure period.
- Dill, cumin and fennel EOs and some of their constituents were the most lethal by fumigation (≥ 80%).
- *A. graveolens* EO and (S)-carvone were considered both dose- and time-effective by contact.
- Parsley fruit EO and *trans*-anethole also showed promising contact toxicity and antifeedant action.

CHAPTER VI–Highlights



- EOs and compounds from the four Apiaceae species influenced *P. unipuncta* development and nutrition.
- Dill and cumin EOs, carvone and cuminaldehyde, disrupted embryogenesis.
- Parsley EOs, and their major compounds, reduced eggs hatchability.
- *trans*-Anethole and cuminaldehyde inhibited the assimilation/conversion of food to biomass.
- Parsley fruit EO showed ovicidal, larvicidal, feeding/growth inhibitory activities.

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CHAPTER II

EOs from Apiaceae species -
A phytochemical study of *Anethum graveolens*, *Cuminum cyminum*,
Foeniculum vulgare and *Petroselinum crispum*

The majority of results presented and discussed in this chapter was included in four publications and two poster communications listed in page xxix.

1. Abstract

The Apiaceae family comprised important plants species, which are used as edible crops (vegetables, herbs and spices), being also sources of various bioactive compounds. Essential oil (EO) components encountered in Apiaceae are very diverse and mainly distributed by monoterpenes, sesquiterpenes, rarely diterpenes, phenylpropanoids, phthalides, octanol and octyl esters, trimethylbenzaldehydes, aliphatic aldehydes (Chizzola, 2010) and organosulfur compounds (Kanani et al., 2011).

This chapter describes the analytical study of the EOs extracted from one or more organs of the following well-known Apiaceae species: ***Anethum graveolens*** (dill), ***Foeniculum vulgare* var. *vulgare*** (bitter fennel), ***Petroselinum crispum* var. *neapolitanum*** (parsley) and ***Cuminum cyminum*** (cumin). In addition, a more comprehensive appraisal of the bitter fennel agronomic potential based on growth parameters and EO production is here presented.

EOs were isolated by hidrodistillation from plant parts/whole plants cultivated *in vivo* and from cumin fruits of different origins. EOs contents were determined whenever possible. In addition, two EOs were purchased and included in the study. The identification of the EOs constituents was performed by gas chromatography/mass spectrometry (GC-MS), by comparison of the respective mass spectra and Kovats retention indices (KI) with those of authentic standards or those of the data base libraries. The quantification of EOs constituents was performed by gas chromatography (GC-FID).

EOs contents (0.10-3.45% w/w) and chemical profiles were highly variable among species. The accumulation of (*S*)-carvone (4 to 61% of total EO content) and the significant decrease of α-phellandrene and dill ether were the most evident changes of dill umbels EOs, from the flowering to the ripe fruit stage. Leaves more stems and infrutescences of bitter fennel were characterized by very high contents of estragole (28 and 64%), followed by α-phellandrene, α-pinene and fenchone. Moreover, some bitter fennel plants presented estragole-dominant profiles (49-54%), whereas other ones accumulated larger amounts of the isomer *trans*-anethole (47-55%). Parsley EOs were extremely rich in hydrocarbon monoterpenes and phenylpropene-derivatives. Allyl-tetramethoxybenzene was a distinctive compound found in parsley infrutescences and fruits, while other ones namely, isopropenyl-4-methylbenzene, *p*-α-dimethylstyrene and 1,3,8-*p*-menthatriene were mostly accumulated in parsley leaves. Cumin fruits EOs showed similar qualitative profiles containing cuminaldehyde as a main constituent. However, the relative content of the several monoterpenes hydrocarbons and the isomers α- and γ-terpinen-7-al were distinct among cumin EOs.

The type and developmental phase of the organs as well as the geographic origin of the plant material had a strong influence on EOs profiles.

Keywords: Bitter fennel, chemical composition variability, cumin, dill, ontogeny, parsley, phenylpropene-derivatives.

2. Introduction

As any other familly of aromatic plant, the Apiaceae members present an appreciable content of monoterpenoids and sesquiterpenoids. Monoterpenes such as pinenes, phellandrenes, are frequently found amongst the major compounds in many species EOs, most often in combination with other monoterpenes (i.e. sabinene, limonene, γ-terpinene) (Table I.8). Though, even within this class, we found numerous reports of uncommon natural-occurring compounds (such as those of Kubeczka, 1982; Fraga et al., 1986; Nitz et al., 1989; Spraul et al., 1992; Saad et al., 1995; Mazzoni et al., 1999; Chen et al., 2000; Cool et al., 2010; Drew et al., 2012). Several guaiane-type sesquiterpene hydrocarbons were identified in *Peucedanum* species (Tesso et al., 2006), while the daucane (carotane) class of sesquiterpenes have been isolated or newly characterized from several species (Ghisalberti, 1994), as for instance *P. crispum* (Spraul et al., 1992; Appendino et al., 1998) and *D. carota* (Mazzoni et al., 1999; Ahmed et al., 2005). Chemical studies of *Ferula* species showed the widespread occurrence of a variety of sesquiterpene compounds (daucanes, humulanes, himachalanes, germacranes, eudesmanes, and guainanes) (González & Barrera, 1995), in addition to characteristic mono- and sesquiterpene coumarins (terpene backbone linked to a coumarin moiety) (El-Razek et al., 2001 and 2003). Sesquiterpene coumarins were also found to occur in other genus of the family, namely *Heptaptera*, *Heraclum*, *Peucedanum*, *Angelica* (Gliszczyńska & Brodelius, 2012). Moreover, the prenyl-furocoumarin-type has been found in *Smyrnium olusatrum* (an edible vegetable commonly known as wild celery or Alexanders), where the furanosesquiterpenoid fraction may constitute 36-50% of the root, leaves and unripe fruits EOs (Mölleken et al., 1998) (Table I.8), or occasionally more (54-76% of the total content) (Maggi et al., 2012).

Studies covering Apiaceae EOs have established that numerous species present high contents of volatile phenylpropanoids (Table I.8 and Fig. I.10). Phenylpropanoids such myristicin, apiole, and dill apiole are typically encountered in root EOs, although their location is not limited to this organ. The occurrence and emission of phenylpropanoids by Apiaceae fruits, for example *trans*-methylisoeugenol, methyleugenol and β-asarone from the carrot fruit (Saad et al., 1995), has revealed the relevant role of these closely related compounds in insect-plant interactions (Guerin et al., 1983). Besides, several phenylpropanoid compounds have been described as exlusive of the family (Kubecza et al., 1989), or genus specific, as for instance those indentified in *Pimpinella* species (Reichling et al., 1991; Başer et al., 2007).

Natural phtalid, and their corresponding derivatives, are another important class of compounds regularly found in EOs of approximately 23 plant families including Apiaceae (Beck & Chou, 2007; Lin et al., 2011). The family have afforded the greatest number of phthalides (around 71 volatile lactones) which have been characterized from 40 diferent sources (Beck & Chou, 2007). The most referenced bioactive natural phtalides (Fig. I.11) were identified from species included in the genera *Ligusticum* (12 species) and *Angelica* (9 species). Others phtalides-enriched species belong to *Apium*, *Cnidium, Levisticum* and *Petroselinum* genus (Lin et al., 2011). In *A. graveolens*

var. dulce EOs, these compounds may represent the major part of the EO extracted from leaves (75%), stalks (57%) and roots (58%), respectively (Sellami et al., 2012).

Moreover, Apiaceae EOs also contain other volatile compounds such as alkanes (Kanani et al., 2011; Bendiabdellah et al., 2012), aliphatic esters (Radjabian et al., 2014), C_{17} polyacetylenes (Asuming et al., 2005; Thiem et al., 2011), sulfur- (Kanani et al., 2011) and nitrogen-containing constituents (Evergetis et al., 2012), with some of them being associated to particular genera (Table I.8).

As suggested by Kapetanos et al. (2008) the analysis of EOs composition could be helpful to provide insight into the biosystematics relationships and substantiated some of the currently accepted taxonomic relationships withing the family. Several classes of compounds have been used as reliable marker to discriminate Apiaceae species at different taxonomic levels (Smitt, 1995; Asuming et al., 2005; Kapetanos et al., 2008; Kanani et al., 2011; Radjabian et al., 2014). The presence of non-terpene aliphatic esters and organosulfur compounds, have found interesting application as chemotaxonomic markers of *Ferula* and *Heracleum* genera, respectively (Kanani et al., 2011; Radjabian et al., 2014). Likewise, Tabanca and co-workers (2005) have analysed the occurrence pattern of some sesquiterpenes and phenylpropanoid derivatives in 19 *Pimpinella* species and discussed their chemotaxonomic significance.

2.1. Aim of the study

In this study we have focused our attention on the EOs production and accumulation by *in vivo* plants of *A. graveolens*, *F. vulgare* var. *vulgare* and *P. crispum* and their phytochemical variations. In addition, the bitter fennel agronomic potential was evaluated based on several growth parameter and on the EO profile of individual plants. Furthermore, the composition of *C. cyminum* EOs were also determined for fruits samples of different origins. Accordingly, the EO content and the chemical variability occurring among tissues and/or during different developmental stages are here presented and discussed.

3. Materials and methods

3.1. Plant material

3.1.1. *Anethum graveolens*

Seed of *A. graveolens* (dill) were purchased (Somelite® plus, N.V. Somers, Co.) for cultures establishment. Soaked seeds were sown in moist sterile paper and left inside glass Petri dishes during seven to 10 days for germination (22 ºC in the dark). Subsequently, seedlings were transferred to small plastic cups containing a mixture of loamy soil and sand (2:1) and maintained under controlled temperature and light (25 ± 2 ºC; 12 h photoperiod). Four to eight-weeks-old young plants were transplanted to definitive soil (30 cm apart in 40 cm spaced rows) and cultivated in an open-air experimental field from July to November 2010[1] (Fig. II.1), without the use of synthetic fertilizers or pesticides. Irrigation was provided daily in the first weeks and every week afterwards. The cultivation and sampling scheme for two groups of plants grown in separated periods are shown in table II.1.

Table II.1. *Anethum graveolens* cultivation: growing phases and sampling.

Time	April /10	May	June	July	Aug	Sep	Oct	Nov /10	Total [b] (Days)
Culture 1 [a]	22					6			150
Sampling after 136 days of development:									
Stems, leaves, green infrutescences and roots									
Culture 2 [a]			16			20	18	30	10	146
Sampling at different phenological phases (Fig. II.1):									
Pre-flowering umbels, flowering umbels (anthesis), green infrutescences [e], umbels with maturing fruits [f], Mature fruits [g].									

Seedling emergence
Plantlets growth [c]
Vegetative growth [d]
Pre-flowering [d]
Flowering [d]
Fruits ripening [d]
Mature fruits [d]

[a] Numbers indicate the day of sowing or sampling; [b] Time of plants' development from germination to seeds maturation; [c] Development in culture room with controlled temperature and light; [d] Development in an open-air field trial; [e] With fruits in a pre-ripening stage; [f] With fully grown ripening fruits; [g] Fruits reached their maturity approximately 45 days after the anthesis. These were collected and stored for 6 months before EO extraction.

[1] Supplementary information concerning the field location and the climatic conditions during the cultivation period is provided in tables B.1 and B.2 of the Appendix B.



Figure II.1 (A-H). Cultivation of *Anethum graveolens* (dill) from April to October 2010.

(A) 72-Day-old plantlets grown in a culture room with controlled temperatures and photoperiod. **(B)** Recently transplanted plants previously grown in controlled condition for the period of 84 days. **(C** and **D)** 125-Day-old flowering plant showing several umbels. **(E)** Detail of a pre-flowering umbel. **(F)** Detail of an inflorescence (flowering primary umbel) with a specialist insect, the swallowtail caterpillar (*Papilio* sp.). **(G)** Green infrutescences, primary and secondary umbels, after 136 days of growth. **(H)** Detail of an umbellule with fully formed schizocarpic fruits with oval and flattened shape (ripening phase).

3.1.2. *Foeniculum vulgare* var. *vulgare*

Dry fruits of *F. vulgare* var. *vulgare* containing mature seeds were collected in Braga (north of Portugal) (41º36´03.8´´N, 8º26´50.25´´W) from a wild population. The varietal identification was based on morphochemical characteristics. Voucher specimens (plant with inflorescences and dry fruits) were deposited at the University of Porto (Portugal) herbarium under the accession number PO1000MFF. Fruits were soaked and sown in moist sterile paper for germination (approx. four weeks at 22 ºC in the dark). After eight weeks of growth in a culture room (25 ± 2 ºC; 12 h photoperiod) in a mixture of loamy soil and sand (2:1), young plants were transplanted to a 7 × 5 m^2 plot, 50 cm apart. Two sets of plants were grown without fertilizers or pesticides, from February 2010 to September 2011, as described in the cultivation scheme (Table II.2). Irrigation was provided daily in the first weeks and afterwards whenever necessary.

Fresh stems with leaves and green infrutescences (premature fruits) were collected from five and eight to nine-month-old plants, respectively (Fig. II.2). A second set of plants from culture 2 (*n*=10) was harvested in 2011 during the fruit maturing stage after 15.5 months of development for an evaluation of some agronomic parameters (Table II.3). The whole plants were air-dried in a cool and shaded location for seven days and processed individually for dry weights determination and EO extraction.

Table II.2. *Foeniculum vulgare* var. *vulgare* cultivation: growing phases and sampling.

Time	Feb /10	Mar	April	May	June	July	Aug	Sep	Oct	Nov /10	Total [b] (Day)
Culture 1 [a]	2					22			12	2	273
Time	May /10	June	July	Aug	Sep	Oct	-	July /11	Aug /11	Sep /11	Total (Day)
Culture 2 [a]	12								6		475

Seedling emergence
Plantlets growth [c]
Vegetative growth [d]
Flowering [d]
Fruits ripening [d]
Mature fruits [d]

[a] Numbers indicate the day of sowing or sampling; [b] Time of plants development from germination to the stage of seeds maturation. [c] Development in culture room with controlled temperature and light. [d] Development in an open-air field trial. Supplementary information concerning the field location and the climatic conditions during the cultivation period is provided in tables B1 and B2 of appendices.

Table II.3. Studied agronomic traits of *Foeniculum vulgare* var. *vulgare* cultivated in a 4 m^2 equivalent field area.

Growth parameters recorded for each plant	- Stems maximum height (cm) - Branches per stem (no.) - Umbels per branches and per stem (no.) - Total fresh weights (g) - Umbels, stems and plants dry weights (g)
Productivity of the culture (per m^2 of cultivated area)	- Plant biomass (on a dry weight basis) - Volume of essential oil (mL)



Figure II.2 (A-H). Cultivation of *Foeniculum vulgare* var. *vulgare* (bitter fennel) from February 2010 to September 2011.

(A) 84-Day-old plantlets ready for transplantation after growing in the controlled environment of the culture room. **(B)** 125 Day-old plants with 40 days of growth in definitive soil. **(C)** Elongation of green vegetative parts, 62 days after transplantation (147-day-old plants). **(D)** Plant at the frutification stage after 187 days (culture 1). **(E)** Plants of culture 2 in a late flowering stage with some infrutescences after 15 months. **(F)** Detail of an inflorescence with flowers in asynchronous anthesis. **(G)** Detail of a green infrutescence (umbel with premature fruits) with a striped shield bug (*Graphosoma lineatum* subsp. *italicum*), a specialist insect (Family Pentatomidae) commonly found in cultivated Apiaceae species. **(H)** Detail of a secondary umbel (umbellet) showing the fully formed schizocarpic and oblong fruits in the ripening stage.

3.1.3. *Petroselinum crispum* var. *neapolitanum*

The culture of *P. crispum* var. *neapolitanum* (Italian giant parsley) was established from purchased seed (N.V. Somers, Co.). After 24 h of soaking, seeds were placed over moist filter paper into glass Petri dishes and left to germinate in the dark (three weeks). Seedlings were grown under controlled temperature and light (25 ± 2 ºC; 12 h photoperiod) into small plastic cups containing a mixture of loamy soil and sand (2:1). Seven weeks later, young plants were transplanted to definitive soil and planted in double rows of 40 cm large. The distance between plants of the same row was 20 cm. Initially irrigation was provided on a daily basis and then regularly during summer.

Leaves and roots were collected five months later, while infrutescences bearing green fruits were sampled from distinct 14-month plants (Table II.4 and Fig. II.3).

Table II.4. *Petroselinum crispum var. neapolitanum* cultivation: growing phases and sampling.

Time	April /10	May	June	July	Aug	Sep	Oct	Nov	-	May /11	June /11	July /11	Total [b] (Day)
Culture 1 [a]	23	10	14					29				27	446

Seedling emergence
Plantlets growth [c]
Vegetative growth [d]
Flowering [d]
Fruits ripening [d]

[a] Time from sowing to sampling; [b] Time of plants development from germination to seeds maturation; [c] Development in culture room with controlled temperature and light; [d] Development in an open-air field trial.
Supplementary information concerning the field location and the climatic conditions during the cultivation period is provided in tables B.1 and B.2 of the Appendix B.

3.1.4. *Cuminum cyminum*

Concerning cumin, we made several attempts to establish a culture of this annual species. The procedures to obtain plantlets (germination and growing) were analogous to those described for the remaining species. However, after four to six weeks of development in a culture room, young plants (four leaves stage and 5-8 cm height) started forming some flowering buds. This early flowering and reduced plant growth have made difficult obtaining a satisfactory amount of biomass and EOs for the subsequent study.

Nevertheless, two small samples of dried cumin fruits were purchased and used for a chemical characterization of the EO. A sample, kindly offered by Doctor Franklin Gregory, was obtained in an Indian traditional market, while the other one, traded as culinary spices by McCormick Co., was obtained in a superstore.

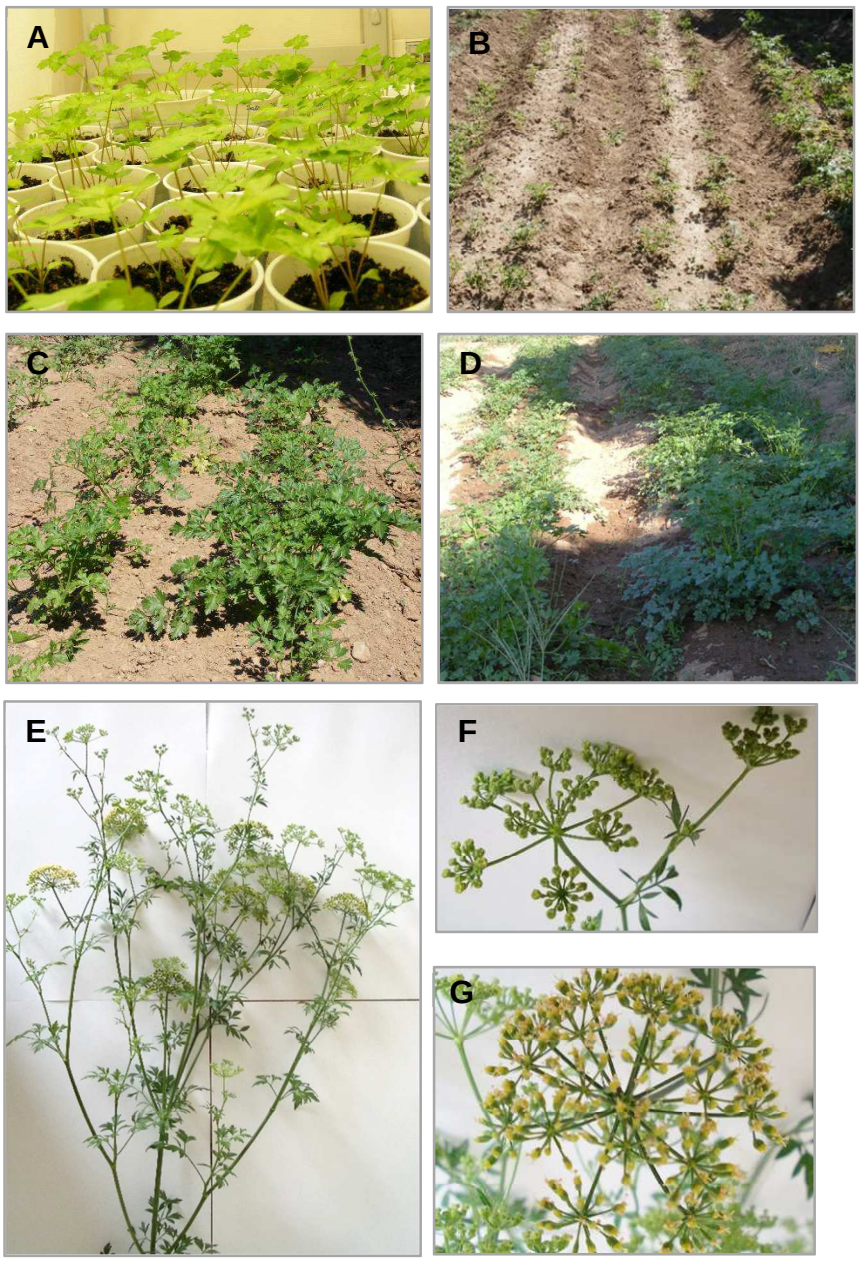


Figure II.3 (A-G). Cultivation of *Petroselinum crispum* var. *neapolitanum* from April to September 2010.

(A) 36-Day-old plants growing in a culture room under controlled temperature and light conditions. **(B)** Young plants growing in definitive soil (30 days after transplantation). **(C)** Plants development after 54 days of cultivation in field conditions. **(D)** 130-Day-old plants with 94 days of cultivation in the field. **(E)** Plants aerial parts with inflorescences and infrutescences harvested after 14 months of development. **(F)** Detail of pre-flowering compound umbels. **(G)** Detail of a young infrutescence.

3.2. EOs extraction

In general, essential oils were isolated from freshly sampled material up to 24 h following collection (excepting the whole bitter fennel plants and the dry fruits of dill and cumin). Plant material was submitted to a 2 h hydrodistillation with 2-3 L of deionized water using a Clevenger-modified apparatus. The recovered EO was separated from the aqueous phase and dried over anhydrous sodium sulphate.

Each plant material was pre-weighted before the hydrodistillation (150-300 g) and its dry weight was extrapolated based on the dry weight contents determined for representative samples conserved at -20 ºC and then submitted to freeze drying (Christ Alpha 2-4 Christ LDC-1m, B. Braun) under 0.025 atm and at -32 ºC for three days.

Whenever possible, EO weight (g) and volume (mL) were determined to estimate EO content per 100 g of dried biomass (% w/w). All EOs were stored in brown sealed vials at -20 ºC until use.

3.3. EOs for biological assays

The EOs availability was a crucial issue of this work. Bearing in mind that considerable quantities would be necessary to perform several bioactivity assessments, and that homogenous and well-defined profiles should be used in order to establish possible relationships between bioactivities and chemical composition, we opted to mix EOs of the same plant and tissue origin. Hence, EOs that had been isolated from identical plant materials were pooled to obtained adequate amounts of EOs with a representative chemical profiles (Table II.5). After this procedure, each mixture was submitted to chemical analysis.

Table II.5. Essential oils from Apiaceae plant species available for chemical analysis and biological activity assessments.

Scientific name	Common name English [a] Portuguese [b]	Origin	Plant parts	EO abbrev.
Foeniculum vulgare var. *vulgare*	Bitter fennel [a] Funcho amargo [b]	Cultivated plants	Stems with leaves Green infrutescences	Fv-L Fv-I
Anethum graveolens	Dill [a] Aneto, endro [b]	Cultivated plants	Stems with leaves Green infrutescences Dried mature fruits	Ag-L Ag-I Ag-F
Petroselinum crispum var. *neapolitanum*	Italian giant parsley [a] Salsa gigante [b]	Cultivated plants	Green infrutescences	Pc-I
Petroselinum crispum	Flat-leaf parsley [a] Salsa [b]	France [c]	Fruits	Pc-F
Cumin cyminum	Cumin [a] Cominho [b]	Iran [c]	Fruits	Cc-F

[a] English common name. [b] Portuguese common name. [c] *Petroselinum crispum* and *Cuminum cyminum* fruits essential oils were purchase from Sigma-Aldrich, Co.

Nevertheless, the quantities of EOs that were obtained from *P. crispum* (parsley) and *C. cyminum* material were scant. As an alternative, we purchased EOs from *P. crispum* and *C. cyminum* fruits (supplied by Sigma-Aldrich Co, St. Louis, MO, USA). Both EO are natural food grade certified products and in compliance with US Food and Drug Administration (FDA) and European Union regulations.

3.4. Fractionation of phenylpropene-derivatives from *P. crispum* EO

The compounds myristicin, apiole, 1-allyl-2,3,4,5-tetramethoxy-benzene and elemicin were separated from *P. crispum* EO by preparative column chromatography in silica gel (Fig. II.4). Approximately 25 g of Merck silica gel 60 of technical grade (particles size of 40-63 µm, porosity 0.71-0.78 mL g^{-1}) was wetted with an apolar solvent (*n*-hexane) prior to its loading in a glass column chromatography. Parsley EO (0.3 or 0.4 g) was diluted in 2 mL of *n*-hexane and applied on top of the wet silica gel column. A mixture of *n*-hexane: ethyl acetate (93:7) was used as eluent. The volume necessary to start the elution of the first compound was slightly superior to the void volume (V_0) [2]. The elution of compound and bands position was monitored from time to time using a small portable lamp emitting UV-light (254 nm). The above-mentioned procedures were repeated 3 times with equivalent amounts of parsley EO (total of 1.4 g of EO).

The composition of the collected fractions (approx. 45 fractions) was monitored by thin layer chromatography (TLC) using Merck silica gel 60_{F254} glassplates (10 × 20 cm) and mobile phases identical to those previously used in column chromatography. Compounds' detection was achieved by observing the TLC plates under UV (254 nm), without any previous revelation step/chemical reaction according to Wagner & Bladt (2009). A purified sample of myristicin (≥ 85%) from parsley leaf essential oil was purchased (Sigma-Aldrich Co.) and used as a standard in the first TLC analysis. Compounds distribution into the stationary phase of the planar chromatography were evaluated by calculating their Retardation factors (R_f):

$$\mathrm{R}f = Migration\ distance\ of\ substances / Migration\ distance\ of\ solvent\ front$$

Fractions containing the co-eluted products myristicin and apiole were recycled and submitted to an additional fractionation step, using a high-purity grade silica gel 60 (particles size of 15-40 µm) and an eluent mixture of *n*-hexane: ethyl acetate (92:8).

In the end, the enriched fractions containing each compound were dried under a nitrogen flux until the complete evaporation of the solvent. The recovered compounds were stored in brown sealed vials at -20 ºC and their purity was appraised by GC analysis.

[2] The void volume of columns was calculated based on columns dimensions according to the equation (**2**) presented in the Appendix C.

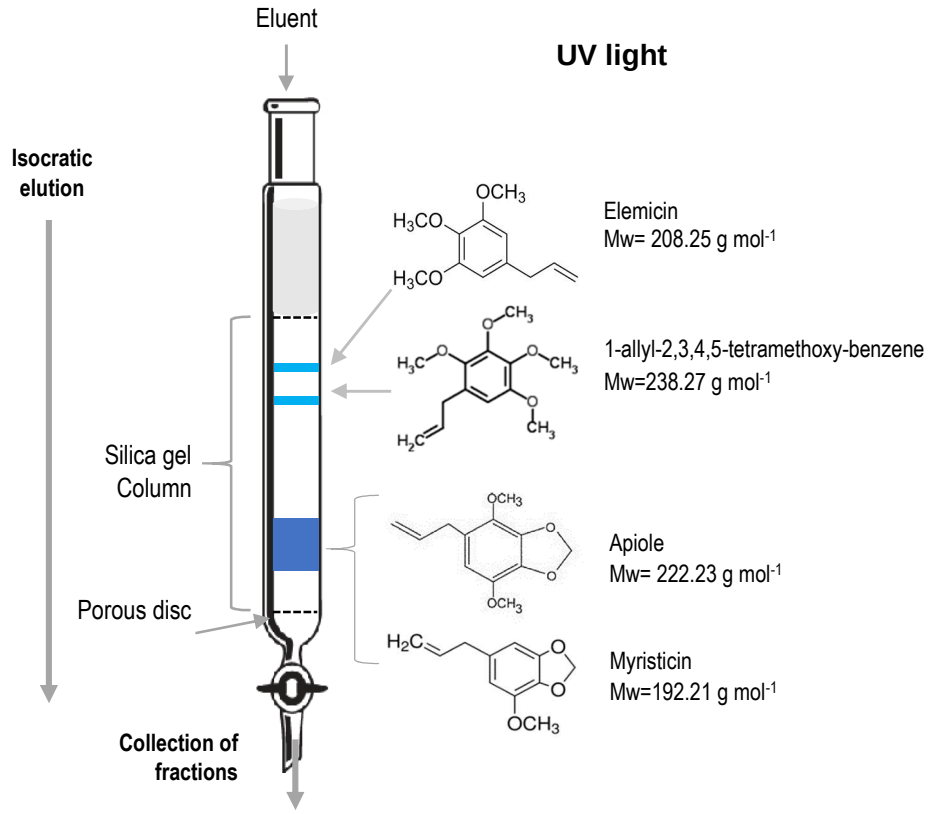


Figure II.4. Illustration of the fractionation procedures of the gravity silica gel column chromatography performed to isolate the four phenylpropanoids composing the *Petroselinum crispum* fruits essential oil.

The compounds are distributed in the silica gel column by their order of elution with an eluent mixture of *n*-hexane-ethyl acetate (93:7). Their detection in the eluted fractions was achieved by TLC. All procedures were performed at room temperature (18-22 °C).

3.5. Qualitative and quantitative characterization of EOs by GC-MS and GC -FID analyses

All EO samples (single, mixtures or purchased samples) were submitted to GC and GC-MS analyses after a dilution in *n*-hexane (10 µL:100 µL) In the same way, the isolated fractions containing purified compounds from parsley EO were diluted in *n*-hexane (2 µL:100 µL) prior to analysis. Methods and analytical conditions are described in table II.6.

Compounds identification was assigned by comparing their KI as well as their electron ionization spectra (MS fragmentation patterns) with libraries. Some constituents were confirmed by comparison with available authentic standards.

Co-injection of samples with a series of *n*-alkanes (C_8-C_{34}) was performed for KI calculation under temperature-programmed GC[3]. The respective KI were compared with those reported in the

[3] Application of the linearized formula **(3)** (Appendix C).

literature, or available in several online databases, for similar analytical conditions and equivalent stationary phases.

MS of compounds were compared with those of authentic standards found in mass spectral libraries (NIST, Mainlib, Wiley and TR) incorporated in the *NIST MS search* program v.2.0, or online mass spectral databases[4] and, in some cases, by comparison with available authentic standards. Quantitative data were obtained from the GC FID analyses which use the normalization procedure for the calculation of peaks' absolute area. The quantification of compounds (means values determined after 3 or more injections and expressed as percentages) was carried out considering only peaks ≥ 0.05% and without correction factors.

Table II.6. Equipment and analytical conditions used for GC and GC-MS.

Equipment and model	**GC** GC focus (Thermo-Scientific, Co)	**GC-MS** Thermo Trace GC Ultra coupled to a Thermo-Finnigan Polaris Q mass spectrometer (Thermo-Scientific, Co)
Detector	FID	ITD
Capillary column	Rtx-5 (30 m × 0.25 mm ID, 0.25 µm film thickness)	Trace TR-5 (30 m × 0.25 mm ID, 0.25 µm film thickness)
Temperature of the injector	300 ºC	300 ºC
Temperature of the detector	300 ºC	300 ºC
Ion-source	-	operating in EI mode at 70 eV at 250 ºC
Mass scan	-	range of 40-400 amu and frequency of 2 scan s^{-1}
Flux repartition	Splitless mode	1/13
Carrier gas	Hydrogen at constant flow (1.0 mL min^{-1})	Helium at constant flow (1.5 mL min^{-1})
Oven program temperature	From 60 ºC to 285 ºC, rising at 3 ºC min^{-1}, isothermal at 285 ºC for 15 min	from 60 ºC to 285 ºC, rising at 3 ºC min^{-1}, isothermal at 285 ºC for 15 min
Software	*Chrom Card data system*	*Excalibur 1.4*
	Thermo-Scientific, Co	Thermo Electron corp.
No. of injections	At least three time and co-injection with *n*-alkanes	Once plus a co-injection with *n*-alkanes
Injected volumes	0.3- 0.5 µL	0.3- 0.5 µL

3.6. Statistical analyses

Correlations between bitter fennel plants growth parameters were evaluated by calculation of Pearson´s coefficients (α = 0.05) using *Prism* vs. 4.00 (Graph Pad software Inc., USA).

[4] Online databases: http://www.massbank.jp/?lang=en; http://www.pherobase.com/; http://webbook.nist.gov/

4. Results

4.1. *Anethum graveolens*: EO content and variation of its chemical composition between different plant parts and during umbels ontogeny

Dill plant material and EOs recovery parameters are described in table II.7. The chemical composition of representative EOs from each type of material (vegetative parts including roots and reproductive organs in distinct developmental stages) is listed in tables II.8 and II.9, respectively.

Table II.7. Summary of *Anethum graveolens* plant material, sampling, essential oil recovery and percentage in tissues.

Plant material	Ext. date (dd/mm/yy) [a]	HD Rep. [b]	Material weight (g) [c]	EO volume (ml)	EO weight (g)	EO content % (w/w) [d]	EO density (g mL^{-1})
Pre-flowering umbels	20/09/2010	1	10.13	0.29	0.278	2.75	0.959
Flowering umbels	18/10/2010	1	29.76	0.93	0.774	2.60	0.832
Umbels with green fruits	30/10/2010	1	41.85	1.25	1.169	2.79	0.935
Umbels with green fruits	6/09/2010	2	43.80	3.20	2.660	3.40	0.831
			20.06	1.90	1.712	2.60	0.901
Umbels with mature fruits	10/11/2010	1	38.48	0.87	0.808	2.10	0.929
Dry fruits	6/05/2011	1	47.50	0.84	0.639	1.34	0.761
Leaves	6/09/2010	4	33.76	0.48	0.385	1.14	0.802
			27.01	0.39	0.281	1.04	0.721
			28.92	0.50	0.352	1.22	0.704
			20.25	0.21	0.160	0.79	0.762
	19/10/2010	2	22.44	0.40	0.345	1.71	0.862
			20.57	0.39	0.353	1.82	0.905
Stems	6/09/2010	3	35.03	0.12	0.105	0.30	0.875
			34.50	0.17	0.126	0.37	0.741
	19/10/2010		41.15	0.33	0.294	0.71	0.891
Roots	6/09/2010	1	31.58	—[e]	—[e]	—	—

[a] EOs extraction dates; [b] Number of hydrodistillations (HD) performed with the same type of material; [c] Equivalent dry weights. EOs were isolated from freshly collected and processed material (excepting for the dry fruits sample) and the equivalent dry weights extrapolated by drying of representative samples; [d] Contents were calculated based on biomass dry weights (mass of EOs per 100 g of dried biomass); [e] The recovered amount of roots EO was very low and could not be measured in terms of volume and mass. The recovery of volatile compounds was achieved by introducing 1 mL of solvent (n-hexane) in the Clevenger apparatus during the hydrodistillation process.

The EO accumulated by umbels in all stages of development correspond to 2.10 to 3.40% of their equivalent dry weights, whilst leaves and stems presented lower contents of EO (0.79 to 1.82% and 0.30 to 0.71%, respectively). The average percentage of EO isolated from fresh reproductive

organs (2.707 ± 0.171%, on a dry weight basis) was considerably superior to the average value determined for fresh vegetative parts (1.011 ± 0.177%, on a dry weight basis). Furthermore, we verified that the storing of ripe fruits for half a year was not favorable causing a significant decrease of the accumulated EO with relation to umbels with ripe fruits (1.34 and 2.10%, respectively).

The chemical analysis of EOs accumulated in different organs of the dill plant resulted in the identification of 97.7 to 99.8% of the constituents (Table II.8 and II.9). Despite their varying amount, the α-phellandrene, β-phellandrene + limonene, carvone and dill ether (also known as 3,6-dimethyl-2,3,3a,4,5,7a-hexahydro-1-benzofuran, 3,9-epoxy-*p*-menthene or anethofuran) were the main volatile compounds produced by dill aerial parts (stems, leaves, flowers, fruits). Phellandrenes were ubiquitous in all types of tissues, including roots. However, their total amount was clearly superior in the youngest organs (flowers and greens parts EOs). The EO obtained from roots presented a dissimilar chemical profile, being constituted by several compounds derived from distinct biosynthetic origins. Apiole (37%), falcarinol (24%) and α-phellandrene (10%) were the chief volatile constituents identified in roots and the principal chemical specimens of the phenylpropanoid, polyacetylene and monoterpenoid classes, respectively. Unlike leaves and stems, the EO composition of umbels in different developmental stages showed significant quantitative differences. Fruit development and ripening was accompanied by a remarkable increase of (*S*)-carvone (13.4-83.8%) while the content of other constituents decreased (α-phellandrene, sum of limonene and β-phellandrene, and dill ether) (Table II.9 and Fig. II.5). Hence, the EO of dill mature infrutescences (umbels with fully-grown and brown fruits) was characterized by a very high content of (*S*)-carvone (61%) in comparison to inflorescences (13 and 4%). Simultaneously, the α-phellandrene followed an opposite trend of variation (Fig. II.5).

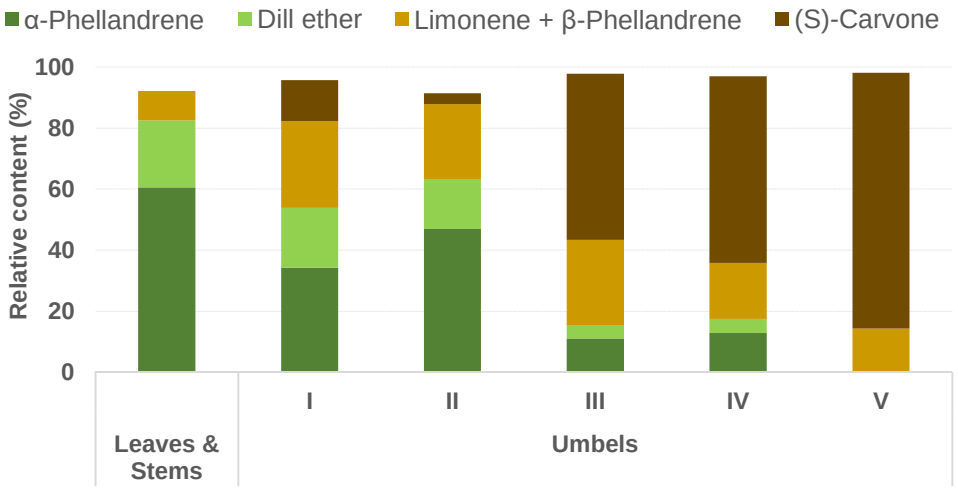


Figure II.5. Main compounds of *Anethum graveolens* essential oils (EOs) produced by greens and reproductive organs and their variation in distinct developmental stages of the umbels.

I-Inflorescences before blooming; II-Fully bloomed inflorescences; III-Green infrutescences with pre-ripened fruits; IV-Infrutescences with ripened fruits; V- Mature and dry fruits stored for 6 months.

Table II.8. Composition of essential oils from stems, leaves and roots of 146-day-old plants of *Anethum graveolens*.

Compounds [a]	**KI**			**Relative content** [d] (%)		
	Rtx-5	TR-5	Ref.	*Stems*	*Leaves*	*Roots*
α-Thujene [b,c]	930	927	931	0.1	0.1	0.1
α-Pinene [b,c]	937	935	939	**2.1**	**1.6**	0.5
Sabinene [b,c]	984	976	975	tr	tr	0.4
Myrcene [b,c]	996	990	990	0.1	–	0.1
α-Phellandrene [b,c]	1018	1007	1002	**60.0**	**60.7**	**10.0**
α-Terpinene [b,c]	–	1016	1018	**4.4**	**4.0**	tr
ortho-Cymene [b,c]	1025	1023	1026	**6.9**	–	0.7
Limonene [c] β-Phellandrene [b,c]	1038	1030	1030 1035	**6.8**	**9.6**	**4.3**
γ-Terpinene [b,c]	1061	1057	1062	0.1	tr	tr
Terpinolene [b,c]	1090	1087	1088	0.2	0.1	**3.6**
Undecane [b,c]	1100	1000	1000	–	–	0.7
1,3,8-*p*-Menthatriene [b,c]	1107	1108	1114	–	–	tr
p-Menth-2-en-1-ol [b,c]	1118	1116	1122	tr	0.1	tr
2,6-Dimethyl-2,4,6-octatriene [b,c]	1127	1126	1127	0.1	–	0.3
(E,Z)-2,6-Dimethyl-2,4,6-octatriene [b,c]	1131	1131	1131	tr	–	0.1
(Z)-3-Undecen-5-yne [b,c]	1142	1141	1146	tr	tr	0.2
Pentyl benzene [b,c]	–	1153	1158	–	–	**1.5**
(E)-3-Undecen-5-yne [b,c]	1155	1154	1161	–	–	
Dill ether [b,c]	1184	1180	1184	**16.4**	**21.9**	tr
(*S*)-Carvone [b,c]	1240	1238	1242	0.2	tr	0.1
Bornyl acetate [b,c]	1281	1279	1285	0.1	tr	–
cis-Sabinyl acetate [b,c]	1329	1326	1325	0.1	0.1	tr
(E) or (Z)-2-Tetradecene [b,c]	1410	1410	1406/1414	0.1	0.1	**2.5**
Myristicin [b,c]	1522	1516	1525	tr	tr	**1.7**
Elemicin [b,c]	1556	1552	1554	–	tr	0.2
Dillapiol [b,c]	1636	1624	1625	–	tr	**8.2**
Apiol [b,c]	1700	1696	1687	–	0.1	**36.7**
Unknown	1733	1724	–	–	tr	0.4
3-N-Buthylphtalide [b]	1755	1748	–	–	0.1	0.7
7,10-Pentadecadiynoic acid [b]	1977	1973	–	–	tr	**1.0**
Unknown	1990	1985	–	–	–	0.5
Falcarinol [b,c]	2052	2052	2056	–	tr	**23.9**
Falcarindiol [b]	2136	2134	–	–	–	0.1
6,9-octadecadiynoic acid methyl ester [b]	2189	2186	–	–	–	0.3
Identified content (± SD)				**97.7** (± 0.04)	**98.5** (± 0.01)	**97.9** (± 0.10)

[a] Compounds listed by order of elution on both nonpolar capillary columns, Rtx-5 and TR-5 (fused silica capillary columns coated with 5% phenyl methyl polysiloxane); [b] Identification based on peaks mass spectra (MS) comparison with MS available in the NIST library and data bases (Wiley, Mainlib, tr, *etc.*); [c] Identification confirmed by Kovats indices calculation and comparison with those reported in the literature and in the online databases (http://webbook.nist.gov/ and http://www.pherobase.com/kovats/); [d] Mean values of peaks percentage area based on three or more injections.
KI-Kovats indices; N.I.- Not identified; –-Not detected; tr- Compound detected below 0.05% and considered as trace amount.

Table II.9. Composition of *Anethum graveolens* (dill) essential oils throughout flower and fruit development.

Compounds [a]	**KI**			**Relative content (%)** [d]				
				Umbels				*Dry fruits*
	Rtx-5	TR-5	Ref.	*Pre-flowering*	*Flowering*	*Green fruits*	*Maturing fruits*	
α-Tujene [b,c]	930	927	931	0.2	0.1	tr	tr	–
α-Pinene [b,c]	942	935	939	**1.0**	**1.3**	0.4	0.4	–
Sabinene [b,c]	964	965	975	0.1	tr	tr	tr	–
β-Pinene [b,c]	974		980	0.1	–			
Myrcene [b,c]	990	992	990	0.5	0.1	tr	–	–
α-Phellandrene [b,c]	1011	1008	1002	**34.2**	**46.9**	**11.1**	**12.8**	0.3
α-Terpinene [b,c]	1022	1019	1018	0.2	**5.0**	–	–	**tr**
ortho-Cymene [b,c]	1025	1023	1026	0.3	–	–	–	–
Limonene [c]	1033	1041	1030	**28.5**	**24.7**	**28.1**	**18.3**	**13.9**
β-Phellandrene [b,c]			1035					
trans-Ocimene [b,c]	1042	1058	1037	tr	tr	tr	tr	–
γ-Terpinene [b,c]	1061	1057	1062	tr	tr	tr	tr	–
Terpinolene [b,c]	1085	1087	1088	0.1	0.1	0.1	0.1	–
2,5-Dimethylstyrene [b,c]	1088	1086	1090	–	–			–
1,3,8-*p*-Menthatriene [b,c]	1107	1108	1114	tr	tr	tr	tr	0.1
Allo-Ocimene [b,c]	1126	1128	1127	tr	0.1	–	–	–
cis Limonene oxide [b,c]	–	1135	1140	–	–	tr	0.1	tr
1-Terpineol [b,c]	1143	–	1146	tr	tr	–	–	–
Dill ether [b,c]	1184	1182	1184	**19.6**	**16.2**	**4.2**	**4.7**	0.1
Unknown	1189	1191	1192	0.2	0.2	0.3	0.8	0.2
trans-Dihydrocarvone [b,c]	1194	1198	1201	0.6	0.5	0.6	tr	0.3
Unknown	1224	1229	–	0.1	–	0.1	–	–
Unknown	1239	–	–	tr	–	0.6	0.7	–
(*S*)-Carvone [b,c]	1253	1255	1242	**13.4**	**3.6**	**54.4**	**61.2**	**83.8**
Carvone oxide [b,c]	1260	–	1263	tr	–	–	–	–
trans-Carvyl acetate [b,c]	1333	1329	1337	tr	tr	–	–	0.1
Unknown	1591	–	–	tr	0.1	tr	tr	–
Unknown	1674	–	–	tr	0.1	tr	tr	–
Unknown	1825	–	–	0.1	0.1	tr	tr	–
Identified content% (± SD)				**99.0** (±0.56)	**98.8** (±0.01)	**99.8** (±0.04)	**99.1** (±0.03)	**98.6** (±0.68)

[a] Compounds listed by order of elution on both nonpolar capillary columns, Rtx-5 and TR-5 (fused silica capillary columns coated with 5% phenyl methyl polysiloxane); [b] Identification based on peaks mass spectra (MS) comparison with MS available in the NIST library and data bases (Wiley, Mainlib, tr, *etc.*); [c] Identification confirmed by Kovats indices calculation and comparison with those reported in the literature and in the online databases (http://webbook.nist.gov/ and http://www.pherobase.com/kovats/) for equivalent stationary phases; [d] Mean values of peaks percentage area based on three or more injections. KI-Kovats indices; –-Not detected; tr- Compound detected below 0.05% and considered as trace amount.

4.2. *Foeniculum vulgare* var. *vulgare:* plants growth, EO content, yield and chemical composition intraspecific variation

A characterization of the vegetative growth was made for each bitter fennel plant before processing and EO extraction (Table II.10). Mature plants in the reproductive stage were morphological organized in one or two stems (2.3 m of average high), highly branched (8-15 branches), with senescent leaves, and supporting a total of 114-302 umbels in different developmental stages. Concerning their dry weight content, plants were constituted by 46% of biomass, which was much superior to green infrutescences (31%) (Table II.11). The umbels' weight relatively to the total plant weight was 6.3 times inferior to that of the stems and stalks. Nonetheless their weight ratio was even among plants (Table II.11). Moreover, we observed significant positive correlations between some growth parameters (Table II.10). The total number of umbels in each stems was strongly related with the numbers of umbels in each branch (r=0.974) and the number of branches per stem (r=900), respectively. Other significant to highly significant correlations (0.670≤ r ≤0.881) were observed between stem total weight and the number of umbels found in branches and stems. In addition, we found an interesting relationship between the amount of umbels per branch and the number of branches in each stems (r=0.806).

Table II.10. Characterization of *Foeniculum vulgare* subsp. *vulgare* var. *vulgare* plants collected at the ripe fruit phase after 15 months of cultivation and correlations [a] between growth parameters.

	Stems Height (cm) [b]	**Branches per stem** (no.)	**Umbels per stem** (no.)	**Umbels per branch** (no.)	**Stems total fresh weight** (g)	**Stems total dry weight** (g)	**Plants' weight** [c]	
							Stalks (%)	**Umbels** (%)
Mean (± SEM)	227 (± 10.3)	11.6 (± 0.64)	169.1 (± 24.29)	13.6 (± 1.53)	494 (± 68.3)	206 (± 37.0)	84.3 (± 1.03)	16.3 (± 1.52)
Stems height (cm) [b]	-	0.304 ^{ns}	0.161^{ns}	0.044 ^{ns}	0.615*	0.565 ^{ns}	0.260 ^{ns}	-0.035 ^{ns}
Branches per stems (no.)	-	-	**0.900*****	**0.806** ***	**0.702****	**0.810****	0.229 ^{ns}	0.002 ^{ns}
Umbels per stem (no.)	-	-	-	**0.974*****	**0.741****	**0.760****	0.310 ^{ns}	-0.135 ^{ns}
Umbels per branch (no.)	-	-	-	-	**0.670****	**0.721****	0.268 ^{ns}	-0.127 ^{ns}
Stems total fresh weight (g)	-	-	-	-	-	**0.881** ***	0.179 ^{ns}	0.027 ^{ns}
Stems total dry weight (g)	-	-	-	-	-	-	0.248 ^{ns}	-0.115 ^{ns}

[a] Pearson correlation coefficient (α = 0.05) not significant (^{ns}) or significant: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; [b] Total number of stems (n = 16). [c] Number of plants (n = 10).

Yields of *F. vulgare* var. *vulgare* plants, fresh vegetative parts and infrutescences EOs, as well as the estimated plant biomass and EO yields per square meter are shown in Table II.11. The average content of EO extracted from individual plants was approximately 1% (w/w), which is significantly inferior to that of fresh stems and leaves (2.3%) and fresh infrutescences (3.4%).

Twelve to seventeen compounds, accounting for 99.1 to 99.9% of the total EO content, were detected (relative contents ≥ 0.05%) in *Foeniculum vulgare* var. *vulgare* EOs. Plants, umbels bearing green fruits, and young vegetative parts accumulated EOs with similar qualitative profiles. Nonetheless, the quantitative differences found between EOs from different plant parts and between EOs from different whole plants were considerable (Table II.12). The EO extracted from green fruits bearing umbels was mainly constituted by estragole (65%) and fenchone (16%), with several monoterpene hydrocarbons (17%) found in smaller amounts. The oxygenated monoterpene, fenchone, was considerably less abundant in vegetative parts (6%) than in infrutescences (16%) and, in both samples, a low content of trans-anethole was detected (2%). In general, individual plants of bitter fennel exhibited some differences in their EOs constituents' relative contents. Nonetheless, the quantitative profiles were more consistent in terms of their monoterpenoids compounds compared to the level of divergence observed for the major phenylpropanoids (Table II.10). The major compounds of bitter fennel plant's EOs were (by order of elution in a Rtx-5 capillary column): α-pinene (1-17%), myrcene (1-2%), α-phellandrene (6-17%), α-terpinene (1-7%), β-phellandrene (4-16%), fenchone (7-16%), estragole (2-54%), and trans-anethole (1-55%). Plants I-IV were characterized by estragole dominant profiles (49-54%) with extremely low amounts of trans-anethole (1-4%), whereas plants V-VII presented inverse proportions of these isomers (47-55% of trans-anethole and 2-3% of estragole).

Table II.11. *Foeniculum vulgare* var. *vulgare*: essential oils (EOs) content and yields.

Plant material	Biomass dry weight (% ± SEM)	EO content (v/w) [c,d] (% ± SEM)	EO content (w/w) [c,d] (% ± SEM)	Yield per square meter		Yield per plant (mLof EO)	EO density [d] (g mL^{-1} ± SEM)
				Biomass dry weight (g ± SEM)	EO (mL ± SEM)		
Green stems with leaves [a]	14.6 ± 0.77	2.51 ± 0.35	2.33 ± 0.23	-	-	-	0.958 ± 0.042
Green infrutescences [a]	31.3 ± 1.50	3.53 ± 0.20	3.43 ± 0.17	-	-	-	0.975 ± 0.009
Entire plants with mature infrutescences [b]	45.8 ± 2.49	1.07 ± 0.23	1.03 ± 0.19	383.8 ± 62.2	2.24 ± 0.27	1.17 ± 0.18	1.048 ± 0.019

[a] EOs were isolated from freshly collected and processed material and the equivalent dry weights extrapolated by drying of representative samples; [b] The whole dried plants were used for EO extraction (n = 7); [c] Contents were calculated based on biomass dry weights (mass and volume of EOs per 100 g of dried biomass); [d] Volume and weight measurements were performed at 20 ºC. Average values for stems with leaves, infrutescences and plants were calculated from 6, 5 and 7 samples, respectively.

Table II.12. Composition of *Foeniculum vulgare* subsp. *vulgare* var *vulgare* essential oils isolated from stems and leaves (St & Lv), green infrutescences (Infr) and whole individual plants (I-VII) harvested during fruit maturation phase (after 15 month of cultivation).

Compounds [a]	KI		Relative content (%) [d]								
	Rtx-5	Ref.	*Lv. & St.*	*Infrut.*	I	II	III	IV	V	VI	VII
α-Thujene [b,c]	928	931	tr	tr	tr	–	tr	–	–	–	–
α-Pinene [b,c]	933	939	**18.0**	**3.8**	0.9	**16.7**	**2.4**	**2.4**	**15.2**	**4.6**	**5.4**
Camphene [b,c]	969	953	tr	–	0.3	0.1	–	–	0.1	–	–
β-pinene [b,c]	978	980	**2.9**	**1.0**	0.9	**1.6**	0.5	0.5	**1.4**	**2.5**	**2.5**
Myrcene [b,c]	984	990	**2.9**	**1.2**	**1.3**	**2.0**	**1.2**	**1.2**	**1.8**	**1.9**	**2.0**
α-Phellandrene [b,c]	1000	1002	**24.3**	**4.9**	**11.1**	**8.0**	**11.0**	**5.7**	**9.9**	**13.7**	**16.6**
α-Terpinene [b,c]	1009	1016	**2.6**	0.2	**3.6**	**1.1**	**4.2**	**7.0**	**1.2**	**1.7**	**1.8**
β- Phellandrene [b,c]	1025	1030	**10.1**	**5.5**	**15.7**	**4.3**	**15.5**	**14.0**	**4.4**	**6.1**	**6.2**
Z-β-ocimene [b,c]	1039	1037	0.1	–	–	0.1	–	0.2	tr	tr	tr
E-β-ocimene [b,c]	1051	1050	0.1	–	–	–	tr	tr	0.1	0.1	0.1
γ-Terpinene [b,c]	1060	1062	–	0.2	0.5	–	0.3	–	–	–	–
Fenchone [b,c]	1096	1087	**6.2**	**15.7**	**9.0**	**9.7**	**11.9**	**12.4**	**7.1**	**16.3**	**14.2**
Fenchol [b,c]	1112	1117	0.1	tr	–	tr	–	–	tr	0.1	0.1
cis-p-Menth-2-en-1-ol [b,c]	1120	1122	–	–	tr	–	0.1	–	tr	0.1	0.1
allo-Ocimene [b,c]	1128	1129	**2.0**	0.1	0.3	**1.0**	0.5	0.5	0.8	0.7	0.9
Camphor [b,c]	1142	1146	tr	0.1	tr	tr	tr	0.1	0.1	0.3	0.3
Terpinen-4-ol [b,c]	1176	1177	tr	0.1	–	–	0.1	–	0.1	0.1	0.1
Estragole [b,c]	1199	1198	**28.4**	**64.9**	**54.2**	**51.7**	**49.0**	**51.6**	**2.5**	**3.0**	**2.0**
endo-Fenchyl acetate [b,c]	1216	1221	tr	–	0.1	tr	–	tr	tr	tr	tr
cis-Anethole [b,c]	1254	1258	tr	tr	tr	tr	–	tr	tr	0.1	0.1
***trans*-Anethole** [b,c]	1295	1287	**1.5**	**2.2**	**1.1**	**3.4**	**2.5**	**3.7**	**55.2**	**48.4**	**47.1**
Myristicin [b,c]	1517	1523	tr	tr	tr	tr	0.1	0.1	tr	0.1	0.2
Monoterpenes hydrocarbons			63.0	16.9	34.6	34.9	35.6	31.5	34.8	31.0	35.5
Oxygen-containing monoterpenes			6.3	15.9	9.1	9.7	12.1	12.5	7.3	16.8	14.8
Phenylpropene-derivatives			29.9	67.1	55.3	55.1	51.6	55.4	57.7	51.4	49.4
Identified content% (± SD)			**99.4** (± 0.03)	**99.9** (± 0.06)	**99.1** (± 0.67)	**99.9** (± 0.01)	**99.4** (± 0.02)	**99.5** (± 0.03)	**99.7** (±0.03)	**99.7** (± 0.07)	**99.5** (± 0.18)

[a] Compounds listed by order of elution on both nonpolar capillary columns, Rtx-5 and TR-5 (fused silica capillary columns coated with 5% phenyl methyl polysiloxane); [b] Identification based on peaks mass spectra (MS) comparison with MS available in the NIST library and data bases (Wiley, Mainlib, tr, *etc*.); [c] Identification confirmed by Kovats indices calculation and comparison with those reported in the literature and in the online databases (http://webbook.nist.gov/ and http://www.pherobase.com/kovats/) for equivalent stationary phases; [d] Mean values of peaks percentage area based on three or more injections.

KI- Kovats retention indices determined for a Rtx-5 capillary column and reference KI found in the literature and databases; *Lv & St*- Essential oil from leaves more stems; *Infr*ut.- Essential oil from green infrutescences; tr- Trace amount with relative content below 0.05%; – - Not detected.

4.3. *Petroselinum crispum*: EO content and chemical variation among different plant parts

The table II.13 shows the results concerning EOs recovered by hydrodistillation from leaves and infrutescences of parsley samples. These parameters were not determined for the roots owing to the little amount of volatiles extracted from this tissue and the high density of the EO droplets that tended to disperse in the water after the vapors condensation.

The accumulation of EO content in parsley plants varied dramatically with the plant part, with EO yields in infrutescences samples (1.02 w/w) being 7 times higher than those of leaves with petioles (0.14% w/w). Moreover, the differences in EOs densities (density of infrutescences EO > density of EOs from foliar tissues) reveals that compounds with high molecular weight are representing a larger fraction of the infrutescences EOs.

Table II.13. Summary of *Petroselinum crispum* var. *neapolitanum* (Italian giant parsley) plant material, sampling, essential oil recovery and percentage in tissues.

Plant material	Ext. date (dd/mm/yy) [a]	HD Rep. [b]	Material weight (g) [c]	EO volume (ml)	EO weight (g)	EO content % (w/w) [d]	Density (g mL^{-1})
Whole roots	29/11/2010	1	88.60	– [e]	– [e]	–	–
Leaves with petiole	29/11/2010	4	50.48	0.075	0.058	0.12	0.773
			37.86	0.055	0.039	0.10	0.709
			36.19	0.075	0.059	0.16	0.787
			48.25	0.085	0.075	0.16	0.882
Mean (± SD)			-	-	-	**0.135 (0.030)**	**0.788 (0.071)**
Umbels with green fruits	28/06/2011	3	88.93	1.20	1.074	1.21	0.895
			73.47	0.49	0.465	0.63	0.948
			31.55	0.44	0.381	1.21	0.867
Mean (± SD)			-	-	-	**1.017 (0.335)**	**0.903 (0.041)**

[a] EOs extraction dates; [b] Number of hydrodistillations (HD) performed with the same type of material; [c] Equivalent dry weights; EOs were isolated from freshly collected and processed material and the equivalent dry weights were extrapolated based on the dry weight content of representative samples; [d] Contents were calculated based on biomass dry weights (mass of EOs per 100 g of dried biomass); [e] The EO could not be measured in terms of volume and mass as consequence of the occurrence of an emulsion with water and the dispersion of the EO. The recovery of volatile compounds was only possible by introducing 1 mL of solvent (*n*-hexane) in the Clevenger apparatus during the hydrodistillation process.

In fact, we have found important chemical differences amongst the studied EOs (Table II.14). Twenty-one to 38 constituents were detected during the analyses of *P. crispum* EOs, and around 19 to 34 compounds, corresponding to 95.1 to 99.5% of total EOs compounds, were identified (Table II.14). In the four characterized EOs, a total of seven to nine compounds were found in amounts

over 1%. Of these major constituents, only three to five occurred at a higher amount (> 10%). α-Pinene (2-23%), β-pinene (2-21%), myrcene (0.2-5%), β-phellandrene (1-40%), 1,3,8-*p*-menthatriene (0.1-11%), and apiole (6-56%) were found in all EOs but their abundance was highly variable amongst the studies organs. The EOs extracted from leaves and infrutescences of the *neapolitanum* variety shared similar qualitative profiles, although the relative content of some of the compounds, namely both pinenes, β-phellandrene as well as apiole, were very distinct. Furthermore, we observed that allyl-tetramethoxybenzene was stored in the reproductive tissues (fruits and umbels bearing green fruits) but that myristicin was not accumulated in umbels of *P. crispum* var. *neapolitanum* during the fruit pre-ripening stage.

Table II.14. Composition of leaves, roots, infrutescences essential oils, extracted from cultivated plants of *Petroselinum crispum* var. *neapolitanum,* and *Petroselinum crispum* fruits EO purchased from Sigma Aldrich Co.

Compounds [a]	**KI**			**Relative content** (%) [d]			
	Rtx-5	TR-5	Ref.	*Roots*	*Leaves*	*Infrut.*	*Fruits* [e]
Tricyclene [b,c]	–	921	926	–	tr	–	–
α-Thujene [b,c]	–	931	931	–	tr	tr	tr
α-Pinene [b,c]	934	936	939	tr	**2.2**	**23.4**	**16.2**
Camphene [b,c]	–	946	953	–	tr	tr	0.1
Sabinene [b,c]	975	968	976	–	**2.1**	–	–
β-Pinene [b,c]	984	977	980	**1.1**		**20.8**	**13.6**
Myrcene [b,c]	991	971	990	0.2	**4.7**	1.8	0.7
α-Phellandrene [b,c]	1000	991	1002	0.2	**1.5**	0.6	0.2
Ortho-Cymene [b,c]	1026	1024	1026	tr	tr	–	**6.6**
β-Phellandrene [b,c]	1028	1029	1030	**1.1**	**40.4**	**10.6**	
Z-β-Ocimene [b,c]	1048	1045	1037	–	0.1	0.1	tr
E-β-Ocimene [b,c]	1055	1054	1050	–	0.5	tr	–
γ-Terpinene [b,c]	1057	1059	1062	0.1	–	0.2	0.3
Terpinolene [b,c]	–	1086	1088	**9.6**	–	–	–
Isopropenyl-4-methylbenzene [b,c]	1091	1086	1080	–	**10.2**	**2.4**	0.6
***p*-α-Dimethylstyrene** [b,c]		1088	1090	–			–
1,3,8-*p*-Menthatriene [b,c]	1113	1112	1114	0.1	**6.2**	**10.6**	0.1
p-Menth-2-en-1-ol [b,c]	1118	1116	1117	–	0.2	0.9	–
allo-Ocimene [b,c]	1127	1123	1126	–	0.1	0.3	–
1,3,5,5-tetramethyl-1,3-Cyclohexadiene [b]	1130	1129	–	–	–	0.2	0.7
p-Cymen-8-ol [b,c]	1183	1178	1183	0.2	**1.3**	0.2	0.1
α-Terpineol [b,c]	1188	1187	1189	0.1	–	0.2	–
Mirtenal [b,c]	1192	1191	1195	–	–	0.3	0.2
Estragole [b,c]	1200	1200	1198	–	0.5	–	–
3,9-Epoxy-p-mentha-1,8(10)-diene [b]	1220	1217	–	–	0.4	–	–
1-Hydroxymethyl-4-isopropylbenzene [c]	1227	1226	1225	–	0.1	–	–
Ascaridole [b,c]	1240	1238	1237	–	0.2	–	–
Phellandral [b,c]	1275	–	1276	–	–	–	0.1
Bornyl acetate [b,c]	1285	1285	1285	–	0.6	–	–
Unknown, M^+=164.96	1309	1309	–	–	0.3	–	–
2-Caren-10-al [b], $[M]^+$ 166.84	1321	1320	–	–	0.8	0.1	–
α-Terpenyl acetate [b,c]	1346	1346	1346	0.1	–	–	–

Table II.14. (*Continued*)

Compounds [a]	**KI**			**Relative content** (%) [d]			
	Rtx-5	TR-5	Ref.	*Roots*	*Leaves*	*Infrut.*	*Fruits* [e]
α-Copaene [b,c]	1369	1368	1371	–	0.2	0.1	–
β-Elemene [b,c]	1380	1384	1388	0.1	0.4	0.6	0.1
cis-or trans-*p*-Mentha-6-en-2,8-diol [b,c]	1386	1391	1389	–	0.6	–	–
E-Caryophyllene [b,c]	1412	1417	1417	–	0.7	tr	0.1
γ-Elemene [b,c]	1427	1427	1430	–	0.4	0.1	–
(+)–Aromadendrene [b,c]	1445	1442	1447	–	0.1	–	–
E-β-Farnesene [b,c]	1454	1455	1458	–	–	–	0.2
γ-Muurolene [b,c]	1466	1465	1477	–	–	tr	0.1
Unknown	1471	1469	–	–	0.1	–	–
γ- Gurjunene [b,c]	1475	1471	1476	–	0.1	tr	0.1
Germacrene D	1479	1481	1480	**1.6**	0.3	–	–
Unknown	1507	1507	–		0.3	–	
Myristicin [b,c]	1529	1527	1523	**22.0**	**13.9**	–	**31.5**
α-Eulemol [b,c]	1551	1552	1551/6	–	0.1	–	–
Elemicin [b,c]	1556	1555	1556	0.3	0.2	–	**3.4**
Unknown	1574	1568	–	**1.5**	–	–	
Caryophyllene oxide [b,c] + Alkylbenzene derivative [b]	1575	1572	1579	–	tr	–	–
Carotol [b,c]	1596	1589	1600	–	0.2	–	**8.6**
1-Allyl-2,3,4,5-tetramethoxybenzene [b,c]	1607	1610	1609	–	–	**1.1**	
Unknown	1612	1611	–	0.2	–	–	–
2-methylene 6,8,8-trimethyl tricycle[5.2.2.0(1,6)] undecan-3-ol [b]	1618	1615	–	–	0.1	–	–
epi-α-Cadinol [b,c]	1634	1632	1639	–	0.2	–	–
epi-α-Eudesmol [b,c]	–	1658	1658	0.2	–	–	–
Apiole [b,c]	1688	1689	1687	**55.5**	**6.1**	**24.3**	**15.9**
N Buthylphthalide [b]	1724	1724	–	0.2	–	–	–
Benzyl benzoate [b,c]	1753	1748	1750	–	0.1	–	tr
9-Octadecyne [b]	1835	1834	–	–	tr	–	–
2-Acetate-1,3-dimethoxy-5-(1-propenyl)benzene [b]	1960	1959	–	–	tr	–	–
Falcarinol [b,c]	2040	2031	2038	**3.2**	–	–	–
Caffeic acid dimethyl ether [b]	2072	2071	–	0.1	–	–	–
Phytol [b,c]	2108	2108	2114	–	0.1	–	–
Identified content % (± SD)				**96.0** (±0.11)	**95.1** (±0.12)	**98.9** (±0.04)	**99.5** (±0.24)

[a] Compounds listed by order of elution on nonpolar capillary columns, Rtx-5 and TR-5 (fused silica capillary columns coated with 5% phenyl methyl polysiloxane); [b] Identification based on peaks mass spectra (MS) comparison with MS available in the NIST library and data bases (Wiley, Mainlib, tr, *etc.*); [c] Identification confirmed by Kovats indices calculation and comparison with those reported in the literature and in the online databases (http://webbook.nist.gov/ and http://www.pherobase.com/kovats/) for equivalent stationary phases; [d] Mean values of peaks percentage area based on three or more injections.
KI- Kovats retention indices determined for two capillary column and reference KI found in the literature and databases; *Infrut*- Essential oil from green infrutescences; tr- Trace amount with relative content below 0.05%.

4.4. *Cuminum cyminum*: EO content and composition in fruits from different origins

Cumin fruits EOs were extracted from dry material for a comparative analysis. The contens of EOs recovered from two distinct samples are shown in table II.15. Comparing to the fruits purchased from India, the EO content (% w/w) of cumin fruits commercialized for seasoning was two times higher.

The chemical study of the aforementioned EOs in parallel with a purchase EO lead to the identification of 18 to 25 volatile constituents accounting for 98.1 to 98.8% of the total composition (Table II.16). We distinguished four to seven major compounds corresponding to 93-97% of the EOs, while several remaining ones (13-17 compounds) occur in proportions below 1%. EOs were characterized by high contents of cuminaldehyde (28-39%) and *p*-mentha-1,4-dien-7-al more *p*-mentha-1,3-dien-7-al (11-52%), and significant amounts of the monoterpene hydrocarbons, β-pinene (0.8-12%), *p*-cymene (4-10%) and γ-terpinene (6-16%) (Table II.16).

Tabela II.15. Summary of *Cuminum cyminum* (cumin) material and essential oils (EOs) contents of dried fruits.

Plant material	**Origin**	**Ext. date** (dd/mm/yy) [a]	**HD Rep.** [b]	**Material weight** (g) [c]	**EO volume** (ml)	**EO weight** (g)	**EO Content %** (w/w) [d]	**Density** (g mL^{-1})
Dry fruits	Unknown	24/05/2010	1	18.91	0.47	0.454	2.40	0.965
Dry fruits	India	31/05/2011	1	19.27	0.22	0.217	1.13	0.986

[a] EOs extraction dates; [b] Number of hydrodistillations (HD) performed with the same type of material; [c] Equivalent dry weights based on the dry weight content of representative samples; [d] Contents were calculated based on biomass dry weight (mass of EO per 100 g of dried biomass).

Table II.16. Composition of *Cuminum cyminum* L. (cumin) essential oils extracted from fruits from different origins.

Compounds [a]	**KI**			**Relative content** (%) [d]		
	Rtx-5	TR-5	Ref.	*Fruits*		
				Unknown [e]	*India* [f]	*Iran* [g]
Cumene [b,c]	923	926	924	–	0.1	–
α-Tujene [b,c]	928	929	931	0.1	–	0.2
α-Pinene [b,c]	935	936	939	0.2	–	0.8
Sabinene [b,c]	969	974	975	tr	0.1	tr
β-Pinene [b,c]	979	980	980	**6.7**	0.8	**12.4**
Myrcene [b,c]	991	991	990	0.5	0.2	0.4
α-Phellandrene [b,c]	1004	1005	1002	0.2	tr	0.8
α-Terpinene [b,c]	1017	1017	1016	0.1	tr	0.1
***p*-Cymene** [b,c]	1026	1027	1026	**3.7**	**6.3**	**10.4**
β- Phellandrene [b,c]	1030	1031	1130	0.3	–	–
γ-Terpinene [b,c]	1063	1064	1062	**10.0**	**6.0**	**15.8**
cis-Sabinene hydrate [b,c]	1068	1069	1068	tr	0.1	–
Terpinolene [b,c]	1088	1089	1088	tr	---	0.7
2,5-Dimethyl styrene [b]	1097	1098	–	0.1	---	
trans-Pinocarveol [b,c]	1138	1140	1139	0.1	0.1	0.2
1,4-Dimethyl-4-acetylcyclohexene [b,c]	1147	–	1152	–	–	0.1
trans-caren-4-ol [b]	1167	1168	–	0.1	0.3	–
Terpinen-4-ol [b,c]	1176	1178	1181	0.1	0.2	0.2
α-Terpineol [b,c]	1184	1189	1189	tr	–	**1.5**
p-Cymene-8-ol [b,c]	1192	1193	1183	**1.3**	0.6	–
Cumin aldehyde [b,c]	1249	1251	1239	**27.7**	**31.4**	**39.4**
Phellandral [b,c]	1275	1278	1275	–	–	0.3
***p*-Mentha-1,4-diene-7al** [b,c]	1284	1286	1278	**2.0**	**52.5**	**9.7**
***p*-Mentha-1,3-diene-7al** [b,c]	1298	1300	1282	**45.8**		**1.7**
Carvacrol [b,c]	1300	1303	1298	–	–	0.3
2-Caren-10-al [b]	1330	1331	–	0.4	0.1	–
Unknown	1337	1336	–	0.1	0.1	0.1
α-Copaene [b,c]	1373	1379	1376	–	0.1	0.1
1,1,6-Trimethyl-1,2-dihydronaphthalene [b]	1377	1380	–	–	–	0.5
E-caryophyllene [b,c]	1416	1419	1418	tr	tr	0.3
Unknown	1433	–	–	–	0.2	–
E-β-Farnesene [b,c]	1453	1456	1458	–	–	0.2
Acoradiene [b,c]	1468	1471	1471	0.1	0.1	**2.3**
Carotol [b,c]	1587	1590	1600	tr	–	0.2
Identified content% (± SD)				**98.5** (±0.27)	**98.1** (±0.04)	**98.8** (±0.03)

[a] Compounds listed by order of elution on nonpolar capillary columns, Rtx-5 and TR-5 (fused silica capillary columns coated with 5% phenyl methyl polysiloxane); [b] Identification based on peaks mass spectra (MS) comparison with MS available in the NIST library and data bases (Wiley, Mainlib, tr, *etc.*); [c] Identification confirmed by Kovats indices calculation and comparison with those reported in the literature and in the online databases (http://webbook.nist.gov/ and http://www.pherobase.com/kovats/) for equivalent stationary phases; [d] Mean values of peaks percentage area based on three or more injections; [e] EO extracted from dry cumin fruits of unknown origin, commercialized by Mc Cormick Co for seasoning purposes; [f] EO extracted from dry cumin fruits cultivated in India; [g] Certified EO from Iran purchased from Sigma Aldrich Co.

KI-Kovats indices; –-Not detected; tr- Compound detected below 0.05% and considered as trace amount.

4.5. Characterization of the EO samples used in the biological assays

The EOs used in several biological assays are described in table II.17 and their major constituents, representing more than 1%, are depicted in figure II.6 (A-D). Mixtures obtained from cultivated plants (dill, bitter fennel and parsley) and the purchased EOs (parsley and cumin) were compared in terms of major classes of compounds and the total percentage of identification. The overall identification of EOs constituents covered about 98.7 to 99.9%. The total number of compounds with relative content above 0.05% was variable amongst EOs, depending on the plant species as well as the plant organ. The diversity of constituents found in *F. vulgare* and *A. graveolens* EOs (mixtures) was low (10 up to 15 compounds), compared to EOs from the other two species.

EOs obtained from different organs of *F. vulgare* var. *vulgare* presented a similar composition, however, differences in the relative contents of the compounds were substantial (Fig. II.6, A). Bitter fennel young vegetative parts (stems more leaves) accumulated higher amounts of several monoterpene hydrocarbons (63%) (mainly α- and β-phellandrenes, and pinenes), and lower contents of phenylpropene-derivatives (30%), whereas the EO from green infrutescences was richer in oxygenated compounds (65% estragole and 16% of fenchone) (Table II.17 and Fig. II.6, A).

EOs of *A. graveolens* followed the same general trend although with some several qualitative differences, namely in the oxygenated terpenoids. A large fraction of dill green infrutescences and fruits EO was constituted by (S)-carvone (67 and 84%, respectively), contrarily to the vegetative part EO where this compound was not detected. Dill ether, an oxygen-containing compound typically found in this species, was detected in both EOs, though in different amounts (22% in leaves + stems, and 1.3% in infrutescences) (Fig. II.6, B). Moreover, phellandrenes and limonene represented a significant part in both dill EOs, roughly the double of their corresponding sum in *F. vulgare* EOs (Fig. II.6, B *vs* A).

Some qualitative and quantitative differences were also found in parsley EOs. Both presented some phenylpropene-derivatives as major compounds (total of 25 and 59%) and high percentages of pinenes (total of 30 and 44% of the EO) (Fig. II.6, C). Nevertheless, the hydrocarbon monoterpenes component was superior in parsley infrutescences EO (72%), whilst the class of phenylpropanoids was prevailing in fruit EOs (Table II.17). Despite the difference in qualitative profiles, the proportion of oxygenated: non-oxygenated compounds in the purchased EOs of cumin and parsley fruits were similar (Table II.17). As aforementioned in the previous section, cuminaldehyde (39%) plus several monoterpene hydrocarbons (total of 48%) were representing the major part of cumin fruit EO (Fig. II.6, D).

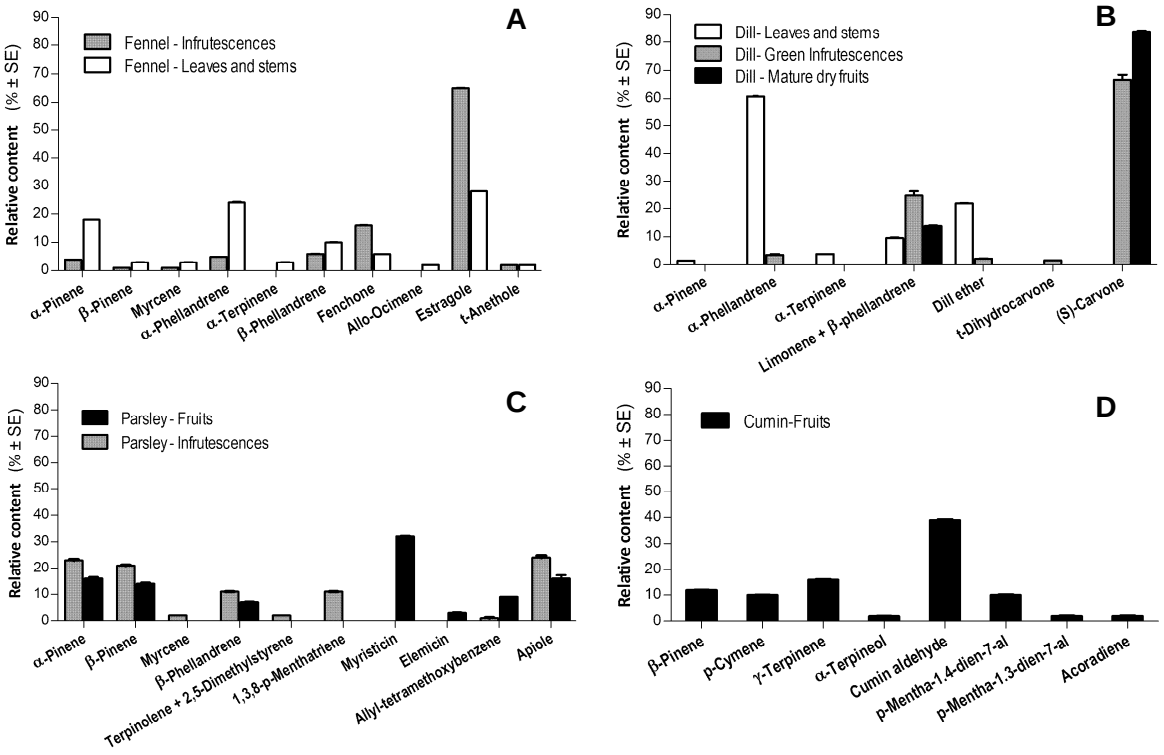


Figure II.6 (A-D). Composition of the Apiaceae essential oils used in bioassays.

(A) Major volatile constituents identified in *Foeniculum vulgare* var. *vulgare* (bitter fennel) green infrutescences and fresh stems with leaves EOs; **(B)** Major volatile constituents identified in *Anethum graveolens* (dill) vegetative parts, green infrutescences and mature dry fruits EOs; **(C)** Major volatile constituents identified in *Petroselinum crispum* (parsley) fruits and green infrutescences EOs; **(D)** Major volatile constituents identified in *Cuminum cyminum* (cumin) fruits EO. Note: Major constituents with relative contents above 1% are presented by order of elution in an Rtx-5 capillary column.

Table II.17. Apiaceae essential oils used in bioassays, identification and classes of compounds percentages.

Scientific name	Abbreviation	Nº Cmpds [a]	Identification cover (%) [b]	MH	OCM	SH	PPD
Foeniculum vulgare subsp. *vulgare* var. *vulgare*	Fv-L	15	99.4	63.2	6.3	0.0	29.9
	Fv-I	13	99.9	16.9	15.9	0.0	67.1
Anethum graveolens	Ag-L	13	98.5	76.1	22.1	0.0	0.1
	Ag-I	9	99.5	30.3	69.2	0.0	0.0
	Ag-F	11	98.8	14.3	84.5	0.0	0.0
Petroselinum crispum var. *neapolitanum*	Pc-I	24	98.7	71.9	1.6	0.8	25.4
Petroselinum crispum [c]	Pc-F	22	99.5	39.1	0.4	0.6	59.4
Cuminum cyminum [c]	Cc-F	25	98.8	41.6	53.3	3.7	0.2

[a] Number of compounds detected on GC-FID analysis with relative peak area ≥ 0.05%; [b] Sum of relative contents of identified compounds; [c] *Petroselinum crispum* and *Cuminum cyminum* fruits essential oils were purchase from Sigma-Aldrich, Co. MH: monoterpenes hydrocarbons; OCM: oxygen-containing monoterpenes; SH: sesquiterpenes hydrocarbons; PD: phenylpropene-derivatives.

4.6. Fractionation of the *P. crispum* EO phenylpropene-derivatives

In the course of the column separation of the *P. crispum* EO a diffuse but deep blue fluorescent band and two minor pale blue bands were observable under UV-light, indicating the presence of myristicin mixed with apiole followed by allyl-tetramethoxybenzene and elemicin. In the TLC analyses all four compounds appeared as dark quenching zones against the green fluorescent background of the F_{254} coated TLC plates, due to the presence of conjugated double-bonds in their structures (Fig. II.7, A and B).

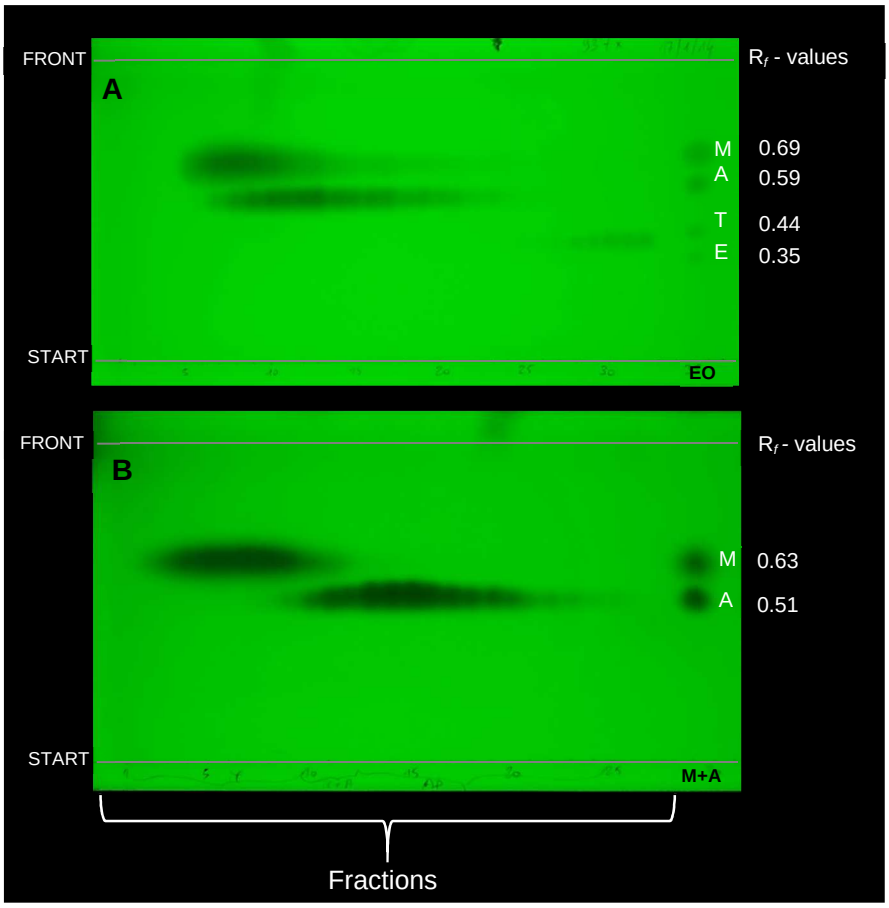


Figure II.7 (A, B). Thin layer chromatography (TLC) plates developed with fractions collected after the fractionation of the *Petroselinum crispum* essential oil phenylpropene-derivatives: myristicin (M), apiole (A), allyl-tetramethoxybenzene (T) and elemicin (E) in a silica gel column.
(A) Chromatogram developed with the collected fractions obtained after a first separation of EO constituents (the TLC photography is approximately half the size of the original TLC-plates). **(B)** Chromatogram developed with the fractions obtained after the second separation of co-eluted compounds (M+A) through silica gel column. Purified myristicin and apiole were recovered from the fractions 1-7 and 12-28, respectively. The co-eluted compounds in fractions 8-11 were once more submitted to the separation procedure.

TLC conditions: Temperature of 20 ± 2 °C; Silica gel 60 F_{254} pre-coated TLC plates; 5-8 µL of volume sample; *n*-hexane: ethyl acetate eluent mixtures as solvent system in the proportion of (A) 93:7 and (B) 92:8; Detection under UV-light (λ 254 nm) and without chemical treatment.

For the first solvent system used (93:7 of *n*-hexane: ethyl acetate) the R_f values of myristicin, apiole, allyl-tetramethoxybenzene and elemicin were 0.69, 0.59, 0.44 and 0.35, respectively (Fig. II.7, A). The TLC developed after the second separation procedure (Fig. II.7, B) showed that the lowering of silica particles size and the slight increase in eluent polarity had both enhancing effects in the resolution of the two compounds myristicin and apiole during column chromatography. The planar chromatography also revealed that the second solvent system (92:8 of *n*-hexane: ethyl acetate) caused an increased retention by the silica for both compounds with the consequent decrease of their R*f* values relatively to the first TLC separation (0.63 and 0.51 *versus* 0.69 and 0.59 for myristicin and apiole, respectively) (Fig. II.7, B vs A).

The performed separation procedures led to the recovery of reasonable amounts of myristicin and apiole at relatively high purity grade, and smaller quantities of the two less abundant phenypropene-derivatives (Table II.18). The recovery percentages of allyl-tetramethoxybenzene and elemicin (26.3 and 45.0%, respectively), were low compared to those recorded for myristicin and apiole (95.9 and 78.8%, respectively). At room temperature the isolated myristicin, allyl-tetramethoxybenzene and elemicin were clear liquids, while apiole formed white-opaque and needles-like crystals.

Table II.18. Final composition of the purified products after a fractionation procedure by column chromatography with silica gel.

		Enriched fractions				
		Myristicin	Apiole	Allyl-tetramethoxy-benzene	Elemicin	Parsley EO (59.4% of PPD)
Compounds	Myristicin (%)	**95.4**	7.3	5.6	2.0	31.5
	Apiole (%)	1.3	**91.9**	5.0	1.9	15.9
	Allyl-tetramethoxybenzene (%)	0.5	0.8	**87.6**	**13.2**	8.6
	Elemicin (%)	2.3	**-**	1.1	**80.0**	3.4
Amount of the fraction [a] (mg)		443.2	190.8	36.2	26.8	-
Recovered amount [b] (mg)		422.8	175.3	31.7	21.4	-
Expected amount [c] (mg)		441.0	222.6	120.4	47.6	832.0 [d]
Recovery (%)		**95.9**	**78.8**	**26.3**	**45.0**	**-**

[a] Weight of the enriched and solvent free fraction determined in an analytical scale.
[b] Compounds' absolute amount within each enriched fraction with basis on their relative content (%) determined by GC analysis.
[c] Value extrapolated from the total amount of each compound existing in the EO submitted to separation with basis on their percentage in the original sample.
[d] Total amount of the PPD (phenylpropene-derivatives) existing in the parsley EO fractionated in the several procedures (1.4 g) and calculated with basis on their percentage in the original sample.

5. Discussion

In the present work, the highest EOs percentages were obtained from fresh green infrutescences of *F. vulgare* var. *vulgare* and *A. graveolens* in the pre-ripening stage with average values of 3.43 and 2.93% w/w, respectively. The variation of the EO content within tissues and/or during development of the three cultivates species was evident. For *A. graveolens* and *P. crispum* var. *neapolitanum* the EO content of infrutescences was considerably superior, 2.9 and 7.5 times more, respectively, with relation to that obtained from the respective vegetative parts. This relative difference was not so marked in *F. vulgare* var. *vulgare* from which 100 g of dry infrutescences gave 1.5 times more EO than vegetative material. Therefore, fennel foliage is also a good source of EO, as it was already reported (Ehsanipour et al., 2012). Overall, the EO content determined for the several tissues of the studied species are more or less consistent with the values described by others authors. Dill, fennel, parsley and cumin fruits usually contain (on a dry weight basis) 2.5-4% (Bailer et al., 2001), 2-6% (da Cunha et al., 2007), 0.6-2.3% (Kurowska & Gałazka, 2006) and 2-4.5% (Amin, 2012) of EO, whereas vegetative parts accumulate 0.2-1.5% (Bailer et al., 2001; Góra et al., 2002), 0.3- 1.5% (Ehsanipour et al., 2012), 0.04-0.4% (Charles, 2012) and 0.1-0.33% (El-Sawi & Mohamed, 2002; Bettaieb et al., 2010), respectively.

Dill, fennel and parsley plants are cultivated not only for their EO but also as fresh herbs for seasoning. However, it has been shown that the harvesting of leaves in dill and parsley can cause a decrease in plants' weight at the flowering stage, a reduction of the umbels and seeds number per plant and a consequent decline of oil yield when compared to a non-harvested culture (Osman & El-Wahab, 2009). Based on that assumption, we have deliberately performed the sampling of dill leaves and infrutescences all at once, and in the case of parsley from separated plants. On the other hand, as it was mentioned in the methods, the irrigation of the three cultures was frequent during the first period of adaptation to field conditions. Additional irrigation was provided during the summer to mitigate the water deficit caused by the insufficient rainfall noticed from May to September in 2010 and 2011[5]. The developmental phase of fruit-ripening and seed filling in the three species was coincident with the hottest season (Tables II.1, 2 and 4). It has been showed that water stress, imposed by restricting the number of irrigations, can impair plant growth, and decrease total biomass, seeds number and EO yields in sweet fennel (Mohamed & Abdu, 2004), and in both plain- and curly-leaf parsley (Petropoulos et al., 2008).

In most cases of EO producing plants, an increase of EO content is observed during development, from bud formation until full bloom (Franz & Novak, 2009). A rapid accumulation started at newly formed fruits and continued until their full maturity (Msaada et al., 2009). Marotti et al. (1993) and Stefanini et al. (2006a) have showed that, when compared to the previous and later stages of fruit ripening, the highest EO yields of sweet and bitter fennel fruits could be obtained at

[5] Supplementary information on climatic conditions is presented in table B.2 of the Appendix B.

the green pre-ripening stage, respectively. Later, Telci et al. (2009) concluded that the content of EO decreases with increasing maturity of fruits. This decrease in EO content during maturation has been attributed to the decline of secretory ducts in fennel ripening fruits (Bernáth et al., 1999), and also along the whole plant (Sousa et al., 2005). This event is common to several Apiaceae species, and a similar trend was reported for *Angelica archangelica* (Varga et al., 2012) and observed herein with *A. graveolens*. Nevertheless, it as been reported that in some species, for instance caraway, carrot, and coriander fruits atain their highest EO content only when fully mature (Sedlakova et al., 2001; Gonny et al., 2003; Msaada et al., 2009;). In bitter fennel, the drying of fruit accompanied by the development of the seed, were point out as the main processes responsible for the fruit wall shrinkage and decrease in oil duct area (Bernath et al., 1999).

The EO content determined for bitter fennel mature plants (1.1 ± 0.2% v/w), harvested after the first year of cultivation during the fructification stage, was much higher than those recorded for wild plants from several Italian locations (0.04- 0.4% v/w, at the full bloom stage) (Piccaglia & Marotti, 2001). This difference might be due to the superior relative weight of umbels found herein (13-25%), with relation to the flowering Italian plants (2-9%) (Piccaglia & Marotti, 2001). According to Telci et al. (2009), and as we observed, the maturation stage and harvesting time have a notable influence on the percentage of EO in *F. vulgare* fruits. In fact, pronounced variations in EO content were reported in fruits of bitter fennel (0.4- 6.8%) (Napoli et al., 2010) and sweet fennel (*F. vulgare* subsp. *vulgare* var. *dulce*) (3-5.8%) (Telci et al., 2009). In our study, plant sampling occurred when leaves were nearly absent, and stalks, a hardened type of material with a low contribution to the overall EO accumulation, represented the major fraction of plants' weight (84 ± 1.0%). Even though the reproductive organs were only a small fraction of plants total weight with a very low weight ratio relatively to stalks (0.19), umbels bearing fruits were the most productive part of the plant in terms of EO accumulation per gram of tissue. These findings suggest that the extraction of EOs from bitter fennel infrutescences bearing unripe fruits, can be more profitable than delaying the harvesting to a more advanced stage of fruit maturation (Table II.11). When compared to other reports of sweet fennel, the EO yield determined herein for the whole plant at the maturity stage (2.24 ± 0.27 mL of EO per m^2) was superior to that of seeds yields (0.54-1.4 g of EO per m^2) achieved by cultivation without soil fertilization (Ehsanipour et al., 2012). The EO yield per bitter fennel plant (1.2 ± 0.2 mL) was within the range or even inferior to yields obtained from seeds of sweet fennel (0.9-1.4 and 1.7-2.0 mL per plant, respectively) described by Mohamed & Abdu (2004) and Azzaz et al. (2009). Moreover, in what concerns the morphology and growth of the bitter fennel culture, plants were considered similar in terms of maximum height, number of branches and umbels per branch (Table II.10). These bitter fennel plants produced much more umbels (169 umbels per plant) than plants of the *dulce* variety grown in non-fertilized soils of Egypt (64 and 70 umbels per plant) (Azzaz et al., 2009) and Iran (41.6-52.8 umbels per plant) (Ehsanipour et al., 2012). It is worth pointing out that plants showing a more complex branching pattern (stems with superior number of branches and with more umbels in each one, resulting in higher number of total umbels) were also the most productive plants (I, IV, V and VII) in terms of accumulated amount of EO (1.1, 1.2, 1.7 and 2.0 mL,

respectively) (data not shown). Besides the predictable positive relationship found between some growth characters, a significant correlation was also established between the number of branches in each stem and the quantity of umbels per branch, suggesting that breeding or selecting for these traits would increase overall culture yield.

Concerning the chemical composition of EOs we found that *A. graveolens* samples presented very simple profiles by comparison with those of other species. Carvone, α-phellandrene, β-phellandrene + limonene, dill ether and pinenes were the most abundant compounds of dill EOs. This result is consistent with previous reports (Porter et al., 1983; Huopalahti & Linko, 1983; Góra et al., 2002; Callan et al., 2007) (Table I.8). Furthermore, depending on the organ and the origin, additional constituents like *cis* and *trans*-dihydrocarvone, carvacrol, *p*-cymene, dillapiol, apiole and myristicin have been occasionally found in variable amounts (Huopalahti & Linko, 1983; Góra et al., 2002). It is worth mentioning that the amount determined for β-phellandrene and limonene (Tables II.8 and II.19) is corresponding to a mixture of both. Based on a first analysis of GC-MS and GC data of three of the characterized EOs lead to the identification and quantification of β-phellandrene (Sousa et al., 2013, 2015). Nevertheless, taking into consideration previous studies concerning the biochemical pathway of the monoterpenes produced in dill (Faber et al., 1997) and caraway (Bouwmeester et al., 1995; Bouwmeester et al., 1998), there are strong arguments in favor of the presence of limonene, as a biogenetic precursor of (+)-(*S*)-carvone, in our samples. In fact, the simultaneous occurrence of limonene and β-phellandrene in variable amounts in dill EOs was previously showed by other authors (Huopalahti & Linko, 1983; Huopalahti, 1985; Faber et al., 1997; Góra et al., 2002). Based on the similarity of β-phellandrene and limonene KIs, we assumed the presence of a mixture of both compounds, even though we have detected a large peak with a MS patterns consistently similar to that of β-phellandrene[6]. The characteristics of the stationary phase, the analytical conditions during GC analyses and the presence of a more abundant constituent (very large peak), are important factors that may have contribute for the co-elution in the column and overlapping of β-phellandrene and limonene MS, which have constrained the identification and quantification of the last one. According to Faber et al. (1997), the two enantiomeric forms (+)-(R)- and (-)-(S)-limonene can be detected in all plant parts and during all stages of plant development, while β-phellandrene, α-phellandrene, dill ether and (+)-(S)-carvone occur as enantiomerically pure compounds.

As we were able to perceive during samples processing the scent of leaves and stems was distinct from the one released from the ripening infrutescences and mature fruits. The chemical analysis of their EO allowed to confirm that vegetative parts contained mainly phellandrenes with limonene and dill ether, which resulted in an herbal, fresh and minty odor, whereas the reproductive

[6] Supplementary information on the chemical structure, chromatographic and mass spectra characteristics of β-phellandrene and limonene and their biosynthetic relationship with (+)-(S)-carvone is provided in table B.4 and figure B.1 of the Appendix B.

organs were extremely rich in carvone, a compound that carries a spicy and sweet fragrance[7]. Huopalahti and Linko (1983) have formerly reported that terpene hydrocarbons, particularly α-phellandrene (34–47%), are the main components of the EO extracted from young herbs. Likewise, the latter compound was the chief component of dill leaves and umbels EOs, accounting for 29 to 66% and 34.5 to 13.8%, respectively (Góra et al., 2002).

The variation in the production/accumulation of volatile secondary metabolites throughout plant development was the most noticeable aspect of the EOs herein studied. We found a clear pattern linking the umbel developmental phase and the chemical variation of dill EO. (*S*)-carvone was almost residual in vegetative material but its content increased throughout the development of dill reproductive organs (3.6 up to 66%) reaching its maximum value in the ripe fruits (84%) (Fig. II.5). Góra et al. (2002) have recognized the same trend from the beginning of blooming until fruits full ripeness, although the (*S*)-carvone contents described therein were much inferior (0.2-22%). According to several authors (Porter et al., 1983; Huopalahti & Linko, 1983; Góra et al., 2002; Callan et al., 2007) and as we could observe, this increase is accompanied by a significant decline in the levels of several constituents (phellandrenes, limonene, dill ether, and pinenes). The amount of dill ether in vegetative parts and flowering umbels (16-22%) was superior to those of maturing infrutescences (4-5%). Likewise, higher contents of dill ether were detected in fresh leaves (from 6 to 20%) and umbels until blooming (22 and 15%) (Góra et al., 2002) and during blooming (about 30%) (Huopalahti & Linko, 1983). Clearly, this compound is distinctive of green structures since its accumulation fall off within the fruit maturation process cumulating in residual storing in dry fruits (0.1%) (Fig. II.5). Given that there is no other commercially available source of dill ether, its presence in dill herb EO (at least 5% or more) is a good indicator of the genuine origin of the EO (Gupta et al., 2012). Furthermore, it was suggested that the early harvest or high plant density could give rise to a more herbaceous EO. By contrast, a late harvest or low plant density should be more appropriate if EOs with richer carvone profiles are desired (Callan et al., 2007).

In order to distinguish different chemical profiles during the cultivation of bitter fennel, we performed a chemical study of EOs accumulated by individual mature plants, and two types of organs collected in previous developmental stages. Taken as a whole, samples were mainly constituted by estragole (2-65%), *trans*-anethole (2-55%) and considerable amounts of α- and β-phellandrene (5-16%, respectively), fenchone (6-14%) and α-pinene (2-17%). Estragole and *trans*-anethole (phenylpropanoid derivatives), and fenchone and limonene (cyclic monoterpenes) have been identified as the major volatile constituents of EOs from *F. vulgare* var. *vulgare* fruits (Napoli et al., 2010) and oleoresins (Barazani et al., 2002; Gross et al., 2009). According to some authors, and as we could observe, the presence of fenchone in high amounts is a distinctive characteristic of the *vulgare* variety of fennel (Miraldi, 1999; Barazani et al., 2002). Most of the studies have confirmed that the major volatile fraction consists of phenylpropenes, estragole and/or *trans*-anethole, but their

[7] The characteristics odor of a selection of volatile compounds commonly found in Apiaceae EOs are provided in table A.4 of the Appendix A.

relative proportion varies according to the population and the chemotype (Table I.8) (Barazani et al., 2002; Napoli et al., 2010).

Besides wide chemical variations of EO constituents have been identified and linked with plant varieties (Marotti et al., 1994), organs (Németh, 2005; Gross et al. 2009), plant ontogeny (Marotti et al., 1994; Gross et al., 2006; Stefanini et al., 2006a; Telci et al., 2009), seasonal variation (Stefanini et al., 2006b) or the existence of chemical polymorphisms (Muckenstrum et al. 1997; Barazani et al. 2002). The chemical polymorphism in bitter fennel has been recurrently documented for natural but also for cultivated populations (Bernáth et al., 1996; Muckenstrum et al., 1997; Barazani et al., 1999; Barazani et al., 2002). Additionally, altitude and latitude have been conjectured as possible factors influencing chemotype differentiation in bitter fennel populations from Israel (Barazani et al., 1999) and chemical variation between wild fennel populations in Italy (Piccaglia & Marotti, 2001), respectively.

In this study, we identified two distinct EO chemical profiles in the bitter fennel plant, based on the opposite estragole/*trans*-anethole ratio (0.93 to 0.98 and 0.04 to 0.06). Individual plants I-IV were characterized by an estragole-dominant profile (49 and 54%) with extremely low amounts of *trans*-anethole (1 to 4%). In contrast, individual plants V-VII presented inverse proportions of these isomers (*trans*-anethole 47 to 55% and estragole 2 to 3%). Estragole and *trans*-anethole are propenilic isomers, differing only in the position of the double-bond in the propenyl side chain. Despite their content variation their sum was nearly constant, implying the occurrence of a biosynthetic regulation. Recent findings indicate that the accumulation of *trans*-anethole and estragole in bitter fennel may be controlled by a single biallelic gene with partial dominance of the estragole allele over the *trans*-anethole allele (Gross et al., 2009). Natural fennel populations with estragole-dominant profiles, ranging between 35 and 89%, were already identified in Portugal (Cavaleiro et al., 1993), Spain (Garcia-Jimenez et al., 2000), Israel (Barazani et al., 2002) and Italy (Piccaglia & Marotti, 2001; Napoli et al., 2010). Moreover, according to Piccaglia and Marotti (2001), a wide number of intermediate chemical forms with variable characteristics may be found as a consequence of a high outcrossing within this species. Eventual crosspollinations with the *dulce* variety are not discarded despite the morphological and phenological evidences supporting the identification of the *vulgare* variety.

Parsley EOs, as well as the odorant profile of parsley leaf, have been subject of several studies (Lamarti et al., 1993; Masanetz & Grosch, 1998; Petropoulos et al., 2004 Kurowska & Gałazka, 2006; Petropoulos et al., 2008; Vokk et al., 2011). The identification completed in this work is substantiated by most of these previous reports (Table I.8). In relation to the quantitative characterization proposed herein, we found the EOs profiles closely comparable to preceding descriptions available in the literature. The composition of leaves EO was similar to the one recently described by Vokk et al. (2011) which comprise 34 compounds and β-phellandrene (22-36%), myristicin (31-43%), *p*-1,3,8-menthatriene (5-10%), and β-myrcene (4.5–9%) as major ones. Likewise, α-pinene (27-41%), β-pinene (19-27%) myristicin (12-42%) 1-allyl-2,3,4,5-tetramethoxybenzene (2-13%) and apiole (0.5-10%) were the chief constituents of fruit EO of several

parsley cultivars (Kurowska & Gałazka, 2006). A more contrasting report was published by Petropoulos et al. (2004) whom have shown that the percentages of β-phellandrene, 1,3,8-*p*-menthatriene, α, *p*-dimethylstyrene, myristicin, β-myrcene and apiole in EOs of leaves are significantly influenced by many factors, including plant variety and the growing stage.

In the present study, phenylpropanoids constituted 59% of parsley fruits EO, whereas the monoterpene hydrocarbons class constituted the major part of the EO extracted from fresh infrutescences (57%) and leaves with petioles (68%). The predominance of these groups above the oxygen-containing monoterpenes or sesquiterpenes is not unusual for this species and was already described for fruits or other plant parts (Lamarti et al., 1993; Petropoulos et al., 2004; Kurowska & Gałazka, 2006). Myristicin and apiole were the most representative phenylpropene-derivatives found herein in parsley EOs, and were previously described as the principal components of the plain leaf parsley EO (64 and 14%, respectively), followed by *β*-phellandrene and 1,3,8-*p*menthatriene (Pino et al., 1997). Both compounds are also very common in roots EOs of other Apiaceae species, namely *Pastinaca sativa* (Lichtenstein & Casida, 1963). The percentage of myristicin varied from 14, 22 and 32% in leaves, roots, and fruits, respectively, but it was totally absent in infrutescences EO of the *neapolitanum* variety. Myristicin is a phenylpropanoid frequently found in considerable amounts in parsley fruits (12-42%) and leaves (31-43%) (Kurowska & Gałazka, 2006; Vokk et al., 2011). Myristicin-rich chemotypes were previously identified and related to the curly leaf or plain leaf type of parsley (Lamarti et al., 1993; Pino et al., 1997; Petropoulos et al., 2004). Apiole accounted for more than half of the volatile fraction of the roots (56%) and 24, 16 and 6% of the infrutescences, fruits, and leaves EO, respectively. The high content of apiole found in *P. crispum* var. *neapolitanum* infrutescences is, in a certain extent, in agreement with a previous report for the giant Italian variety (Lamarti et al., 1993). Like we reported here, apiole is commonly found in root parts of some parsley varieties (Petropoulos et al., 2004), though the fruit of the turnip-rooted parsley (var. *tuberosum*) is an important source of this compound too (16 to 75%) (Kurowska & Gałazka, 2006).

As we described herein, the phenypropene-derivatives (myristicin, apiole, 1-allyl-2,3,4,5-tetramethoxybenzene and elemicin) constitute an important fraction of parsley EOs. However, unlike many others EO constituents these are quite less purchasable. High-grade analytical standards, aimed for accurate chemical analyses (MS, NMR, IR), are available in the market as small samples (mg), though, smaller amounts could not fill the estimated needs of our upcoming work. Hence, the purchasing of these products was not an affordable option considering the substantial quantities of chemicals generally required to complete several biological assessments and dose-response assays, which frequently exceeds the gram unit. In order to ensure their availability, we performed column chromatography (CC) separations of EOs to obtain enriched fractions in these four volatile phenypropanoids.

Despite the lower amount of EO loaded each time on the column (1.2-1.6% w/w of the silica), the co-elution of myristicin and apiole (Fig. II.7, A) may have occurred as consequence of several factors, namely, structural similarity, approximate molecular weights, high amounts in the extract, low interaction with silica particles, defective column packing or separation conditions, *etc*.

Consequently, a second fractionation with some changes in the procedure was required to attain a satisfactory separation (Fig. II.7, B). From those results we deduce that the use of a high-grade silica with smaller particles and the slight increase in the eluent polarity have enhanced the interaction of those two compounds with the stationary phase, improving their separation. Notwithstanding the fact that this preparative method was time-consuming and that significant amounts of allyl-tetramethoxybenzene and elemicin were retained into the matrix, the CC technique still was cost-effective and appropriate to supply satisfactory amounts of myristicin and apiole.

Despite some quantitative discrepancy, the characterization of the three *C. cyminum* fruits EOs revealed a great similarity regarding the qualitative profiles. The most abundant compounds were common to all samples and in accordance with previous reports concerning the same organ (Table I.8). Nonetheless, the purchased EO of cumin fruits from Iran presented lower percentages of cuminaldehyde, distinct contents of the terpinen-7-al isomers (*p*-mentha-1,4-dien-7-al and *p*-mentha-1,3-dien-7-al), and a richer fraction of monoterpenes hydrocarbons (42%). The composition of cumin fruits EOs herein described is consistent with previous studies (Beis et al., 2000; Iacobellis et al., 2005, Park et al., 2008). The terpene hydrocarbons (β-pinene, *p*-cymene, γ-terpinene) and the oxygen-containing monoterpenes, more precisely, aldehydes (cuminaldehyde, *p*-mentha-1,3-dien-7-al, *p*-mentha-1,4-dien-7-al) and alcohols (cuminyl alcohol, α-terpineol, terpinene-4-ol), have been the most reported EOs' constituents of cumin fruits (Bettaieb et al., 2011; Bettaieb et al., 2012; Amin, 2012). Furthermore, according to our results and as previously described by Beis et al. (2000), menthane type monoterpenoids and some sesquiterpenoids (β-caryophyllene, β-farnesene), may be detected in lower content. Several compounds, namely, safranal, perilla aldehyde, camphor, myrtenol, β-bisabolene, diepi-α-cedrene, and 1-phenyl-1,2-ethanediol, have been identified in cumin fruits EOs (Bettaieb et al., 2011 and 2012) but were not detected in our samples. Overall, the oxygenated-containing monoterpenes and the monoterpene hydrocarbons were the most important classes of compounds quantified, corresponding to 53-85% and 14-42% of the EOs total content, respectively. In contrast, Bettaieb et al. (2011) reported the prevalence of terpenic hydrocarbons (54-58%).

6. Conclusion

The present investigation gives an overview about EOs contents and constituents produced by the four selected umbelliferous crop species. The results obtained indicate that EO accumulation and composition are species-specific, but quantitative profiles within the same species are also highly variable and dependent on plant tissues/organs and their developmental stages. Others factors influencing EO content and composition were: plant cultivars/subspecies, plant material and/or origin.

We highlight the important changes observed in *A. graveolens* EOs throughout fruit development. Some volatile constituents were specifically present in ripe fruits but were almost absent in vegetative tissues and unripen fruits. The increase in (*S*)-carvone in dill reproductive organs during ripening could be part of a defense strategy against fruits damage and herbivory, and/or to attract seed dispersal organisms.

The study of *F. vulgare* var. *vulgare* EOs from individually processed plants revealed the occurrence of intra-populational variations with possible chemotypic differentiation. This chemical variability within cultivated plants derived from the same natural population was not unexpected taking into account that seeds were harvested from a wild population. As procedures and environmental conditions were similar for all plants, we consider that the chemical profile variability observed among plants is unlikely attributable to climate, cultivation, light exposure, harvesting time, or material processing. Hence, our results suggest that two chemotypes, estragole or *trans*-anethole dominant, may co-exist in the wild population originally studied, possibly as a result of genetic polymorphism and/or previous cross pollinations. On the other hand, and as we could verify after comparing with previous results (Sousa, 2009), natural populations can exhibit higher levels of plasticity, and different patterns of chemical variability may be observed as a response to cultivation and/or changing environment (Barazani et al., 2002). From a defense strategy perspective, chemical variability is a biological advantage. However, for extensive cultivation and commercialization, the occurrence of more than one chemotype and/or chemical variation between lots of raw material may compromise the batch consistency and/or quality of the EO, which are essential aspects if we consider regulatory and marketing issues.

In comparison with the other Apiaceae species studied, parsley was the less attractive species in terms of EO yield. The EO content of the foliage was especially low and the volatile component of parsley infrutescences was somewhat lower than expected (average value of 1.02% w/w). Although this lower productivity may justify, in part, a lack of interest in investigating its applications (as we noticed in the literature in what concerns the biopesticidal activities), this plant should not be undervalued as it offers a variety of compounds with great potential.

This work bring into focus important aspects that have to be taken in consideration, such as the distinction of plant material, the adequate time of harvesting and the accurate knowledge of chemical profiles variation during plants' life cycle. Bearing in mind that the bioactivity of EO depends on their chemical composition, which varies with plant part used for extraction, harvesting time, plant age, pedological aspects and growing conditions (Olle & Bender, 2010), an appropriate documentation concerning plants' development, sampling and processing is of great importance to ensure a better understanding of EOs bio production and quality, and therefore to decide which is the most adequate harvesting time.

7. References

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CHAPTER III

Larvicidal, molluscicidal and nematicidal activities of EOs and compounds from *Foeniculum vulgare* subsp. *vulgare* var. *vulgare*

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1. Abstract

Plant-based products, namely essential oils (EOs), are environmentally friendly alternatives for the control of disease vectors, hosts and/or parasites. Here, we studied the general toxicity and biopesticidal potential of EOs and phenylpropanoids from *Foeniculum vulgare* var. *vulgare* (bitter fennel), a perennial plant well adapted to temperate climates. EOs/compounds toxicity was tested against a freshwater snail and potential intermediate host of *Fasciola hepatica* (*Radix peregra*), a mosquito and former European malaria vector (*Anopheles atroparvus*) and one of the most damaging plant-parasitic nematodes, a root-knot nematode (*Meloidogyne javanica*).

Lethal concentrations ($LC_{50;}$ LC_{90}) of EOs from infrutescences/stems with leaves and some of the respective compounds were calculated by probit analysis. All displayed noteworthy activity against *R. peregra* adults (LC_{50} 21-39 µg mL^{-1}) and *A. atroparvus* larvae (LC_{50} 16-56 µg mL^{-1}). *trans*-Anethole revealed acute nematicidal activity after 24 and 48 h (LC_{50} 310 and 249 µg mL^{-1}, respectively) and estragole (1000 µg mL^{-1}) showed some effectiveness against *M. javanica* hatching and juveniles after 15 days.

Although additional toxicological studies against nontarget organisms are required, this study demonstrates that bitter fennel is a productive source of molluscicides and larvicides, and thus a potential sustainable biological agent to control particular host species, namely freshwater snails and mosquitoes.

Keywords: Biopesticides, bitter fennel essential oils, *Anopheles atroparvus*, *Meloidogyne javanica*, *Radix peregra*, brine shrimp lethality assay

2. Introduction

Throughout the world, populations struggle with effective control of multiple harmful species (such as disease vectors, hosts and parasites). For instance, freshwater snails and mosquitoes, intermediate or definitive hosts of pathogenic agents, play a crucial role in the transmission of diseases, causing high mortality and morbidity (acute or chronic) to human populations, livestock and domestic animals. In agriculture, plant-parasitic nematodes (over 4100 species) can have a substantial negative impact and are responsible for major damage to economically important crops (Decraemer & Hunt, 2006), with an estimated consequent economic loss of US$100 billion worldwide (Oka et al., 2000a).

The use of synthetic pesticides is undesirable because of their persistence in the environment and collateral effects in humans and other nontarget organisms. Less toxic and environmentally acceptable substitutes are therefore needed, which has created a significant market opportunity for alternative products such as botanical pesticides (Ntalli & Caboni, 2012; Regnault-Roger et al., 2012). In the actual context of research and development, plant extracts have been considered excellent candidates for integration in pest management strategies. In particular, volatile constituents of EOs are among the most promising compounds for biorational pest control (Shaaya & Rafaeli, 2007), and their activity against numerous pests has been widely studied. One of these is *trans*-anethole, a flavoring agent of great economic importance in food and cosmetic industries (Hunault et al., 1989), mainly obtained from *Foeniculum vulgare* Miller (Apiaceae), a medicinal and aromatic plant with high agroindustrial value. The variety *vulgare* (Miller) Thellung (*F. vulgare* Miller), commonly named bitter fennel, is a perennial plant with bitter fruits native to the Mediterranean basin (Hunault et al., 1989). *Foeniculum vulgare* EOs and their main constituents have been recognized for their insecticidal (Chantraine et al., 1998; Kim & Ahn, 2001; Traboulsi et al., 2005; Ebadollahi, 2011) and nematicidal (Oka et al., 2000b; Ntalli et al., 2011) activities. However, the effect of this plant product on freshwater snails is unknown, and only a few studies have evaluated its effect on disease vectors and pests. Freshwater snails, mosquitoes and nematodes are some relevant organisms representing the aforementioned classes, respectively.

Freshwater snails of the Lymnaeidae family have been described as the intermediate hosts of trematodes, including *Fasciola hepatica* Linnaeus and *F. gigantica* Cobbold (liver flukes). Fasciolids are parasites that cause endemic fascioliasis in cattle and a consequent decrease in animals' productivity (Correa et al., 2010). Moreover, fascioliasis is an important human disease of worldwide distribution. At least 2.4 million people are infected in more than 70 countries (WHO, 2014). In Europe, including the Azores (islands), the freshwater snail *Galba* (sin *Lymnaea*) *truncatula* Müller is the primary intermediate host of the common liver fluke *F. hepatica* (Martins, 1991). Nevertheless, *Radix* (sin *Lymnaea*) *peregra* Müller infections have also been reported in some affected areas where *G. truncatula* is less abundant, evidencing its potential role as a fascioliasis vector (Relf et al., 2009).

The mosquito *Anopheles atroparvus* van Thiel (Culicidae) is an efficient malaria vector, widely distributed in Europe (Odolini et al., 2012). In the early twentieth century, mosquitoes included in the *A. maculipennis* complex, namely *A. atroparvus*, *A. sacharovi* Favre and *A. labranchiae* Falleroni were responsible for most of the malaria transmission in the European continent (Jetten &Takken, 1994). Eradication programs were effective in the control of the disease in Europe. However, nowadays there is an increasing concern over the reappearance of malaria in regions where it was eradicated, namely in Europe, as a consequence of environmental changes (Jetten & Takken, 1994; Martens et al, 1999; Sainz-Elipe et al., 2010; Odolini et al., 2012).

The species included in the genus *Meloidogyne* (Meloidogynidae) are important plant-parasitic nematodes with worldwide distribution and broad pathogenicity for a wide variety of plant cultures (Oka et al., 2000a). *Meloidogyne* spp., also known as root-knot nematodes, were recently classified as the most damaging plant-parasitic nematodes in a top-ten list (Jones et al., 2013) and *Meloidogyne javanica* (Treub) Chitwood is one of the four major species of this genus (Moens et al., 2009).

In the present study, we propose an appraisal of *F. vulgare* subsp. *vulgare* var. *vulgare* EOs and two phenylpropanoid compounds (*trans*-anethole and estragole) biopesticidal potential, take into account their molluscicidal, larvicidal and nematicidal properties against three species: *R. peregra*, *A. atroparvus* and *M. javanica*. Moreover, the general toxicity of these plant-products was assessed using *Artemia salina* Linnaeus (Artemiidae) as a test organism.

3. Materials and methods

3.1. EOs

Two EOs isolated from different organs of bitter fennel plants, were used for several biological assessments. EOs from green vegetative parts including leaves more stems (Fv-L) and green infrutescences bearing premature fruits (Fv-I) were obtained from fresh material cultivated during the years of 2010 and 2011. Further details about the origin, cultivation procedures, sampling dates, EOs extraction and chemical analyses were described in the Chapter II, section 3.1, 3.2 and 3.5. The chemical composition of both EOs (Fv-L and Fv-I) was previously described in the respective chapter (Table II.12).

3.2. Volatile constituents and chemicals

The two most frequently reported major volatile compounds of fennel EOs, estragole and *trans*-anethole, were included in this study. Estragole (1-Allyl-4-methoxybenzene, 98%), *trans*-anethole [*trans*-1- Methoxy- 4 - (1-propenyl) benzene, 99%] and Tween® 20 were purchased from

Sigma-Aldrich (Aldrich Chemical Co., St. Louis, MO, USA). Solvents, *n*-hexane, and ethanol were of reagent grade (≥ 99.9%).

3.3. Molluscicidal activity against *Radix peregra*

3.3.1. Adult molluscs

Adult snails of *R. peregra* (1.34 ± 0.20 cm length) were collected from water tanks (used for cattle breeding) or small freshwater bodies found nearby in the *Povoação* locality (southwest of São Miguel Island, Azores Archipelago) from February to August 2012 and 2013. Animals were maintained in a polyethylene aquarium (35.7 cm x 23.5 cm x 13.4 cm) containing 3 L of spring water (pH: 7.2±0.15; Total mineral content: 202±3.1 mg L^{-1}; dissolved Ca^{2+}: 4.4 mg L^{-1}) permanently aerated with air pumps, and fed with lettuce leaves (*Lactuca sativa* L.).

3.3.2. Preparation of EOs/compounds emulsions by ultrasonication

Emulsions were prepared according to the following method: EOs/compounds were initially diluted in pure ethanol (300 mg mL^{-1}). The dispersion of the oil fraction into a small volume of spring water was achieved using an ultrasonic homogenizer (sonopuls HD 2200 from BANDELIN) coupled to a 3 mm microtip (25-30% power; 5 s on-off cycles; 2 min) to obtain stock emulsions (33 mg EO per mL^{-1}). Different volumes were pipetted, according to the doses to be tested, and vigorously mixed with a final volumes of spring water (220 mL). Emulsions were left to stabilize 15 min before use.

3.3.3. Dose-response assay

The molluscicidal activity of *F. vulgare* EOs and two pure compounds against adults of *R. peregra* was evaluated by immersion test following WHO standard methods for laboratory screening of molluscicides (WHO, 1965). EOs/compounds emulsions were freshly prepared in concentrations ranging from 10 to 50 µg mL^{-1} (Table III.1) following the procedure described in the previous section 3.3.2. Four replicates of ten snails (*n*=40) were used for each concentration of EOs/compounds and for controls (0.1 µL mL^{-1} ethanol in spring water).

After 24 h, the mortality was recorded and confirmed by absence of reaction, namely by prodding snails with a needle. The surviving snails were rinsed in spring water to remove residues, transferred to new beakers containing an identical volume of spring water and fresh lettuce leaves, and allowed to recover for an additional 24 h, after which snail mortality was recorded again. All

experiments were performed in an incubator with controlled temperature (23 ± 1 ºC) and relative humidity (70 ± 5%) and under natural light:dark photoperiod.

3.4. Larvicidal activity against *Anopheles atroparvus*

3.4.1. Mosquito rearing

The laboratory colony of *A. atroparvus* was established from immature specimens captured in the Águas de Moura locality (Setúbal, Portugal) and has been maintained at the Instituto de Higiene e Medicina Tropical (IHMT) insectaria since 1989. Adults are fed with 10% (w/v) sucrose daily and, twice a week, with blood meals from anesthetized rats (*Rattus rattus* L.). Petri glass dishes containing dechlorinated tap water are kept inside the cage for oviposition. Larvae are nourished with a mixture of Tetra Menu and biscuits (1:1). The rearing temperature, relative humidity, and photoperiod in the insectaria are 26 ± 1 ºC, 70 ± 10%, and 13 h:11 h (light:dark), respectively.

3.4.2. Dose-response assay

Larvicidal activity of EOs/compounds against *A. atroparvus* was evaluated following the WHO laboratory guidelines with modifications (WHO, 2005). Stock solutions of EOs/compounds were first prepared as concentrated emulsions (0.5 mg mL^{-1}) by stirring 0.5 g of EOs, estragole or *trans*-anethole with 10 mL 10% (w/v) Tween® 20 in deionized water. Subsequently, five optimized concentrations of EOs/compounds (Table III.1) were prepared by mixing stock solutions with dechlorinated tap water (225 mL). Emulsions and control solutions of Tween® 20 (at the maximum concentration used in each experiment and equivalent to 0.04 mg mL^{-1}) were distributed in disposable plastic beakers (330 mL) and allowed to stabilize. Fifteen minutes later, 25 third-instar larvae of *A. atroparvus*, previously collected and kept in Petri glass dishes with 25 mL dechlorinated water, were gently placed into the emulsions.

Larvae mortality was determined after 24 and 48 h of continuous exposure (with no food provided). Motionless larvae, even when gently prodded with a pipette, were considered dead or moribund. Four replicates per concentration/control were run simultaneously and three independent experiments (total of 300/concentration) were conducted under laboratory-controlled conditions (25 ± 1 ºC; 50 ± 10% relative humidity; 13 h of photoperiod).

3.5. Activity against *Meloidogyne javanica*

3.5.1. *Meloidogyne javanica* isolate

The *M. javanica* isolate was kindly provided by collaborators from the Nematology Laboratory, University of Coimbra (Portugal), where this species is currently maintained on tomato plants, *Solanum lycopersicum* L. cv. Easypeel, in a growth chamber at 25 ± 2 ºC. The isolate has been previously characterized according to perineal morphology pattern (permanent slides deposited here), biochemical analysis of its isoesterase phenotype and molecular characterization of mtDNA *COII* and 16S rRNA genes regions (Abrantes et al., 2008; Maleita et al., 2012).

For the present study, the characterized isolate was maintained on a susceptible tomato cultivar (*S. lycopersicum* L., cv. "Tiny Tim") in the laboratory of the Biology Department, University of Minho. Plants were cultivated in pots containing sterilized sandy loam soil (50% of sand) in a growth chamber under controlled conditions (25 ± 2 ºC; 12 h photoperiod). The soil was moistened daily with tap water and fertilized once a week. Young plants, with four to five leaves, were inoculated with five *M. javanica* egg masses each. Eggs and juveniles in the second stage (J_2) were obtained 30 to 35 days later from the infected tomato roots (Figure III.1 A and B).

3.5.2. EOs/ compounds dispersion in water by ultrasonication

The concentrations of Fv-I and compounds required for biological assessments against *M. javanica* were considerably higher than those used in the other bioassays. Accordingly, an alternative process was used to avoid using substantial amounts of solvent and/or emulsifier to disperse the organic phase into water. Ultrasonication allows dispersion of EO microdroplets, forming homogeneous O/W mixtures. Bitter fennel EOs from infrutescences (3000 µg mL^{-1}), estragole and *trans*-anethole (1500 µg mL^{-1}) were homogenised with deionized water using high intensity ultrasounds generated by a standard 13-mm probe (amplitude set at 25-30%; on/off cycles of 20 s for 4–5 min) attached to a 400-W ultrasonic processor (VCX 400, Sonics & Materials, Inc., USA). Subsequent concentrations were obtained by dilution into deionized water and gentle agitation before use.

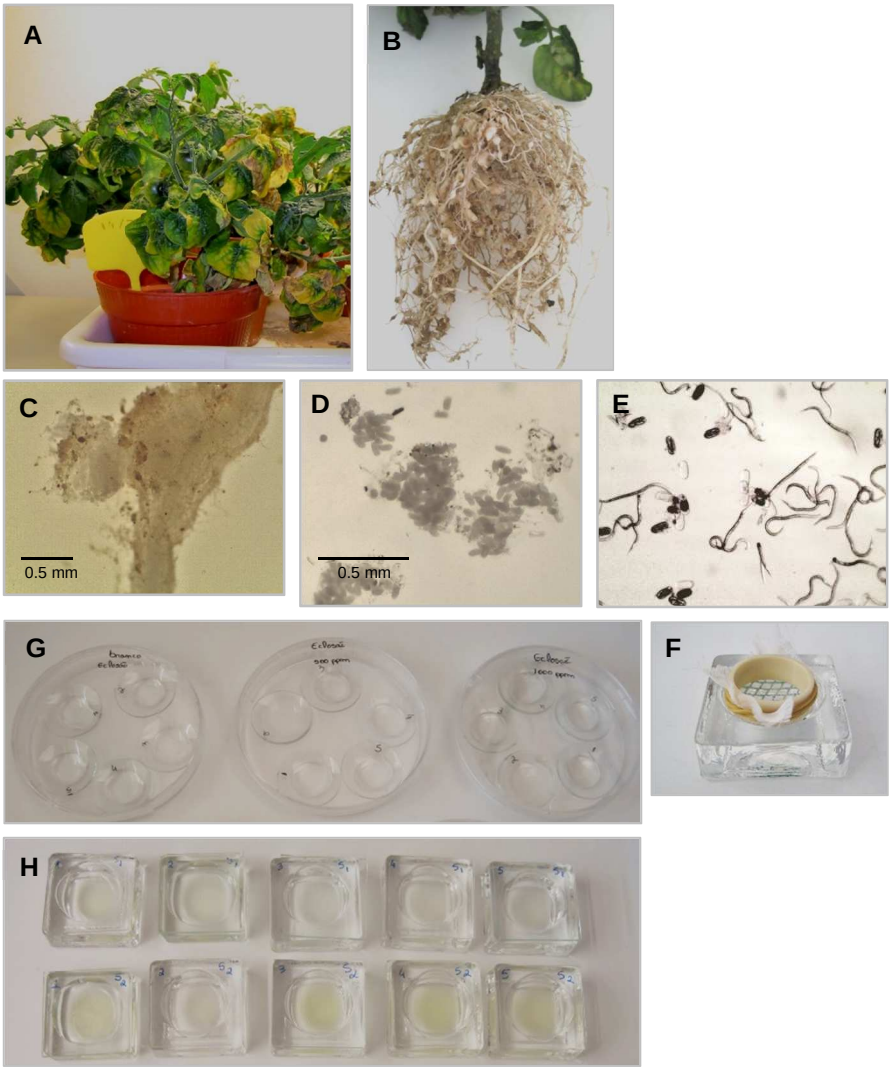


Figure III.1 (A-H). Maintenance of *Meloidogyne javanica* on a *Solanum lycopersicum* susceptible cultivar and isolation of eggs masses from the galls for hatching and nematicidal assays (pictures A, B, G, H and I were kindly provided by Cláudia Silva).

(A) Aerial parts of the infected tomato plants; **(B)** Infected tomato roots with galls; **(C)** Observation of infected root with a separated egg mass and **(D)** eggs with the stereomicroscope; **(E) U**nhatched and newly hatched J_2 (juvenile nematodes in the second stage); (**F)** System used for the hatching of J_2. Eggs masses are placed in a staining glass vial filled with distilled water and retained by a nylon net (with 30 µm holes), which is sustained in the liquid by a sieve and a plastic ring (2.5 cm of diameter and 0.9 cm high); **(G)** Hatching inhibition assay performed into small watch glasses; **(H)** Nematicidal assay performed into staining glass vials**.**

3.5.3. Hatching inhibition and nematicidal activity on second stage juveniles by continuous exposure

The chronic toxicity of Fv-I and the two standard compounds, estragole and *trans*-anethole, on eggs and J_2 nematodes was evaluated by long-term exposure assays (360 h). The egg masses were separated from previously washed infected tomato roots and transferred to small glass staining vials containing distilled water.

Approximately 20 eggs of *M. javanica* (with J_2 in a similar stage) (Fig. III.1, D) were carefully picked under a stereomicroscope and placed in watch glasses containing 100 µL of test solution (Fig. III.1, G). Inhibition of hatching by Fv-I, *trans*-anethole and estragole was assessed for three distinct doses: 1000, 2000, 3000 µg mL^{-1}; 500, 1000, 1500 µg mL^{-1} and 250, 750, 1000 µg mL^{-1}, respectively. Five replicates per concentration/controls with distilled water were included in each assay. All watch glasses were placed into sealed Petri dishes to prevent evaporation and maintained at 23 ± 1ºC in the dark. Hatched juveniles were counted every 24 h for 15 days (totaling 360 h of exposure).

Nematicidal activity against J_2 nematodes was also evaluated. The juveniles were obtained from egg masses maintained in water on a muslin support (Fig. III.1, F). The J_2 hatched during the first hours were discarded, and only individuals from the following 24 h were recovered. Groups of 20 J_2 nematodes (Fig. III.1, E) were collected with the aid of an eyelash, transferred to glass staining vials (5 mL capacity) containing 1 mL of solution and covered with an appropriate glass plate (Fig. III.1, H). Fv-I, *trans*-anethole and estragole were tested using 500, 1000, 3000 µg mL^{-1}, 250, 500, 750 µg mL^{-1} and 500, 750, 1000 µg mL^{-1}, respectively. Assays consisted of 5 replicates per concentration/controls with distilled water. J_2s were incubated at 23 ± 1 ºC in the dark, and mortality was followed every 24 h for 15 days (360 h). To distinguish mortality from immobility, immobile juveniles were individually transferred to a microscopic glass slide with a cavity containing 100 µL of distilled water. After a 1 h recovery, nematodes showing mobility were placed back into their respective glass staining vials, whereas those not reacting to touch with an eyelash were considered dead.

3.5.4. Dose-response assay

In the continuous exposure experiment, only estragole and *trans*-anethole showed acute nematotoxicity against J_2 of *M. javanica* in the first 48 h. Based on these results, the pure compounds were assayed in a range of five optimized concentrations (Table III.1) to estimate lethal concentrations at 24 and 48 h. Preparation of O/W mixtures and experimental procedures were as described above for long-term-assays (3.5.2).

Table III.1. Concentrations used in the dose-toxicity assays for determination of bitter fennel essential oils, *trans*-anethole and estragole lethal concentrations against the four organisms.

	Essential oil (µg mL^{-1})		**Compound** (µg mL^{-1})	
Species	**Infrutescences (Fv-I)**	**Stems and Leaves (Fv-L)**	***trans*-Anethole**	**Estragole**
Radix peregra	20, 25, 30, 40,45, 50	10, 20, 30, 40, 50	10, 20, 30, 40, 50	20, 30, 35, 40, 50
Anopheles atroparvus	25, 50, 75, 100, 150	25, 50, 75, 100, 150	12, 15, 20, 40, 50	25, 50, 75, 100, 150
Meloidogyne javanica	500, 1000, 2000, 3000	–	300, 350, 500, 700, 1000	750, 1000, 1250, 1500, 1750
Artemia salina	62.5, 75, 100, 125, 200, 250	75, 100, 125, 250, 300	75, 100, 125, 250, 300	125, 250, 375, 500, 625, 750

3.6. Brine shrimp lethality assay

The brine shrimp lethality assay was used to assess toxicity of Fv-I, Fv-L, estragole and *trans*-anethole. *Artemia salina* is a saltwater crustacean species frequently used as a test organism for toxicological assays, namely for preliminary evaluation of bioactive natural compounds (Sam, 1993; Solis et al., 1993). *Artemia salina* cysts (Dohse Aquaristik GmbH & Co. KG, Gelsdorf, Germany) were placed in a glass container filled with filtered natural seawater under constant aeration during 48 h, for hatching. Active nauplii were collected, and the second instar shrimp larvae were used in the bioassays. EOs/compounds toxicity against *A. salina* was evaluated in 96-well cell culture plates, adapting the methodology described by Solis et al. (1993). A range of 5–6 optimized concentrations (Table III.1) was obtained by adding stock solutions (1 mg mL^{-1}; EOs/compounds previously dissolved in ethanol and then in water) to wells containing filtered natural seawater. A 100 µL-suspension of nauplii containing 10-15 organisms was added to each well, and the covered multiwell plate was incubated at 25 °C. After 24 h, nauplii were examined under a stereo microscope (×12.5) and mortality recorded (non motile nauplii were considered dead). Acetone (100 µL) was added to determine the total numbers of nauplii. All experiments included 4–5 replicates and controls (ethanol at 12.5 µL mL^{-1}).

3.7. Statistical analysis

Mortality data recorded in dose-response experiments were corrected using Abbott's formula [1](Abbott, 1925). LC_{50} and LC_{90} (concentrations required to cause 50 and 90% mortality) and

[1] The formula (3) provided in the Appendix C.

their confidence limits, at 24 and 48 h, were determined by probit analysis using IBM *SPSS statistics* package 20.0 (IBM corp., USA).

Cumulative hatching and cumulative mortality of *M. javanica* eggs and juveniles in long-term exposure assays were calculated for each treatment and concentration. Areas under the curves of cumulative percentage hatching (AUCPH) and cumulative percentage mortality (AUCPM) were determined by integration of areas with *Prism* software 4.00. AUCPH/AUCPM mean values and the average accumulated hatching/mortality recorded at the end of the experiments were analyzed by one-way-ANOVA followed by Tukey's multiple range post-test (p = 0.05), without data transformation. Both criteria were used to discriminate concentration effects, as previously described by Pérez et al. (2003).

4. Results

4.1. Determination of lethal concentrations (LCs)

LCs of fennel EOs and phenylpropanoids against *R. peregra*, *A. atroparvus, M. javanica* and *A. salina* are shown in tables III.2 and III.3, respectively.

The tested EOs/compounds displayed noteworthy insecticidal and molluscicidal activities, with LC_{50} values ranging from 16 to 56 µg mL^{-1} and 21 to 39 µg mL^{-1}, respectively. No statistical differences were found between LCs determined for both EOs and estragole against *A. atroparvus* third-instar larvae. Comparing compounds activities, *trans*-anethole was 3.6–4.0 and 2.3–3.8 times more active than estragole against *A. atroparvus* and *M. javanica*, respectively (Table III.3). However, no differences were found concerning their molluscicidal activity after 48 h, including the 24 h recovery period. It is interesting to note that the root-knot nematode was much less affected by treatments than the other organisms; consequently, LC values were considerably higher (from 249 to 1736 µg mL^{-1}) (Table III.3).

Regarding the general toxicity assessment, Fv-I was significantly more toxic to *A. salina* than other treatments, and *trans*-anethole was 2-2.3 times more active than its isomer.

Table III.2. Toxicity of *Foeniculum vulgare* subsp. *vulgare* var. *vulgare* essential oil(s) to *Radix peregra* adults, *Anopheles atroparvus* 3rd instar larvae, *Meloidogyne javanica* second stage juveniles, and *Artemia salina* nauplii.

Species	**Time** (h)	**LC_{50}** [a,b] (95% CL)	**LC_{90}** [a,b] (95% CL)	**Slope** ± SEM [a,b] (95% CL)	***H*** [a, c]
R. peregra	24	33.5 c (32.0 – 35.1)	52.7 b (48.7 – 58.4)	6.51 ± 0.51 c (5.50 – 7.51)	2.50
	48 [d]	27.5 b (26.1 –29.0)	45.7 ab (41.2 – 53.3)	5.82 ± 0.65 c (4.55 – 7.10)	2.11
	24	39.0 cd (33.1 – 49.9)	145.6 cd (95.5 – 311.4)	2.24 ± 0.34 a (1.58 – 2.90)	0.87
	48 [d]	21.2 a (19.6 – 22.9)	42.0 a (38.2 – 47.3)	4.33 ± 0.33 bc (3.69 – 4.97)	0.57
A. atroparvus	24	51.9 d (47.5 – 56.2)	110.0 c (98.8 – 125.7)	3.9 ± 0.30 b (3.33 – 4.53)	1.40
	48	46.1 d (41.8 – 50.2)	101.5 c (91.0 – 116.5)	3.7 ± 0.30 b (3.15 – 4.31)	1.79
	24	53.0 d (48.7 – 57.3)	109.0 c (98.4 – 124.0)	4.1 ± 0.31 bc (3.48 – 4.71)	2.24
	48	50.8 d (46.4 – 55.1)	108.4 c (97.4 – 124.0)	3.9 ± 0.30 b (3.30 – 4.48)	2.37
M. javanica	24	> 3000	> 3000	-	-
	48	> 3000	> 3000	-	-
A. salina	24	102.6 e (94.6 – 110.9)	252.3 d (219.8 – 303.6)	3.28 ± 0.28 ab (2.73 – 3.83)	1.08
	24	149.6 f (134.4 – 166.7)	474.5 e (380.5 – 654.6)	2.56 ± 0.26 a (2.04 – 3.08)	1.65

[a] Estimated LC_{50}, LC_{90}, 95% confidence limits (CL) and *H* were determined by probit analysis based on mortalities after 24 h and 48 h of exposure and values are expressed in µg mL^{-1} of essential oils required to cause animals' death; [b] LC values and slopes within a column followed by the same letter are not significantly different based on non-overlapping of 95% CL; [c] *H*, heterogeneity factor (χ^2/ degree-of-freedom). [d] The 48-h toxicity against *R. peregra* corresponds to 24 h of exposure followed by a 24-h recovery period.

Table III.3. Toxicity of *trans*-anethole and estragole to *Radix peregra* adults, *Anopheles atroparvus* 3rd instar larvae, *Meloidogyne javanica* second stage juveniles, and *Artemia salina* nauplii.

Species	**Time** (h)	**LC_{50}** [a,b] (95% CL)	**LC_{90}** [a, b] (95% CL)	**Slope** ± SEM [a,b] (95% CL)	***H*** [a, c]
R. peregra	24	nd	nd	-	-
	48 [d]	29.9 b (28.2 – 31.6)	49.2 b (45.4 – 54.9)	5.93 ± 0.53 bc (4.89 – 6.97)	2.35
	24	39.2 c (37.4 – 41.4)	64.2 c (57.9 – 74.3)	5.99 ± 0.59 bc (4.83 – 7.16)	2.03
	48 [d]	28.8 b (27.4 – 30.1)	44.1 b (41.5 – 47.6)	6.93 ± 0.57 c (5.81 – 8.05)	2.12
A. atroparvus	24	15.7 a (14.8 – 16.7)	26.9 a (24.2 – 31.5)	5.5 ± 0.59 bc (4.21– 6.37)	1.04
	48	15.7 a (14.8 – 16.7)	26.9 a (24.2 – 31.5)	5.5 ± 0.59 bc (4.21– 6.37)	1.04
	24	56.1 d (52.2 – 60.0)	107.4 d (97.5 – 122.1)	4.5 ± 0.39 b (3.79 – 5.30)	0.51
	48	56.1 d (52.2 – 60.0)	107.4 d (97.5 – 122.1)	4.5 ± 0.39 b (3.79 – 5.30)	0.51
M. javanica	24	309.9 f (182.8 – 384.7)	633.4 efg (500.7 – 1231.9)	4.13 ± 0.76 abc (1.72 – 6.54)	2.95 [e]
	48	248.9 f (205.3 – 282.1)	532.7 e (482.3– 613.0)	3.88 ± 0.51 ab (2.88 – 4.88)	0.19
	24	1129.1 h (1086.0 – 1174.1)	1735.5 h (1626.7 – 1884.3)	6.86 ± 0.49 c (5.90 – 7.83)	1.15
	48	954.5 g (986.4 – 921.4)	1228.1 g (1178.4 – 1292.9)	11.71± 0.93 d (9.89 –13.53)	0.15
A. salina	24	129.2 e (115.8 – 143.1)	391.8 e (322.7 – 517.9)	2.66 ± 0.27 a (2.13 – 3.19)	1.58
	24	296.4 f (268.1 – 324.2)	794.9 f (696.1 – 943.7)	2.99 ± 0.24 a (2.52 – 3.46)	0.69

[a] Estimated LC_{50}, LC_{90}, 95% confidence limits (CL) and *H* were determined by probit analysis based on mortalities after 24 h and 48 h. Values are expressed in µg mL^{-1} of compounds required to cause animals' death; [b] LC values and slopes within a column followed by the same letter are not significantly different based on non-overlapping of 95% CL; [c] *H*, heterogeneity factor (χ^2/ degree-of-freedom); [d] The 48-h toxicity against *R. peregra* corresponds to 24 h of exposure followed by 24-h recovery period; [e] Significant heterogeneity, χ^2 (8. 851) is above the χ^2 critical value of 7.815 (3 degree-of-freedom and p >0.05). nd - Not determined.

4.2. Hatching inhibition and nematicidal activities against *Meloidogyne javanica*: Long-term exposure assays

The inhibitory hatching effects of *F. vulgare* var. *vulgare* infrutescence EOs, *trans*-anethole, and estragole on *M. javanica* exposed to three different doses for 360 h are illustrated in figure III.2, along with the results concerning the nematicidal activity against J_2, for the same time of exposure.

After 15 days of treatment, only *trans*-anethole and estragole caused clear ovicidal effects (hatching inhibition > 90%), at 1500 and 1000 µg mL^{-1}, respectively (Figure III.2, B and C). Inhibitory activities above 50% were recorded for bitter fennel EO (2000 and 3000 µg mL^{-1}) (Figure III.2, A), *trans*-anethole (1000 and 1500 µg mL^{-1}), and estragole (1000 µg mL^{-1}).

The EO from infrutescences displayed little nematicidal activity against J_2 juveniles throughout time (Figure III.2, D). Lethal activity above 80% was only recorded for higher doses of EO (1000 and 3000 µg mL^{-1}) in the last 72 h of the experiment, even though significant differences in their cumulative effects (AUCPM) were observed during the experimental period. Furthermore, satisfactory nematicidal activities (> 50% mortality before 48 h) were observed for *trans*-anethole tested at 500 and 750 µg mL^{-1} (Figure III.2, E), and estragole (1000 µg mL^{-1}) (Figure III.2, F). *trans*-Anethole at 750 µg mL^{-1} was the only treatment displaying a remarkable acute nematicidal activity within the first 24 h (> 80% mortality). Cumulative mortality values registered for 500 and 750 µg mL^{-1} of *trans*-anethole were not statistically distinct, although differences in their lethal effect during the course of the experiment (i.e., AUCPMs) were pronounced (Figure III.2, E).

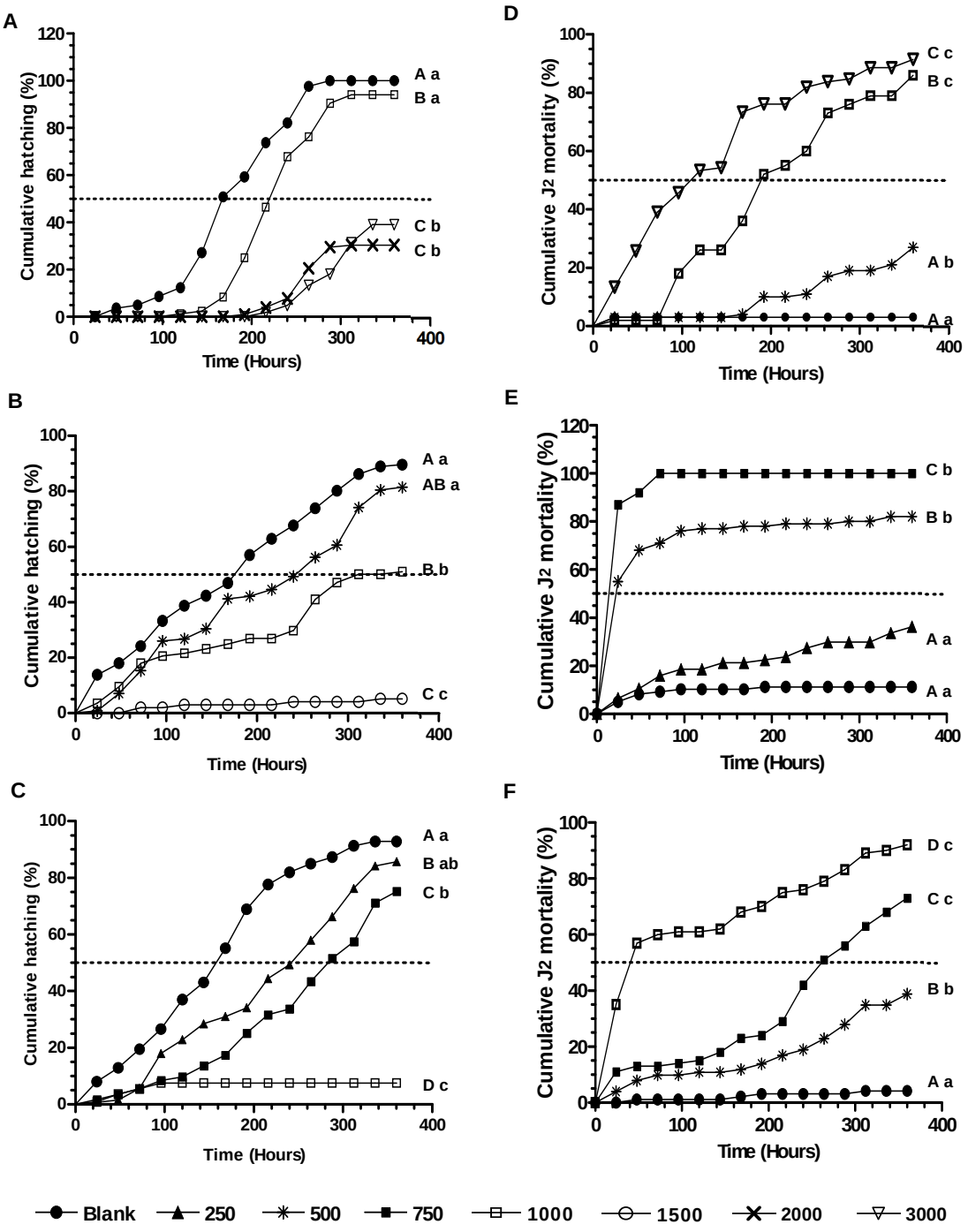


Figure III.2 (A-F). Activities of *Foeniculum vulgare* var. *vulgare* essential oil and two pure compounds, against *Meloidogyne javanica*, for 360 h of exposure to three different concentrations (µg mL^{-1}). Cumulative percentage of hatched eggs exposed to fennel infrutescences essential oil **(A)**, *trans*-anethole **(B)** and estragole **(C)**. Cumulative mortality of J_2 nematodes exposed to fennel infrutescences essential oil **(D)**, *trans*-anethole **(E)** and estragole **(F)**.

Curves from each treatment followed by the same upper-case letter do not differ significantly in their AUCPH/ AUCPM and those followed by the same lower-case letter do not differ significantly in their final cumulative hatching/mortality based on one-way ANOVA variance analysis with Turkey's multiple post-test ($p > 0.05$).

5. Discussion

As a complementary approach or alternative to synthetic pesticides, EOs and volatile compounds of numerous plant species are currently under consideration as components of crop protection products as well as in repellent formulations to prevent disease transmission (Shaaya & Rafaeli, 2007; Regnault-Roger et al., 2012; Suwansirisilp et al., 2013). Several nematicidal candidates of botanical origin have also been proposed and thoroughly reviewed (Andrés et al., 2012; Ntalli & Caboni, 2012). The use of endemic plants as sources of botanical products is a common practice, especially in poorer countries. In our opinion, the survey and identification of local species suitable for cultivation as potential sources of biological pesticides comprise a preemptive and sustainable strategy to meet forthcoming needs for the control of vectors, hosts and parasites in a specific area of the globe. Therefore, we have performed this comparative study on the biopesticidal activity of this fennel variety considering that its cultivation presents some advantageous aspects, namely this variety is widespread, well adapted to the temperate climates of the Mediterranean region, hemicryptophyte with low requirements for nutrient and water, resistant to pests and highly productive. The EOs selected for toxicity assessments (EOs from infrutescences and stems with leaves) were characterized by distinct quantitative profiles (Table II.12). Despite its minor content in the tested EOs, t*rans*-anethole was included in this study not only because of its structural/biosynthetic relationship with estragole, but also because of its abundance in some of the plants EOs (Table II.12), as discussed in Chapter II.

In general, EOs and compounds showed distinct performances against the tested organisms (Table III.2). As an exception, LCs of EOs for *A. atroparvus* larvae after 24 and 48 h were not statistically different. Adult snails were highly susceptible to EOs and estragole, while *trans*-anethole was more active than its isomer against the remaining species (LC_{50} varying from 16 to 310 mg L^{-1} at 24 h) (Table III.3). In fact, compounds showed contrasting activities, with *trans*-anethole being 2-2.3, 3.6-4.0 and 2.3-3.8 times more toxic against *A. salina*, *A. atroparvus* and *M. javanica*, respectively, than estragole (Table III.3). Previous reports have demonstrated the strong larvicidal activity of fennel EOs against other mosquito species (LC_{50} inferior to 50 mg L^{-1}), which was attributed to the major compound *trans*-anethole (Chantraine et al., 1998; Traboulsi et al., 2005; Pitasawat et al., 2007). In contrast to the present findings, Oka et al. (2000b) and Ntalli et al. (2011) showed that *F. vulgare* EO exhibit high activity against *M. javanica*. The distinct EO profiles (*trans*-anethole and limonene were the major EO compounds in both studies), distinct methodology for the preparation of solutions and/or different susceptibilities of field populations are the most likely explanations for such differences. Considerable toxicity to J_2 of *M. incognita* was also attributed to *F. vulgare* flowers EO, after 24 h (100 mg L^{-1} caused 86% mortality) (Hibrahim et al., 2006).

Potential molluscicides of vegetable origin are often tested against *Lymnaea acuminata,* another intermediate host. *Myristica fragrans* (Jaiswal & Singh, 2009), *Ferula assafoetida*, *Syzygium aromaticum* and *C. carvi* (Kumar & Singh, 2006) were identified as having molluscicidal properties

against the former species (LC_{50} < 100 mg L^{-1}). On the other hand, some aromatic plants of the Apiaceae family showed satisfactory larvicidal activities against *A. aegypti* (Lee, 2006) and *C. pipiens* (LC_{50} values from 40 to 100 mg L^{-1}) (Evergetis et al., 2009). Likewise, EOs extracted from *Lavandula gibsoni* and *Plectranthus mollis* (Lamiaceae) were very active against *C. quinquefasciatus*, *A. aegypti* and *A. stephensi* larvae (LC_{50}=25 to 63 mg L^{-1}) (Kulkarni et al., 2013). Moreover, EOs from *Thymus serpyllum*, *Amyris balsamifera* and *Juniperus virginiana* exhibited noteworthy larvicidal activities against the three former mosquito species (LC_{50} < 20 ppm, after 12 h) (Amer & Mehlhorn, 2006). EOs from *Satureja montana* exhibited remarkable nematostatic activity against *M. javanica* second-stage juveniles (LC_{50} =41 mg L^{-1} after 72h) (Andrés et al., 2012). Several species of *Mentha* sp. and *Thymus* sp. (Lamiaceae) have also proven their efficacy against *M. javanica* (LC_{50} ranging from 300 to 204 mg L^{-1}) (Andrés et al., 2012) and *M. incognita* (LC_{50} between 300 and 1000 mg L^{-1} for EO or water extract from mints), another root-knot nematode of utmost importance (Caboni et al., 2013a). EOs from *Carum carvi*, *F. vulgare*, *Mentha rotundifolia*, and *M. spicata* showed the highest nematicidal activities among 27 plants included in an *in vitro* assessment against *M. javanica.* In addition, strong effects on *M. javanica* hatching and juveniles' immobilization were also reported by the same authors, for the pure constituents *trans*-anethole, carvacrol, thymol, and (+)-carvone at concentrations superior to 125 µL L^{-1} (Oka et al., 2000b). Studies about the effect of plants on nematodes are currently increasing and significant progresses were undertaken in the last decade to investigate the nematicidal activity of plant EOs and their constituents. However, their MOA against nematodes is still not fully understood (Andrés et al., 2012).

Several EO constituents and plant volatiles have been assayed for their molluscicidal, larvicidal and nematotoxic activities. The notable activity of *trans*-anethole (LC_{50} =16 mg L^{-1} after 24 h) against *A. atroparvus* found in this study is in agreement with a previous report using *A. aegypti* (LC_{50} = 15 mg L^{-1}) (Chantraine et al., 1998). Likewise, a significant nematotoxic effect of *trans*-anethole against *M. javanica* was previously reported (199 µL L^{-1} after 48 h) (Oka, 2001). This compound was also effective as a nematicide against *M. incognita* (140 mg L^{-1} after 48h) (Ntalli et al., 2010), while 200 µg mL^{-1} estragole caused paralysis of 50% of J_2 of the same species (Ntalli et al., 2011). Thymol, *trans*-anethole, pulegone and eugenol displayed good larvicidal activities against *A. aegypti* third-instar larvae (LC_{50} values from 27 to 83 mg L^{-1} after 24 h) (Waliwitiya et al., 2009). The purified compounds myristicin (Jaiswal & Singh, 2009), limonene and eugenol (Kumar & Singh, 2006) were identified as noteworthy molluscicides against *L. acuminata* (LC_{50} = 1.5, 5.8 and 11 mg L^{-1} after 24 h, respectively). Generally, oxygenated compounds, namely ketones (L-carvone and pulegone), alcohols (geraniol and terpinen-4-ol), and phenols (eugenol, carvacrol and thymol) have revealed good nematotoxic properties (LC_{50} from 115 to 754 mg L^{-1} after 24 h) (Ntalli et al., 2010; Caboni et al., 2013a). Volatile aromatic aldehydes, including benzaldehyde derivatives, have been recognized for their outstanding nematotoxicity against J_2 of *M. javanica* (50% of paralysis for concentrations between 13 and 33 µL L^{-1} after 48 h) (Oka, 2001), and *M. incognita* (LC_{50} between 11 and 167 mg L^{-1} after 24 h) (Caboni et al., 2013b).

Based on LC_{50} and LC_{90} evaluations, Fv-I was significantly more active than Fv-L and the phenylpropanes derivatives tested separately, against *A. salina* nauplii. The toxicity screening performed with *A. salina* suggests that EOs and *trans*-anethole may be considered more toxic (LC_{50} between 103-150 µg mL^{-1}) than estragole (LC_{50} = 296 µg mL^{-1}). According to Padmaja et al. (2002) the general toxic activity of plant extracts can be considered strong when their LC_{50} against *A. salina* range from 0 to 100 ppm, and moderate when values vary between 100 and 500 ppm.

In the present work, it was not possible to establish direct relations between the bioactivity of each bitter fennel EO against *R. peregra, A. atroparvus* and *A. salina* and the individual activity of tested phenylpropanoids. Notwithstanding, it is important to emphasize that individual assessment of the various constituents is not necessarily useful to elucidate the toxicity of such complex mixtures (the whole probably not being the sum of its parts), and that synergisms/antagonisms between constituents ultimately define the specificity of the mixtures. In fact, Ntalli et al. (2011) stated that no clear or linear relation could be established between the main chemical constituents and the nematicidal activity of the tested EOs. Though, it has been shown that *trans*-anethole had, in most cases, synergistic interactions in binary combination with several other compounds, while combinations with estragole conduced to additive effects or antagonisms on *M. incognita* J_2 motility (Ntalli et al., 2011).

6. Conclusion

The present study demonstrates the efficacy of *F. vulgare* subsp. *vulgare* var. *vulgare* EOs, and two of its compounds as promising biochemical agents for control of a potential fascioliasis intermediate host, *R. peregra*, and one European malaria vector, *A. atroparvus*. The molluscicidal activity was significant as respective LC_{90}s were lower than 100 mg L^{-1} after 24h of exposure (WHO, 1983). The toxicity screening performed against *A. salina* nauplii suggests that EOs, *trans*-anethole and estragole are moderately toxic (Padmaja et al., 2002).

However, the use of bitter fennel EO do not seems an attractive solution for the control of *M. javanica* populations, because the concentrations required to decrease hatching and juvenile survival are considerably high. Moreover, the high resistance of *M. javanica* J_2 before hatching was evidenced. Nonetheless, it is worth mentioning that direct use of dried bitter fennel infrutescences as a soil amendment to prevent the root penetration of *M. javanica* J_2, and, consequently, to effectively reduce the population size could be a good alternative (Silva, 2012). Bitter fennel EOs and its phenylpropene-derivatives also showed promising properties as natural mosquito larvicides and molluscicides. Furthermore, the compound *trans*-anethole, which has been recognized for its several biopesticidal activities, could be incorporated into insecticidal and molluscicidal formulations. The preliminary results presented herein give a strong indication of the potentiality of tested EOs and compounds, especially as molluscicides, which justify further investigations.

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CHAPTER IV

Molluscicidal activity of four Apiaceae EOs against the freshwater snail, *Radix peregra* (Gastropoda: Lymnaeae)

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1. Abstract

Freshwater snails (Gastropoda: Lymnaeae) play a crucial role in transmission of fascioliasis and other trematodiases of major medical and veterinary relevance. *Radix peregra* is a European freshwater snail and a susceptible intermediate host of *Fasciola hepatica*, the causing agent of fascioliasis.

Essential oils (EOs) extracted from *Anethum graveolens* (dill), *Cuminum cyminum* (cumin), *Foeniculum vulgare* var. *vulgare* (bitter fennel) and *Petroselinum crispum* (plain leaf parsley) were characterized by GC-FID and GC-MS. Seven EOs and 11 constituents were first screened through a single-dose assay against *R. peregra* egg masses (0–48 h-old) and snails (juveniles and adults). EOs from parsley, cumin and bitter fennel (leaves and stems) were highly active towards embryos and adults at 50 mg L^{-1}. Subsequently, dose and time-lethality assays were performed against adult in order to determine lethal parameters ($LC_{50;\,90}$ and $LT_{50;\,90}$). Estimated LC_{50s} (48 h), varied from 13.7 to 46.5 mg L^{-1}, with *P. crispum* fruits EO exhibiting the most significant activity. EOs from cumin fruits and bitter fennel infrutescences, and cuminaldehyde, were the most time-effective treatments when assessed by continuous exposure (LT_{50} at 50 mg L^{-1} = 15.1, 19.3 and 19.5 h, respectively). Snails' exposure to bitter fennel EOs for 8 and 16 h still was effective for the control of adults ($LT_{50} \leq 25$ h).

The present study uncovers the potential of the four well-known Apiaceae species as natural sources of biomolluscicides. As far as we know, this is the first report of the molluscicidal activity of parsley, dill and cumin EOs against a host freshwater snail.

Keywords: Alternative intermediate host, fascioliasis, biomolluscicides, bitter fennel, cumin, dill, parsley

2. Introduction

As intermediate hosts of plant-borne trematodes, snails of the family Lymnaeidae (Gastropoda: Pulmonata) play a key role in the transmission of important zoonotic diseases. Lymnaeids species are freshwater pulmonate snails (breathing air directly via the lung-like mantle cavity) that can inhabit a variety of wet and aquatic habitats, from large lakes to small temporary ponds (Watson & Dallwitz, 2005). Around 20 species of freshwater snails have been described as intermediate hosts of digenean trematodes (Correa et al., 2010), including species of the genus *Fasciola* (Digenea: Fasciolidae) the causing agents of fascioliasis. The occurrence of *Fasciola hepatica* Linnaeus, also known as the sheep fluke or common liver fluke, is extended to the five continents, while the species *F. gigantica* Cobbold is found predominantly in tropical areas in Asia and Africa (Mas-Coma et al., 2005). Both liver flukes parasites a broad range of herbivorous mammals. The definitive hosts are primarily cows, sheep, goats, buffaloes, but also several other species (namely donkeys, horses, camels, llamas, pigs, *etc*.) including man (WHO, 1995; Mas-Coma et al., 1997; Mas-Coma et al., 2005; Correa et al., 2010). Fascioliasis is recognized as a major veterinary problem of vertebrate domestic livestock, causing decrease of animals' productivity and considerable economic losses (Correa et al., 2010). Furthermore, it is one of the most important disease with higher public-health impact (Savioli et al., 2010). The spreading power of fascioliasis is related to the remarkable adaptive abilities of fasciolids to colonize new environments, and to infect different intermediate and definitive hosts species all over the world (Mas-Coma et al., 2005). Moreover, climate changes and abnormal environmental phenomena may greatly influence host-parasite interactions, and by consequence the epidemiological patterns of fascioliasis and other trematodiases (Mas-Coma et al., 2009; Paull & Johnson, 2011). After been underestimated for decades, trematodiases have attained the status of emerging/re-emerging parasitic disease. In 2005, the World Health Organization (WHO) estimated that more than 56 million people worldwide were infected with foodborne trematodes (Daumerie & Savioli, 2010).

The natural and primary intermediate host of *F. hepatica* in Europe, is *Galba* (sin. *Lymnaea*) *truncatula* (Bargues et al., 2001). Besides, further Lymnaeids from European populations have been identified as alternative intermediate hosts of this fasciolid under special natural conditions (Dreyfuss et al., 2000; Bargues et al., 2001). Young specimens of *Radix* (sin. *Lymnaea*) *peregra* Müller (Lymnaeidae) were found to be susceptible to *F. hepatica* infection in laboratory conditions (Sindou et al., 1991; Dreyfuss et al., 1997). Moreover, *R. peregra* infection by the giant liver fluke *Fascioloides magna* (Fasciolidae) in localities of Czech Republic, suggest that this species may also display a role as a fascioloidosis vector in cervids and domestic ruminants (Faltynkova et al., 2006), besides been a facultative host of *F. gigantica* in near east, middle east, and southern states of the old Soviet Union (Bargues et al., 2001). The prevalence of this species in some habitats where the *G. truncatula* is less abundant, and the occurrence of cattle fasciolosis in some of these area, gives additional evidences of its potential as an alternative or secondary intermediate of the common liver flukes (Relf et al.,

2009). In the Azorean island of S. Miguel, the density of *R. peregra* is effectively increasing in some locations where *G. truncatula* populations were previously found (Teixeira et al., 2011), including areas with identified cases of fasciolosis (Martins, 1991).

As all plant-borne trematodes, including flukes, require aquatic intermediate hosts to accomplish part of their life cycle, the elimination of this essential link is the most effective way of preventing transmission of zoonoses caused by trematodes, such as fascioliasis and schistosomiasis (WHO, 1995; Singh et al., 2010). The drainage of water bodies and/or treatment with synthetic molluscicides (i.e. niclosamide, sodium penthaclorophenate, copper sulphate, *etc*.) are possible measures stated by the World Health Organization for the control of these intermediate hosts (WHO, 1995). Most of the attempts of chemicals application have been to eliminate cases of animal fascioliasis (WHO, 1995). Organization (WHO) recommends niclosamide to be used in management programs, although, its high cost, toxicity to non-target organisms and the possible development of resistance in the mollusk rises some concerns (WHO, 1993). In order to mitigate the impact on environment (accumulation of chemicals residues on aquatic systems and organisms), several efforts have been developed to discover molluscicidal products of natural sources (Singh et al., 2010) that could present lower risks. In that context, with basis on extensive history of uses, effectiveness, low cost and reduced toxicity, plants have been considered as ideal and sustainable sources of bioactive chemicals to control snails' populations (WHO, 1983; Sigh et al., 1996). Furthermore, the biotechnological production of plant-based pesticides might become a developing segment of the bio industry (Singh et al., 2005).

The most frequently assessed plants species are mostly included in the Euphorbiaceae, Asteraceae, Leguminosae, Phytolaccaceae, Apocyanaceae and Solanaceae families (WHO, 1983; Singh et al., 2005). The significant molluscicidal activity of plant extracts against harmful snail species have been related to the presence of tannins, alkaloids, diterpenoids, triterpenoids, steroids, flavonoids and/or saponins (WHO, 1983; Singh et al., 2005). Nevertheless, others plant families, namely Apiaceae (Umbellifereae), have received less attention. Apiaceae species have shown a large spectrum of activities against insect species (Evertis et al., 2009; Yeom et al., 2012; Evertis et al., 2013; Kim et al., 2013; Sousa et al., 2013), and were found to influence the neurophysiology and feeding behavior of the field slug, *Deroceras reticulatum* (Dodds et al., 1999; Birkett et al., 2004), but these remain undervalued with regard to their potential as freshwater snails control agents. Thus far, extracts of *Trachyspermum ammi*, *Ferula assafoetida* and *Carum carvi* were formerly tested against the liver flukes natural host, *Lymnaea acuminata* (Singh et al., 1997; Kumar & Singh, 2006). However, to the extent of our knowledge, studies concerning the potential of Apiaceae EOs for the control of trematodes intermediate hosts are by far scarcest.

In the present work, the application potential of EOs extracted from four well-known and worldwide cultivated Apiaceae species [*Anethum graveolens* L., *Cuminum cyminum* L.*, Foeniculum vulgare* Mill., and *Petroselinum crispum* (Mill.) Nym ex A.W. Hill] as biomolluscicides was appraised. For this purpose, we used *Radix peregra* Müller, a facultative intermediate host of several trematodes

in Europe, including *Fasciola hepatica*, to perform screening assays against several stages of this freshwater snail (embryos, newly hatched juveniles and adults) and to investigate dose- and time-dependent activities on adults. Furthermore, the time-activity study was accomplished with three distinct exposure duration to ascertain whether any of the treatments effectiveness was affected by this parameter and in which extent. In an attempted to relate activities with EOs composition, some volatiles constituents were included in the assessments.

3. Materials and methods

3.1. EOs and chemicals

The Apiaceae EOs previously described in table II.5 were herein assessed for their molluscicidal activity. In the present study several individual pure terpenoids and phenylpropene-derivatives, previously identified as constituents of the EOs, were also assayed. *trans*-Anethole (99%), (S)-(+)-carvone (96%), cuminaldehyde (98%), estragole (98%), (+)-fenchone (99.5%), γ-terpinene (≥ 97%), (-)-β-pinene (99%), (-)-α-pinene and (+)-α-pinene (98%) were purchased from Sigma-Aldrich and Fluka (Aldrich chemical Co., St. Louis, MO, USA) and the compounds myristicin (≥ 95%) and apiole (≥ 90%) were separated from parsley fruit EO by column chromatography fractionation procedures as described in Chapter II (section 3.4). The compound $CuSO_4$ (pro analysis grade, purity 99%) purchased from Merck) was used as a positive control. Solvents (*n*-hexane, ethyl acetate and ethanol) were of reagent grade (≥ 99.9%).

3.2. Activity against *Radix peregra*

3.2.1. Animals

Adult snails of *Radix peregra* (1.34 ± 0.20 cm in length) were caught in small freshwater bodies nearby water tanks used for cattle breeding, in the *Povoação* locality (Southwest of São Miguel island, Azores Archipelago, Portugal) (Fig. IV.1, A and B). The species identification has been confirmed by a malacologist, Professor Frias Martins A., and specimens (accession number DBUA-MT1533) are deposited in the molluscs collection of the Biology Department at the University of the Azores (Ponta Delgada, São Miguel island). The animals were maintained as described in the chapter III, section 3.3.1 and were acclimatized under laboratory conditions (natural photoperiod and 23 ± 1ºC) for at least 72 h (Fig. IV.1, C). In order to prevent any contamination, the aquarium was cleaned three times a week by removing excrements and dead snails, and by renewing the water (Fig. IV.1, D). Some adult snails were outbred in the laboratory (Fig. IV.1, E).

Prior to the ovicidal assays, freshly laid egg masses (0 to 48 h-old) were observed under the stereoscopic microscope to confirm their viability and ascertain their initial stage of embryonic development (4-8 cells) (Fig. IV.1, F and G). In addition, several egg masses were maintained under constant artificial aeration in a separated container until hatching (Fig. IV.1, H and I). Juvenile snails with 48 to 72 h-old, were selected for the molluscicidal assays (Fig. IV.1, J and K).

3.2.2. Ovicidal activity

To assess the ovicidal activity of EOs/compounds*, R. peregra* egg masses with less than 48 h (Fig. IV.1, G) were exposed to single-dose treatments of EOs/compounds (50 mg L^{-1} of spring water). Masses containing around 32 (± 9.3) viable snail embryos were placed in a volume of 10 mL of freshly EOs/compounds emulsions prepared as described in the chapter III, section 3.3.2. Each treatment included at least 6 replicates (6-well plates). Two sets of negative controls were included and performed simultaneously, one with spring water and another with ethanol at the concentration equivalent to the one existing in EOs/compounds emulsions (0.1 µL mL^{-1}). Egg masses were incubated under controlled temperature (23 ± 1 ºC) and relative humidity (70 ± 5%), and exposed to natural photoperiod. Eggs were daily inspected to follow their development. The ovicidal activity of treatment was evaluate by calculation the percentage of hatching (snail emerged from the capsule) 15 days later when the hatching in the control groups was completed (≥ 95%). Embryos were considered dead whenever cells became disaggregated and if they remained unhatched at the end of the experiment.

3.2.3. Single-dose screening assays against juveniles and adults

The single-dose screening for EOs/compounds molluscicidal activity was performed against juveniles and adults of *R. peregra* following the immersion method recommended by the WHO (1965; 1983). Groups of ten juveniles, were gently placed in 6-well plates (10 per wells) with the help of a micropipette and a large tip. After removing the water, 10 mL of emulsions (10 mg L^{-1}) were carefully dispensed in each well. Concerning the immersion of the adults, groups of ten snails were transferred into glass Petri dishes and distributed randomly by disposable plastic beakers containing 220 mL of EOs/compounds stabilized emulsions (at 50 mg L^{-1} of spring water). To prevent adults from falling out or escape, cups were covered with a perforated plastic film or a Petri dish (Fig. IV.2, A, B and C). Emulsions were freshly prepared as described in the chapter III, section 3.3.2.

A positive control with copper sulphate was included in both assays at the same concentration used for EOs/compounds. Two sets of negative controls, one with spring water and the other with ethanol at the concentration equivalent to one used in emulsions (0.02 mL L^{-1} for juveniles, and 0.1 mL L^{-1} for adults), were performed simultaneously. Four to six replicates of ten snails per EOs/compounds/controls were included in each single-dose assay ($40 \leq n \leq 60$).

Snails' mortality was first determined after 24 h of exposure (Fig. IV.2, D). The surviving snails were rinsed with spring water to remove emulsions residues, transferred into new cups containing an identical volume of spring water and fed with fresh lettuce leaves. Following a 24 h recovery period, the percentage of dead juveniles and adults was determined once again. The mortality was monitored by observation with a stereo microscope. Juveniles' death was confirmed by absence of heartbeat, whereas adults were considered dead when the deterioration of the tissues was evident (foot discoloration and fluids leakage) (Fig. IV.2, E) or if they failed to react when prodded (typical withdrawal movements). Experiments were carried out under natural photoperiod at 23 ± 1 ºC and 70 ± 5% of RH.

3.2.4. Dose–response assay against adults (LCs)

The information obtained in the previous assay allowed to establish a range of concentrations for determining the LC values of the most lethal treatments (adults' mortality > 80%, after the recovery period). Furthermore, despite the first indication of its lower molluscicidal activity revealed in the preliminary assay, the compound (*S*)-(+)-carvone was also considered for further evaluations. Owing to several reasons (high availability, lower cost, and higher solubility in water) this oxygenated monoterpene exhibit great applicability in the context of plant-based biopesticides. Moreover, its bioactive potential will be discussed in the following chapters (V and VI).

The selected EOs and compounds were tested in the range of 10 to 60 mg L^{-1}, following the procedures described in the previous sections 3.2.2 and 3.2.4. The positive control ($CuSO_4$) was tested at concentrations ranging between 0.1 and 1 mg L^{-1}. To establish a dose-response relationship, four to six replicates of ten snails per concentration, and at least four concentrations per EO/compound were used ($160 \leq n \leq 300$). The mortality percentage was recorded in the end of exposure (24 h) and after the 24 h recovery period to determine the concentrations causing 50 and 90% of snails' mortality (LC_{50} and LC_{90}).

3.2.5. Time–-response assay against adults (LTs)

A time-lethality evaluation was performed for the most active EOs/compounds and (*S*)-(+)-carvone following the methodology above described. For this time-dependent study we assessed the effects of treatment as consequence of distinct times of exposure. The single concentration of 50 mg L^{-1} was chosen with basis on its proximity to most of LC_{90} values (excepting for Pc-F and carvone) determined after the 24 h recovery period. Following the same criteria, the $CuSO_4$ was assessed at 0.75 mg L^{-1}.

The time-lethally was assessed by continuous and short-term exposure (8 and 16h). Adult snails that were exposed to treatments during 8 and 16 h were rinsed with spring water and left to recover in

clean beakers containing fresh water and lettuce leaves. Subsequently, the mortality was monitored frequently, every 2 h or every hour if acuter and rapid effects were noticed. Snails continuously submitted to treatments were monitored every 2 h and the mortality percentage recorded up to 90% of death. Times of all three experiments started counting from the beginning of the exposure. We used four replicates (n = 40) for each assays and per treatment.

3.3. Statistical analysis

Data obtained in the single-dose assays (hatching and mortality percentages) were transformed by the arcsine function prior to the analysis of variance by one-way ANOVA. Means of treatments were separated at 5% significance level by LSD test, available on IBM *SPSS statistic package* (version 20.0 for Windows, IBM corp., USA, 2011). Mortality percentages recorded in dose- and time-dependent experiments were corrected with the Abbott´s formula (Abbott, 1925) whenever mortality in the negative control exceeded 5%. LC_{50} and LC_{90} after 48h (24 h of exposure more 24 h of recovery) were determined by probit analysis according to Litchfield and Wilcoxon (1949), using IBM SPSS *statistic package*. The same linear model was applied to time-response data, to determine EOs/compounds LT_{50} and LT_{90} (times required to cause 50% and 90% of adults' mortality) at the dose of 50 mg L^{-1}. Pearson qui-square (χ^2) test was used to assess the goodness-of-fit. A good fit of data to the model was found whenever χ^2 values were lower than χ^2 critical values for n-2 degrees of freedom, at 5% of significance level. Treatments' activity was considered significantly different whenever the 95% confidence intervals of lethal concentrations or lethal times failed to overlap.



Figure IV.1 (A-K) Collection of freshwater snails, *Radix peregra*, in Southwest of São Miguel Island (Azores Archipelago) and their maintenance in the biology department at the Azores University.

(A) *Radix peregra* adults were collected in water tanks used for cattle breeding and from small ponds found nearby in the southwest of São Miguel Island. **(B)** After cautious washings, animals were transported with water into a plastic container. **(C)** Adult snails were in a polyethylene aquarium with spring water and lettuce. (**D)** After being acclimatized to the lab condition, adults of the same size were used for bioassays. **(E)** Observation of adult snails (× 6 magnified with a stereo microscope). The arrow is indicating an egg mass attached to the shell of one reproductive individual (bottom). **(F)** Observation of egg mass under low magnification with a stereo microscope (× 6 magnified) to select the viable embryos. (**G)** Examination of an egg mass containing viable embryos at the four-cell and eight-cell division stages (×12 magnification). **(H)** Developing snail in the trochophore stage (× 50 magnified). **(I)** Juvenile surrounded by the egg capsule, in its latter stage of development before the hatching event (× 50 magnification). The eye spots, heart, foot and the spiral shell are already formed. **(J)** and **(K)** Observation of newly hatched juveniles (× 50 magnified) used in bioassays. The large and thin arrows are showing the radula and the eyes, respectively.

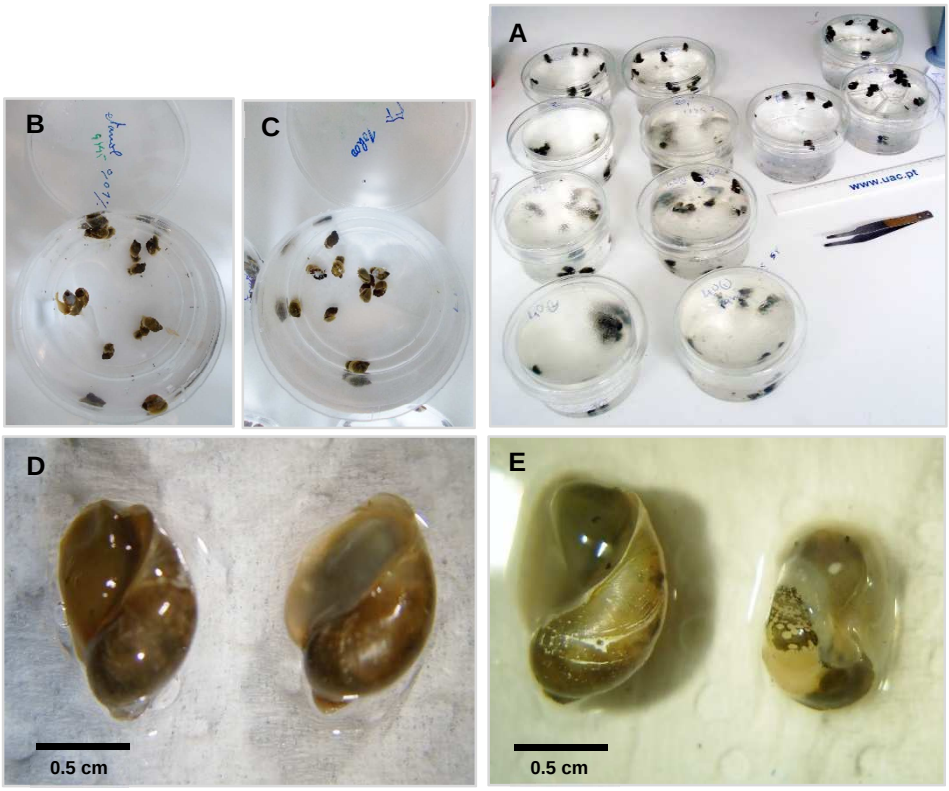


Fig. IV.2 (A-E) Evaluation of the molluscicidal activity of essential oils/ compounds and their effects on *Radix peregra*.

(A) The immersion test performed with adult snails; **(B)** Control with ethanol (0.1 mL L^{-1}); **(C)** Single-dose assay with bitter fennel EO; **(D)** Alive and dead snails observed after 24 h of exposure to ethanol (left) and bitter fennel EO (right), respectively; **(E)** Dead snails after 24 h of exposure to EOs plus 24 h of recovery period. On the left: individual with a swelled foot showing discoloration and deterioration of the tissue with fluids leakage. The soft body of a dead snail is shown on the right side.

4. RESULTS

4.1. Ovicidal and molluscicidal activity

The lethal activity recorded for the seven EOs and eleven volatile compounds tested against *R*. *peregra* eggs, juveniles and adult are presented in figure IV.3 (A-C). In general, EOs from the four Apiaceae species and their major compounds displayed distinct toxicities against the embryonic and post-embryonic stages of *R. peregra* when tested at a unique dosage.

The ovicidal activities recorded for treatments (Fig. IV.3, A) were highly variable (hatching varied from 0 to 96%) and significantly distinct (F-value= 49.378, *df* = 133, *P* = 0.000). In particular, Pc-F, (-)-β-pinene, Cc-F, S-(+)-carvone, (-)-α-pinene, myristicin, (+)-α-pinene, cuminaldehyde, Pc-I and Fv-L (cited by decreasing order of activity) exhibited the most interesting effects against juveniles hatching (mean hatching values ≤ 25%). Nonetheless, only the first two treatment showed comparable values of mortality comparatively to the positive control. On the contrary, Fv-I and γ-terpinene showed no toxicity against the eggs at the concentration tested (not distinct from the negative controls). Satisfactory or moderate ovicidal activities were attributed to the remaining treatments [Ag-I, (+)-fenchone, apiole, *trans*-anethole, Ag-L and estragole] for which the hatching ranged from 37 to 71%.

As shown in the figure IV.3 B and C, newly emerged and adult snails showed different susceptibility to EO and compounds. Snails' susceptibility to Fv-I and several compounds, namely trans-anethole and estragole were dissimilar. Treatments displayed different mortality rates against both forms of *R. peregra* as a result of the different doses tested (10 mg L^{-1} and 50 mg L^{-1}). When comparing results from both molluscicidal assays at 24 h, differences observed among treatment concerning the activity against adults (*F*-value = 60.326, *df* =102, *P* = 0.000) were less significant than those evidenced by the assay performed with juveniles (*F*-value = 94.125, *df* =99, *P* = 0.000). At the end of the experiments (48 h), mortalities varied from 3 to 59% (*F*-value = 42.624, *df* = 102, *P* = 0.000) and 3 to 100% (*F*-value = 42.318, *df* = 97, *P* = 0.000) for juveniles and adults, respectively (Fig. IV.3, B and C). Moreover, EOs from dill, parsley, cumin and bitter fennel (leaves more stems), as well as the compound apiole, were the most active treatments against young snails at 24 h. Their activity was accentuated after the recovery time (23 to 59% of death). Besides, in the second evaluation, we observed that cuminaldehyde attained a similar activity. Consistently, these treatments were highly effective against adults. In addition, Fv-I and the compounds *trans*-anethole, estragole, γ-terpinene and (-)-β-pinene exhibited a satisfactory activity against adults (> 70%). We have observed moderate to noteworthy increases of toxicity for the majority of compounds against juveniles and adults, respectively, during the recovery period (comparison of the 24 and 48 h values in Fig. IV.3, B and C).

4.2. Dose-related lethality

The toxicity of seven EOs and six individual compounds against adults of *R. peregra* was dose-dependent, which allowed the estimation of lethal concentrations within acceptable confidence limits (CLs) through the probit analysis (Table IV.1). In general, most of the data fitted well in the assumptions of the linear model. However, results obtained for the control ($CuSO_4$) and cuminaldehyde were less homogenous (higher *H*) resulting in a lower goodness-of-fit (χ^2 were slightly superior to the critical values). For the total period of the experiment (48 h), LC_{50} and LC_{90} values of EOs/compounds ranged from 13.7 to 46.5 and 29.2 to 83.1 mg L^{-1}, respectively. Slopes of the log-probit regressions varied from 3.2 (±0.28) to 9.5 (±0.83), according to the treatment.

With basis on LCs values and the non-overlapping of CLs we have established, with a probability of 95%, that Pc-F was the most toxic treatment to be tested (both LCs < 30 mg L^{-1}) 24 h after the interruption of the exposure, while S-(+)-carvone was the least active. By comparing the CLs of remaining treatments, we found that Pc-I, Fv-L, Ag-I and γ-terpinene were slightly more active than (-)-β-pinene, Ag-L, Fv-I, *trans*-anethole and estragole, and significantly more lethal to adults' snails than cuminaldehyde and Cc-F (LC_{50} of 34.7 and 38.8 mg L^{-1}, respectively). Even though, when considering the LC_{90} parameter, the statistical differences concerning the toxicity of these aforementioned treatments, which do not include the control, Pc-F and (*S*)-(+)-carvone, were not so marked. Moreover, cuminaldehyde, Cc-F and estragole showed linear regressions with the highest slopes (9.5, 9.2 and 7.5, respectively). Despite the fact these treatments were not the most actives, their LC_{50} and LC_{90} values were significantly closer which indicates that a larger number of individual responded to a certain increase of concentration, comparatively to the response induced by others treatments. In addition, we noticed that Cc-F and Ag-I showed parallel regression lines (equivalent slopes) with their major compounds cuminaldehyde and (*S*)-(+)-carvone, respectively.

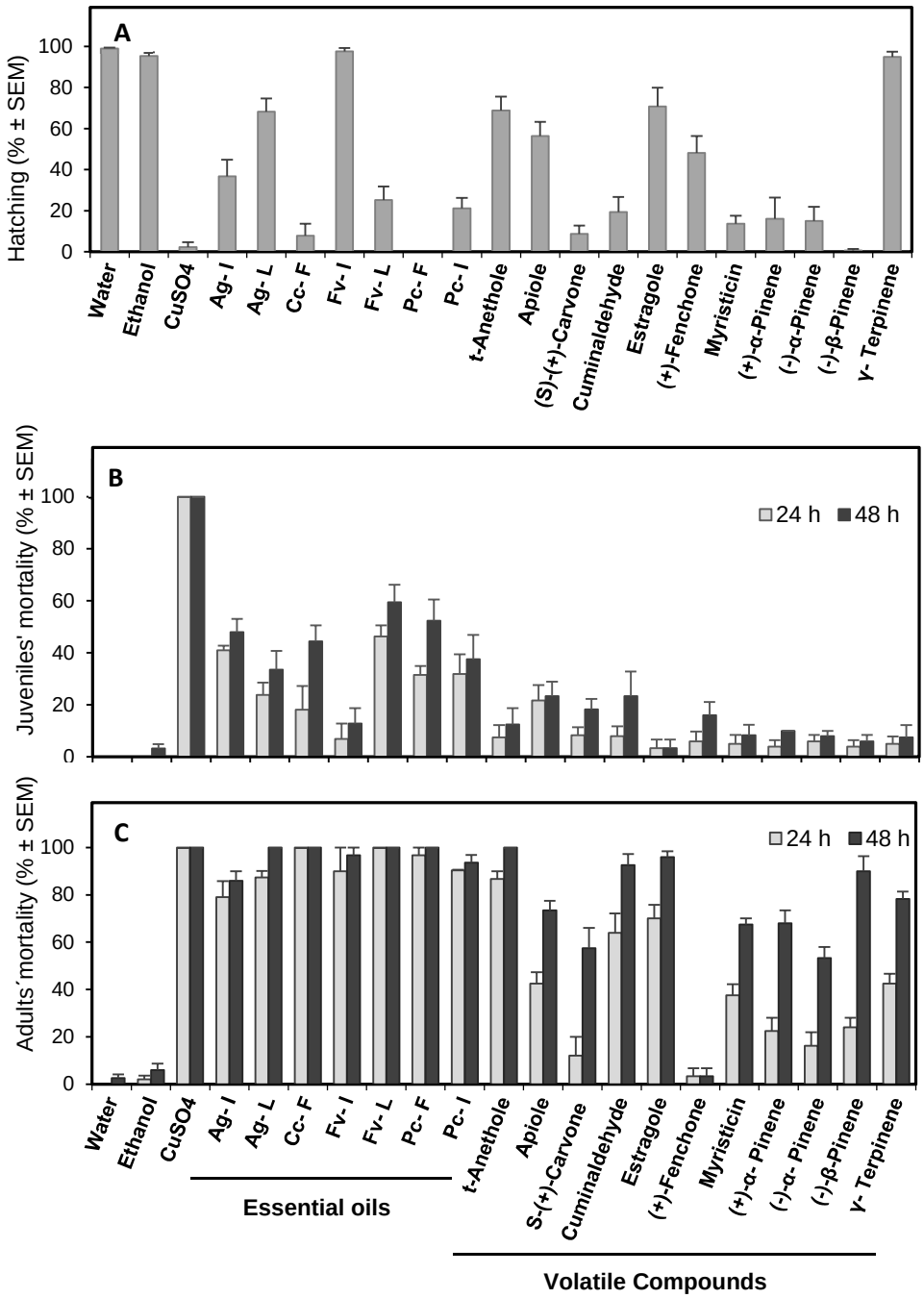


Figure IV.3 (A-C) Lethal activity of *Anethum graveolens* (Ag), *Cuminum cyminum* (Cc), *Foeniculum vulgare* var. *vulgare* (Fv) and *Petroselinum crispum* (Pc) essential oils (EOs) and 11 volatile compounds on *Radix peregra* embryos **(A)**, juveniles **(B)** and adults **(C)**.

(A) Emergence of juveniles after 15 days of exposure to 50 mg L^{-1} of EOs/pure constituents; **(B)** Lethality of EOs/compounds (10 mg L^{-1}) against juveniles after 24h of exposure followed by a 24h recovering period (48 hours); (C) Lethality of EOs/compounds (50 mg L^{-1}) against adults after 24h of exposure following a 24h recovering period (48 hours). Note: The final concentrations of ethanol (negative control) were of 0.02 mL L^{-1} for assay with juveniles, and 0.1 mL L^{-1} for eggs and adults. The positive control ($CuSO_4$) was tested at 10 mg L^{-1} against juveniles, and 50 mg L^{-1} against eggs and adults. I: Infrutescences; L: Leaves more stems; F: Fruits.

Table IV.1. Estimated LC_{50} and LC_{90} of Apiaceae essential oils and some volatile compounds against adults of *Radix peregra,* after 24 hour of exposure followed by a 24 h recovery period.

Treatments	Conc. (mg L^{-1})	Dose-dependent lethality			
		LC_{50} (95% CL) [a]	**LC_{90}** (95% CL) [a]	**Slope** ± SEM (95% CL) [a]	***H*** [b]
$CuSO_4$	0.1, 0.2, 0.25, 0.3, 0.5, 0.75	0.26 (0.21 – 0.32) a	0.65 (0.48 – 1.17) a	3.2 ± 0.25 a (2.72 – 3.70)	3.10
EOs					
Ag-I	20, 30, 40, 50, 60	22.3 (20.1 – 24.2) d	40.9 (37.3 – 46.3) c	4.9 ± 0.54 bcd (3.83 – 5.93)	1.01
Ag-L	20, 25, 30, 40, 50	26.4 (24.7 – 27.9) ef	42.6 (39.5 – 46.8) c	6.2 ± 0.56 de (5.09 – 7.27)	1.51
Cc-F	30, 35, 40, 45	38.8 (37.6 – 40.3) h	53.6 (49.8 – 60.0) d	9.2 ± 1.08 f (7.05 – 11.28)	0.15
Fv-I	20, 25, 30, 40, 45, 50	27.5 (26.1 – 29.0) f	45.7 (41.2 – 53.3) cd	5.8 ± 0.65 cde (4.55 – 7.10)	2.11
Fv-L	10, 20, 30, 40, 50	21.2 (19.6 – 22.9) cd	42.0 (38.2 – 47.3) c	4.3 ± 0.33 abc (3.69 – 4.97)	0.57
Pc-F	10, 20, 30, 40	13.7 (12.1 – 15.1) b	29.2 (26.1 – 33.7) b	3.9 ± 0.36 abc (3.18 – 4.60)	1.80
Pc-I	10, 20, 25, 30, 40, 50	18.5 (16.9 – 20.0) c	40.5 (36.7 – 45.8) c	3.8 ± 0.30 ab (3.19 – 4.36)	0.52
Compounds					
trans-Anethole	20, 30, 40, 50	28.3 (26.4 – 30.1) fg	49.2 (45.0 – 55.5) cd	5.3 ± 0.51 bcd (4.34 – 6.34)	2.34
S-(+)-Carvone	30, 40, 50, 60	46.5 (43.8 – 48.6) i	83.1 (72.8– 102.3) e	4.9 ± 0.62 abcd (3.63 – 6.07)	1.57
Cuminaldehyde	30, 35, 40, 50, 60	34.7 (29.8 – 38.4) gh	47.4 (42.2 – 63.0) cd	9.5 ± 0.83 f (7.89 – 11.15)	3.40
Estragole	20, 30, 35, 40, 50	28.7 (25.7 – 31.9) fg	44.1 (38.8 – 55.7) cd	7.5 ± 0.60 def (5.81 – 8.04)	2.11
(-)-β-Pinene	20, 30, 40, 50	26.0 (24.2 – 27.7) ef	44.5 (41.0 – 49.7) c	5.5 ± 0.52 cd (4.48 – 6.53)	1.79
γ-Terpinene	10, 20, 30, 40, 50	23.1 (20.9 – 25.2) de	58.3 (50.6 – 70.4) d	3.2 ± 0.28 a (2.63 – 3.73)	1.69

[a] LC_{50}, LC_{90} values and 95% confidence limits (CL) are expressed in mg L^{-1} of essential oil/compound required to cause snails' death. LC values and slopes within the same column followed by the same letter are not significantly different based on non-overlapping of the 95% CL. [b] *H*, Heterogeneity factor (χ^2/df).

4.3. Time-related lethality

The toxicity of seven EOs and six volatiles compounds against *R. peregra* varied throughout time. All time-mortality data obtained by the continuous and the 16 h exposure evaluation were homogenous and fitting the probit regression model (Table IV.2). The continuous exposure allowed to determine both lethality parameters for all treatments. Whenever possible (if

accumulated mortalities were lower than 90% but superior to 50% and no heterogeneity of data was recorded) the LT_{50} of these treatment was determined (Table IV.2). The LT_{50} calculated for the continuous, the 16 h and the 8 h exposure varied from 15.1–34.3, 20.1–32.5 (excluding carvone) and 23.0–134.7 hours, respectively, while LT_{90} varied from 21.6–49.3, 24.6–54.5 and 33.0–271 hours, respectively. Moreover, the lethal parameters after 8 h of contact for Ag-I, Ag-L, Cc-F, *trans*-anethole, cuminaldehyde and γ-terpinene could not be estimated within a reasonable time of observation, nor within acceptable confidence limits.

With basis on the determined LT_s values (and respective 95% CLs) Ag-I, Cc-F, Fv-I, Fv-L, Pc-F, *trans*-anethole and cuminaldehyde and the positive control showed the acutest time-related activity on 50% of snails when these were continuously exposed (LTs_{50} below 22 h). With the exception of Pc-I and (S)-(+)-carvone, treatments were active against 90% of individuals within 21.6 to 38.8 h of exposure, nonetheless, differences in their time-effectiveness were significant. Furthermore, for the 16 h exposure, Cc-F, Fv-I, Fv-L, Ag-I, *trans*-anethole, cuminaldehyde, as well as the positive control, exhibited a 90% lethal effect within a significantly lower extent of time (total of 24.6 to 32.1 h) relatively to the other treatments (37.7 to 54.5 h). In addition, Pc-I and (S)-(+)-carvone were found to be the least active treatment (LT_{90} of 49 h) in the evaluation succeeding the 16 h-treatment. After the 8 h short-term exposure, only the control and both bitter fennel EOs (Fv-I and Fv-L) were still exhibiting the most satisfactory time-related activity, causing the death of 50% of snails within 23 to 25.6 h (total time from the beginning of the experiment). In opposition, Ag-I, Ag-L and γ-terpinene were considerably less effective through time, with accumulated mortalities inferior to 25% after 120 h of monitoring.

Furthermore, the time-related lethality of some treatments was significantly influenced by the duration of snails' exposure. The results obtained after an 8 h exposure show that times required to kill 50 and 90% of individuals were generally superior for all treatment with relation to the other two assessments (excepting the positive control and the $LT_{50\ of}$ FV-L). When comparing the 16 h and the prolonged exposure, $CuSO_4$, Fv-L, *trans*-anethole, cuminaldehyde and estragole, did not showed significant increases of their LTs (based on the overlapping of their CLs), although the confidence intervals for some treatments were less coincident. Concerning the time-dependent lethality observed on 50% of individual, we have showed that the lethal effects of Fv-I, Pc-F, Cc-F, Ag-L and (*S*)-(+)-carvone was delayed for 2, 4, 5, 10 and 64 h (higher LT_{50}), respectively, when animals were exposed for a shorter period of 16 h. Times necessary to attain 90% of lethality were found to decline significantly when the treatments of Cc-F, Ag-I, Pc-F, (-)-β-pinene and γ-terpinene were continued (less 3, 11, 13, 14 and 20 h, respectively), comparatively to the 16 h exposure.

Supplementary information on the time-dependent toxicity and on EOs/compounds lethality is given in the section 8 of this chapter – "Supplementary data".

Table IV.2. Estimated LT_{50} and LT_{90} of Apiaceae essential oils and some compounds against adults of *Radix peregra,* assessed for different exposure periods to 50 mg L^{-1}.

Treatments	Time-dependent lethality											
	8 hours exposure				16 hours exposure				Continuous exposure			
	LT_{50} (95% CL) [a]	LT_{90} (95% CL) [a]	Slope (± SEM)	H [b]	LT_{50} (95% CL) [a]	LT_{90} (95% CL) [a]	Slope (± SEM)	H [b]	LT_{50} (95% CL) [a]	LT_{90} (95% CL) [a]	Slope (± SEM)	H [b]
$CuSO_4$ [c]	25.4 a (24.7 – 26.0)	33.0 a (31.8 – 34.8)	11.3 ± 1.15 e (9.1 – 13.6)	1.36	20.5 a (20.0 – 21.1)	28.1 a (26.5 – 30.5)	9.4 ± 0.98 f (7.5– 11.3)	1.13	20.7 c (20.3 – 21.1)	26.1 c (25.1 – 27.5)	12.6 ± 1.08 e (10.5 – 14.7)	0.97
Ag-I	<25%, after 120 h				21.7 abcd (20.4 – 23.0)	42.4 cde (37.8 – 50.1)	4.4 ± 0.45 bc (3.5 – 5.3)	1.54	21.1 cd (20.4 – 21.7)	31.3 ef (29.7– 33.6)	7.4 ± 0.52 b (6.4– 8.5)	0.67
Ag-L	<20%, after 120 h				32.5 f (27.6 – 38.6)	–	1.5 ± 0.22 a (1.0 – 1.9)	0.39	22.8 ef (21.9 – 23.7)	38.6 gh (35.9 – 42.5)	5.6 ± 0.40 ab (4.8 – 6.4)	0.83
Cc-F	70%, after 140 h [d]				20.1 a (19.8 – 20.5)	24.6 a (23.9 – 25.6)	14.6 ± 1.17 g (12.3 – 16.9)	0.83	15.1 a (14.6 – 15.5)	21.6 a (20.6 – 22.9)	8.2 ± 0.64 bc (7.0 – 9.5)	0.33
Fv-I	25.7 a (24.6 – 26.7)	40.6 b (37.5 – 45.9)	6.5 ± 0.77 d (5.0 – 8.0)	0.86	21.4 bc (20.9 – 21.7)	25.6 a (24.8 – 26.7)	16.3 ± 1.68 g (13.0 – 19.6)	0.38	19.3 b (18.7 – 19.8)	24.1 b (23.5 – 25.0)	13.1 ± 1.19 e (10.8 – 15.5)	1.03
Fv-L	23.0 a (12.2 – 26.9)	53.0 bc (41.3 – 183.4)	3.5 ± 0.61 c (2.4 – 4.7)	2.40	21.4 abc (20.2 – 22.6)	26.8 a (24.9 – 30.8)	13.1 ± 1.0 fg (11.1 – 15.1)	2.05	21.0 cd (20.3 – 21.5)	28.6 cde (27.3 – 30.5)	9.5 ± 0.80 bcde (7.9 – 10.9)	1.55
Pc-F	60.0 bc (39.6 – 115.4)	–	0.6 ± 0.18 a (0.2 – 0.9)	0.17	25.8 e (24.8 – 26.7)	41.5 cd (38.5 – 45.9)	6.2 ± 0.54 cd (5.1 – 7.2)	0.17	21.6 de (21.1– 22.1)	28.7 de (27.7 – 29.9)	10.4 ± 0.70 cde (9.1 – 11.8)	1.28
Pc-I	Nt				< 50%, after 140 h				28.9 h (27.7 – 30.1)	49.2 i (45.3 – 54.7)	5.5 ± 0.34 a (4.8 – 6.2)	0.60
trans-Anethole	<35%, after 120 h				21.3 bc (20.7 – 21.9)	29.3 a (27.8 – 31.7)	9.3 ± 0.96 ef (7.4 – 11.2)	0.70	21.1 cd (20.7 – 21.5)	27.7 cd (26.6– 29.0)	10.9 ± 0.71 de (9.5 – 12.3)	0.77
S-(+)-Carvone	Nt				98.7 g (94.6 – 103.6)	–	9.0 ± 1.00 de (7.0 – 11.0)	0.67	34.3 i (33.1 – 35.7)	49.3 i (46.3 – 53.4)	8.2 ± 0.63 bc (7.0 – 9.4)	1.26
Cuminaldehyde	<30%, after 120 h				20.9 abc (20.0 – 21.6)	32.1 ab (29.4 – 36.9)	6.8 ± 0.88 cde (5.1 – 8.6)	0.85	19.5 b (18.9 – 20.1)	28.4 cde (26.9 – 30.8)	7.9 ± 0.74 bc (6.4 – 9.3)	1.11
Estragole	50.4 b (43.2 – 65.0)	271 c (159.0 – 775.6)	1.7 ± 0.30 b (1.2 – 2.3)	0.68	24.1 e (23.1 – 25.2)	37.7 bc (34.8 – 42.2)	6.6 ± 0.63 de (5.4 – 7.9)	1.06	23.1 f (22.4 – 23.9)	32.8 f (31.1 – 35.2)	8.4 ± 0.60 bcd (7.3 – 9.6)	0.53
(-)- β-Pinene	134.7 c (100.7 – 229.0)	–	1.0 ± 0.16 ab (0.6 – 1.3)	0.07	24.4 de (22.8 – 25.8)	49.1 de (45.1 – 54.7)	4.2 ± 0.33 bc (3.6– 5.4)	0.59	25.4 g (24.6 – 26.3)	35.6 fgh (33.4 – 38.7)	8.7 ± 0.67 bcd (7.4 – 10.1)	1.15
γ-Terpinene	<25%, after 120 h				23.2 cde (21.4 – 24.9)	54.5 e (47.0 – 68.2)	3.5 ± 0.39 b (2.7 – 4.2)	0.78	23.3 f (22.5 – 24.1)	34.9 fg (32.7 – 37.8)	7.32 ± 0.52 b (6.2 – 8.4)	0.51

[a] LT_{50}, LT_{90} values and 95% confidence limits (CL) expressed in hours required to cause death to adult snails. LT values and slopes within a column followed by the same letter are not significantly different based on non-overlapping of the 95% confidence limits (CL); [b] H, Heterogeneity factor (χ^2: degree-of-freedom); [c] For the positive control, the time-activity relationship was assessed at 0.75 mg L^{-1}. [d] LTs could not be determined due to a high heterogeneity of data.

5. Discussion

The research of plant-based products and their application as prophylactic measures to control intermediate hosts' populations have been the subject of several review (Mcllough et al., 1980; Marston & Hosttetmann, 1985; Singh et al., 1996a; dos Santos & Sant' Ana, 2000; Singh et al., 2005; Singh et al.., 2010). As it can be conclude through a brief survey of the literature, most of screening for plant-based molluscicides have been targeted on freshwater snail species with major roles in transmitting trematodiasis diseases, namely *Biomphalaria glabrata* (dos Santos & Sant´ana, 2001; Schall et al., 2001; Radwan et al., 2008) and *B. alexandrina* (Radwan & El-Zemity, 2007; Radwan et al., 2008; Bakry, 2009; Salama et al., 2012), two Planorbidae species involved in the transmission of Schistosomiasis, and the lymnaeids *L. acuminata* (Singh et al., 1996b; Singh et al., 1997; Kumar & Singh, 2006, Shukla et al., 2006; Upadhyay & Singh, 2011) and *G. truncatula* (Mendonça et al., 1993; Hammami et al., 2011), both intermediate hosts of the liver flukes.

In the current work, we distinguished parsley and cumin fruits EOs and the compounds myristicin, (*S*)-(+)-carvone, cuminaldehyde and pinenes, as the strongest products affecting the eggs hatchability (Fig IV.3, A). This study also demonstrated that several treatments [Pc-F, Cc-F, myristicin and (-)-β-pinene] were able to cause severe embryonic death. Embryos exposed to these products underwent little development, but not beyond the initial stages of differentiation, with most of them presenting a disintegrated structure, similarly to what have been described by others authors (dos Santos & Sant' Ana, 2001; Adenusi & Odaibo, 2008). Thus, we infer that these EOs/compounds displayed an effective biocidal activity against embryos at the tested concentration (50 mg mL^{-1}) by disrupting embryogenesis and affecting the eggs viability.

The EOs from dill, parsley, cumin and bitter fennel (leaves more stems), as well as the compound cuminaldehyde and apiole, were the most active against young and adult snails (Fig IV.3, B and C, respectively). Activity against both adult and egg stages is of upmost importance for an efficient control of host snails' populations. As revealed in this study, snails in different developmental stages can exhibit a variable susceptibility to chemicals. Young snails of *R. peregra* and *B. glabrata* were found to be more susceptible to molluscicides than elder ones when exposed to some plant EOs (Teixeira et al., 2011) and methanol extracts (Bakry, 2009), respectively. Moreover, egg masses can be as susceptible as adults. This fact has been reported for *Biomphalaria pfeifferi* when exposed to *Dalbergia sissoo* extracts (Adenusi & Odaibo, 2008). On the contrary, other plants that have a proven and notable toxicity to snails, namely *Euphorbia splendens* and *Phytolacca dodecandra*, revealed however a less satisfactory ovicidal activity at levels that are lethal to adult (Schall et al., 1998; McCullough et al., 1980). This contrasting biological activity of plants towards snails' embryos and adult form is certainly the outcome of a differentiate susceptibility to several plant species as established by dos Santos & Sant´Ana (2000). Nevertheless, some additional aspects may possibly have great influence on extracts/ natural compounds action over snails' embryos. We presume that

compounds' ability to disperse into the gelatinous matrix of the egg mass and across the capsule membrane to finally penetrate into the egg is a conditioning factor which rely on the physical and chemical properties of these molecules.

From the screening assay we also observed that, from the onset to the end of the recovery period, treatments´ lethality to juveniles and adults could moderately or substantially increase (mortality rates of newly hatched snails and adults increased 1.2 to 3.2 and 1.2 to 4.8 times, respectively). Among all treatments, Cc-F and Fv-S+L appeared to be acting more acutely against adults since death was occurring within the 24 h exposure period even before washing and recovery. In addition, we acknowledge that the concentration defined for the assays with adults was more suitable than the concentration tested against juveniles (dosage was five-fold superior) because of the higher lethal effects observed in the first situation. Even though juveniles' mortality might have been low in some cases, four EOs have caused juveniles mortality rates proximate to 50% (Fig IV.3, B). Therefore, we assume that the LC_{50} of Ag-I, Cc-F, Fv-L and Pc-F at 48 h should be close to 10 mg L^{-1}, whereas for the EOs/compounds displaying a lower toxicity the LC_{50} values should be much superior. Although the estimation of LCs for the young snails was not included in this work we can tell that LCs values of Pc-F (one of the most active against the newly hatched snails) were low [LC_{50}=10.5 (9.6–11.7) mg L^{-1} and LC_{90}= 23.1(18.7–32.8 mg L^{-1}) for juveniles] (data not shown), but not much distinct than LCs values exhibited by this EO against adults [LC_{50}= 13.7 (12.1–15.1) mg L^{-1} and LC_{90}= 29.2 (26.1–33.7) mg L^{-1})] (Table IV.1). Similarly, the EO extracted from leaves of *Hedychium gardnerianum* (Zingiberaceae) have been found to exhibit the same effectiveness against juveniles and adults of *R. peregra* (Teixeira et al., 2011).

The variability of bioactivity among treatments observed in the preliminary assay have highlighted the importance of further investigation for some of them. As it was evidenced by the probit analyses, the toxic effects of EOs and six compounds were time and dose-dependent. The estimated LC_{50} of EOs (13.7 to 38.8 mg L^{-1}) were below the threshold value (40 mg L^{-1}) which differentiate the plant crude extracts with the highest molluscicidal potential, and thus eligible to be employed against molluscs populations (WHO, 1993). In order to properly appraise their bioactivity, we considered that dose-related evaluation could be associated with a time-study approach. In fact, if we judge treatments potency from both perspectives, we conclude that Pc-F was the most effective product as regards the dose-activity criteria, but its activity was more delayed through time when the exposure occurred for 8 h and 16 h. On the contrary, when compared to Pc-F, Cc-F induced a less prolonged death process to adults, with high lethality recorded within significant short times, despite its much lower LC_s values (2.8 and 1.8 times inferior, respectively). Although we acknowledge that any interpretation of these results is speculative, we suppose that Cc-F mode-of-action may be impairing more acutely important system of homeostatic regulation (water balance, neurohormonal control, excretion, *etc*.) than Pc-F, displaying a more rapid failure of organism. On the other hand, we should keep in mind that the intake of EOs constituents, the kinetic of the process and their bioavailability in the tissue might conceivably be different for each chemical and natural mixture.

With basis on the determined LTs values and respective confidence limits, we have established some differences concerning the time-related activity of EOs/compounds, within each assessment. With the exception of the control, the LTs determined for the shortest exposure were moderately to considerably superior and statistically distinct to their counterpart determined for the other exposure assessments. Fv-I and Fv-L EOs maintained their effectiveness even after a short-term exposure of 8 h, at the concentration used (50 mg L^{-1}). As we could notice, animals' susceptibility toward Cc-F, Fv-I at 50 mg L^{-1} was manifested earlier (LT_{90} within the first 24 h) when these were continuously exposed, being significantly distinct from others EOs and all volatile compounds. Regardless of these differences in LTs values, defined with based on the overlapping criteria, we ascertained that LT_{50}s and LT_{90}s values were not so distant when comparing a short-term treatment (16 h) and a constant exposure. In fact, if we ignore results obtained for (*S*)-(+)-carvone, LT_{50}s and LT_{90}s ratios (16 h/continuous exposure) varied from 1 to 1.4 and 1 to 1.6, respectively. Furthermore, the different slope values of the linear regressions have evidenced the higher sensitiveness into the snail population to small increases in the concentration of Cc-F, cuminaldehyde and estragole (Table IV.1). Consequently, the distance between their LCs values was markedly lower than those found for other treatments, which have reflected in inferior $LC_{90/50}$ ratios for Cc-F, cuminaldehyde and estragole (1.3 to 1.5) comparatively to remaining ones (1.5 to 2.5). Similarly, snails' death throughout time (after 16 h and continued exposure) was quite more synchronized in response to bitter fennel EOs and trans-anethole (all $LT_{90/50}$ ratios were below 1.4)[1]. Similar time-dependent study has been previously performed against *R. peregra* with EOs from plant species of the Macaronesia flora found in Azores (Teixeira et al., 2011). Besides, the accumulated toxicity throughout time can be manifested by a decrease of the LCs values, as it has been established for *Azadirachta indica* (Meliaceae) (Singh et al., 1996)*, Ferula assafoetida*, *Syzygium aromaticum, Carum carvi* (Kumar & Singh, 2006), *Myristica fragrans* (Jaiswal & Singh, 2009) and several bioactive compounds (Price & Berry, 2008). The progressive accumulation of the active compound(s) into the snail body and/or their metabolization into more toxic forms by the organism enzymatic system have been suggested as possible events responsible for these time-related toxicity (Kumar & Singh, 2006; Jaiswal & Singh, 2009). However, it is known that molluscicides (like niclosamine and copper-based compounds) depletion take place after the initial application into water (Muir & Yarechewski, 1982; Francis-Floyd et al., 1997). Even though this could represent an advantage for the environment, such rapid degradation may be a constraint for the application of molluscicides from natural origin. For example, extracts of *Phytolacca dodecandra* (Phytolaccaceae) and its active compound (oleanolic acid glucoside) are rapidly biodegraded with a consequent loss of the molluscicidal potency after a few days (McCullough et al., 1980).

One key question in this work was: how long the exposure to some of the most dose-active treatments should last to impair homeostatic control mechanisms and to trigger irreversible physiological injury? For most cases (except for carvone and Pc-I) we conclude that a 16 h exposure

[1] Supplementary information is provided in the section 8- "Supplementary data".

at a 50 mg L^{-1} might be sufficient to control 90% of *Radix peregra* adult population, although this reduction in the duration of the exposure might have significantly increased the lethal times of OEs from cumin and parsley fruits. Our results also indicated that a great effectiveness can be achieved by submitting *R. peregra* adult snails for 8 h to the bitter fennel EOs characterized herein (rich in estragole, fenchone and α-pinene and α- and β-phellandrenes). For which concerns dill EOs, *trans*-anethole, cuminaldehyde, and γ-terpinene we are impelled to suggest that an 8 h period of contact with these active EOs/compounds was not enough to cause an irreversible state of toxicity and unrecoverable injuries to snails' organism. However, we do not discard that sublethal toxicity over these organisms might well be a longstanding event with eventual effects on metabolism, reproduction, *etc*. We suppose that under the tested circumstances, where the uptake and accumulation of the toxic chemicals in the tissues was interrupted after 8 h, being followed by a period in clear water, most of the individuals exposed to dill EOs, *trans*-anethole, cuminaldehyde, and γ-terpinene were able to recover from the acute symptoms. These findings are consistent with those of Price & Berry (2008) whom reported the knockdown effects caused by acute lethal doses of the volatile compounds citral, geraniol and eugenol to *Planorbis corneus* and the ability of this freshwater snail to recover after a brief 5 h exposure.

The best-studied molluscicides of plant origin are undoubtedly from *P. dodecandra* (Lemma et al., 1972), *Anacardium occidentale* (Anacardiaceae) (Sullivan et al., 1982) and *Ambrosia maritime* (Asteraceae) (Shoeb & El-Eman, 1976). Moreover, the latex extracted from many Euphorbiaceae and Apocynaceae species have shown outstanding activities, often superior to synthetic molluscicides, against *L. acuminata* (24 h LC_{50} ranging from 0.1 to 5 mg L^{-1}) (Singh et al., 2010 and references therein). Crude and aqueous extracts of numerous plant have been studied for their molluscicidal properties (Singh et al., 2005; Fakry, 2009). However, many of these plant species are also harmful to other organisms since they have been discovered because of their potent poisoning effects on fish (McCullough et al., 1980). Moreover, ethanolic extracts from Annonaceae species have revealed some promising activity against the snail host of *Schistosoma mansoni*, *B. glabrata* (dos Santos and Sant' Ana, 2001). In addition, significant molluscicidal activities (LC_{50} < 100 mg L^{-1}) against *L. acuminata* were attributed to extracts from *Trachyspermum ammi* (Singh et al., 1997), *Syzygium aromaticum* (Myrtaceae), *Ferula asafetida, Carum carvi* (Kumar & Singh, 2006), and *Myristica fragrans* (Myristicaceae) (Jaiswal & Singh, 2009).

Works including plants EOs have been undertaken by several authors, although to a lesser extent, against *B. truncatus* (Khallouki et al., 2000; Lahlou & Berrada, 2001; Lahlou et al., 2001; Lahlou, 2003; El-Kamali et al, 2010), *B. glabrata* (Coutinho et al 2007; Tavares et al., 2007) and *B. alexandrina* (Salama et al., 2012). EOs have been described in the literature as molluscicides at very acceptable doses (LC_{50} < 100 mg L^{-1}). EO from *Citrus aurantium L.* var. *valencia* (Rutaceae), *Origanum compactum* (Lamiaceae) and *Cedrus atlantica* (Pinaceae) were found extremely effective (LC_{50}=0.28, 0.44 and 0.47 ppm, respectively) (Lahlou & Berrada, 2001; Lahlou, 2003). The EO from leaves of *Xylopia langsdorffiana* (Annonaceae) also showed a very significant molluscicidal activity (LC_{90}= 5.6 mg mL^{-1}) (Tavares et al., 2007). Furthermore, the remarkable activity of EOs constituent

towards shistosomiasis and fascioliasis intermediate hosts have been confirmed in several studies. Myristicin, limonene, *trans*-anethole, and eugenol were previously established as potent molluscicides against *L. acuminata* (24 h or 48 h $LC_{50} \leq 10$ mg L^{-1}) (Kumar & Singh, 2006; Jaiswal & Singh, 2009). Thymol, carvacrol, β-citronellol, geraniol, *trans*-anethole, pulegone, and eugenol displayed significant toxicity towards *B. alexandrina* (72 h LC_{50} ranging from 14 to 77 µg mL^{-1}) (Radwan & El-Zemity, 2007; Radwan et al., 2008), while thymol geraniol and carvacrol also exhibited potent knockdown effect on *Bulinus truncatus* (Lahlou & Berrada, 2001). Radwan et al. (2008) have demonstrated that the activity of aliphatic monoterpenoids (geraniol and β-citronellol) and aromatic compounds (carvacrol and thymol) could be greatly enhanced if mixed with piperonyl butoxide (a synergist compound), attesting to be as effective as the copper sulfate against *B. alexandrina*. Concerning this copper-based molluscicide, it is worth mentioning that its activity recorded herein against *R. peregra* (LCs of $CuSO_4$ < 1 mg L^{-1}) was significantly acuter than EOs and volatiles compounds (LC_{90} was 45 to 128 times superior). The LC_{50} of copper sulfate on *R. peregra* was very close to that determined against *B. glabrata* (48-h LC_{50}=0.48 mg L^{-1}). Despite, their great effectiveness, the application of copper compounds in freshwater ecosystems, either as algicides, aquatic plant herbicides or as molluscicides, is strongly disclaim due to adverse effects over a variety of non-target aquatic species (Oliveira-Filho et al., 2009).

Some physiological and biochemical effects of plant-derived products on molluscs have been reported in the literature. The behavioral responses observed in the present study are in accordance with those described by several authors for pulmonate snails exposed to different plant extracts and constituents (McCullough et al., 1980; dos Santos & Sant' Ana, 2000; Lahlou & Berrada, 2001; Adenusi & Odaibo, 2008; Price & Berry, 2008). Significant and rapid changes in *B. alexandrina* behavior were previously described by Salama et al. (2012). According to these authors, snails showed obvious sign of distress with muscular and spiral twisting, suggesting some neurotoxic activity of EOs from the two Lamiaceae species (*Thymus capitatus* and *Marrubium vulgare*) at the neuromuscular level. Price & Berry (2008) have demonstrated that millimolar concentrations of EOs compounds have a blocking effects on action potentials of the buccal ganglion neurons, besides impairing the response to some neurotransmitters (octopamine, dopamine and acetylcholine). It has been concluded that some EO constituents, namely eugenol, citral and geraniol, can display a reversible knockdown effects against *Planorbis corneus* (Price & Berry, 2008). However, with basis on their findings, these authors suggested that those compounds could be triggering nonspecific effects rather than direct receptor targeting. In our study, we have noticed that higher dosages elicited acute symptoms (increased mucous secretion and unresponsiveness to stimuli) in the first hours of exposure to EOs and compounds. Those symptoms where followed by swelling of the cephalopedal mass with hemolymph leakage, protrusion of the snail body, discoloration of the tissues and disintegration of the foot epithelium. On the contrary, snails exposed to copper salt manifested strong avoiding behavior by attempting to evade out of the solution and, in a second phase, an absence of responsiveness and a retraction of the foot into the shell.

Currently, there are some evidences of metabolic and biochemical changes produced by plant extracts and compounds on snails' tissues. Extracts of *Euphorbia pulcherima* in water were found to significantly alter the levels of total protein, total free amino acid and nucleic acids in *L. acuminata* (Singh & Singh, 2010). Sublethal doses of plant-based molluscicidal induced a severe reduction of glycogen in the soft tissues of *B. alexandrina* with an increase of glucose levels in hemolymph (Bakry, 2009). Enzyme assay performed with homogenized tissues have revealed that some products could cause an increase in *L. acuminata* protease activity (Singh & Singh, 2010) and/or a decrease of acid and alkaline phosphatase (Tripathi et al., 2004; Jaiswal et al., 2009; Singh & Singh, 2010; Upadhyay & Singh, 2011), lactic dehydrogenase (Shukla et al., 2006) and succinate dehydrogenase activities (Bakry, 2009). Moreover, many of these plant-products also showed significant inhibitory activity of acetylcholinesterase (AchE) in the nervous tissue (Tripathi et al., 2004; Shukla et al., 2006; Jaiswal et al., 2009; Upadhyay & Singh, 2011) and *B. alexandrina* (Bakry, 2009). For instance, the compound myristicin extracted from *M. fragrans* and herein identified in parsley fruits, was established as a potent inhibitor of AChE (IC= 0.03 mM) and acid phosphatase (0.07 mM) in *L. acuminata,* showing a competitive-noncompetitive and competitive inhibition, respectively (Jaiswal et al., 2009).

In the present study, we attempted to relate the biological activity of natural mixtures to those of some individual constituents. Interestingly, our results show that *R. peregra* susceptibility to EOs and compounds is variable and visibly depend on its developmental stage. The biocidal effects observed for EOs in each assay were somewhat consistent with the activity determined for the compounds tested Cc-F and cuminaldehyde activities were coherent in terms of LCs, with parallel dose-mortality rates (same slopes), and ovicidal activity. Thus, cuminaldehyde reflected EO activity in both aspects while γ-terpinene seemed not. Such finding strongly indicate that most of Cc-F activity is likely attributable to this major compound found at 39% but also to β-pinene (12%), rather than γ-terpinene which constitute 16% of the cumin EO. Nonetheless, we admit that other compounds (i.e. *p*-cymene, p-mentha-1,4-dien-7-al, *etc*.) comprised in the remaining part of the EO (1/3) could be important in terms of synergistic interaction with cuminaldehyde, contributing for the acute dose-activity and time-effectiveness of the cumin fruits EO. Interestingly, PC-F and PC-I showed comparable toxicity in terms of LCs and dose-dependent increases in snails' mortality, despite the considerable differences of their composition. The results obtained in the unique dose assays indicate that the ovicidal activity of Pc-F was influenced by the pinenes and myristicin activities, while apiole seem to have had a higher impact in parsley EOs toxicity against adults that it did against embryos (Fig. IV.3, A and C). Contrasting results were also recorded for bitter fennel EOs, since Fv-L was significantly more active than Fv-I against eggs and juveniles. The activity of Fv-L against embryos might be reflecting an interaction of α-pinene (18% of this EO) with other monoterpene hydrocarbons (specifically phellandrenes) identified in greater amount in this EO. On the other hand, estragole was effective against adults but almost inactive towards eggs and juveniles. This could explain, to a certain extent, why both EOs only showed equivalent effectiveness against the adult form regardless of their distinct quantitative profiles. This relation is evidenced by the coherent values of LCs and regressions slopes exhibited by estragole and FV-I (containing 65% of estragole). On the contrary, we have found

that the dose and time-dependent toxicity of Ag-I against adults was not so much consistent with the toxicity of (S)-(+)-carvone which represent the major part of this EO (2/3), whereas both were coherent in terms of ovicidal activity. These results evidence that *R. peregra* susceptibility to Ag-I and (S)-(+)-carvone is variable depending on the developmental stage and that toxicity towards adults is more likely to be attributed to other non-tested compounds, which were found in considerable amounts in both dill EOs (α- and β-phellandrene and limonene accounted for 70 and 28% in leave more stems and infrutescences EOs, respectively), rather than largely to this major compound.

It is important to emphasize that the effects described and discussed herein may be a consequence of the high variety of chemicals and distinct proportion of bioactive compounds existing in the mixtures. More comprehensive evaluations including combinations of identified major and minor constituents have proved to be helpful to researchers (Ntalli et al., 2010; Koul et al., 2013; Kumrungsee et al., 2014). Therefore, we find indispensable to orientate our efforts in this direction to understand the toxicity of such complex mixtures and the nature of their interactions (additive, synergistic or antagonistic) in each situation.

6. Conclusion

The present findings have evidenced the strong potential of the tested EOs/compounds against the trematodes intermediate host, *R. peregra.* EOs showed LC_{50} values below 40 mg L^{-1}, which remains in the defined range of the highly molluscicidal extracts, according to WHO (1995) and Singh et al. (1996b). Moreover, the ovicidal effect recorded for EOs at levels that are lethal to adult snails is of great advantage. Our results indicate that effectiveness can be achieved within a shorter time of treatment which clearly is an appealing aspect of this type of alternative product considering their high biodegradability and volatile nature. Therefore, we suggest that the application of the studied EOs as natural molluscicide agents could be profitable natural alternatives considering their significant bioactivity of in a time-effective mode, the oil productivity of these plant species and the availability of these products in the market. For some EOs, the bioactivity recorded was related with the individual activity of the compounds included in the study.

The outcome of this work is encouraging, lacking, however, field testing under natural conditions are necessary to confirm the bioactivity observed in this lab study. Furthermore, it is indispensable to carry out an extensive toxicological evaluation on non-targeted organisms and aquatic ecosystem where this freshwater snail proliferates. Being aware that the efficacy of some molluscicides may be adversely influenced by light, combinations of salts in solution, pH, and action of the microbiota (McCullough et al., 1980; Muir & Yarechewski, 1982), we shall consider to perform some assays concerning the physical chemical stability of these products under several conditions. Further studies, making use of controlled release systems, which are already been applied in some commercialized pesticides, are also under consideration.

7. References

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8. Supplementary data

Table IV.3. Effects of Apiaceae essential oils/pure constituents on *Radix peregra* eggs viability (after 15 days of exposure to 50 mg L^{-1}), against juveniles (at 10 mg L^{-1}) and adults (50 mg L^{-1}), after 24h of exposure following a 24h recovering period (48 hours).

Treatments	Hatching (%± SEM) [a]	Juveniles Mortality (%± SEM) [a]		Adults Mortality (%± SEM) [a]	
	15 Days	24 h	48 h	24 h	48 h
Water	99.3 ± 0.5 g	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	2.5 ± 1.6 a
Ethanol [b]	95.3 ± 1.9 g	0.0 ± 0.0 a	3.2 ± 1.6 a	2.0 ± 1.6 a	6.0 ± 2.6 a
$CuSO_4$ [c]	2.3 ± 2.3 a	100.0 ± 0.0 h	100.0 ± 0.0 h	100.0 ± 0.0 j	100.0 ± 0.0 f
EOs					
Ag-I	36.8 ± 8.0 bcd	41.0 ± 1.8 fg	48.0 ± 5.0 efg	79.0 ± 6.7 fg	85.9 ± 4.1 cd
Ag-L	68.3 ± 6.4 ef	23.8 ± 4.8 cde	33.6 ± 7.2 cde	87.3 ± 2.7 g	100.0 ± 0.0 f
Cc-F	7.9 ± 5.7 a	18.2 ± 9.1 bc	44.4 ± 6.1 efg	100.0 ± 0.0 j	100.0 ± 0.0 f
Fv-I	97.6 ± 1.6 g	5.8 ± 3.9 a	11.0 ± 5.9 ab	90.0 ± 10.0 hi	96.7 ± 3.3 ef
Fv-L	25.3 ± 6.6 abc	46.3 ± 4.5 g	59.4 ± 6.8 g	100.0 ± 0.0 j	100.0 ± 0.0 f
Pc-F	0.0 ± 0.0 a	31.6 ± 3.4 def	52.4 ± 8.1 fg	96.7 ± 3.3 ij	100.0 ± 0.0 f
Pc-I	21.2 ± 5.6 ab	31.9 ± 7.5 ef	37.5 ± 9.4 def	90.3 ± 0.3 gh	93.6 ± 3.2 de
Compounds					
trans-Anethole	68.8 ± 6.8 ef	7.5 ± 4.8 ab	12.5 ± 6.3 ab	74.0 ± 8.1 g	90.0 ± 6.3 f
Apiole	56.4 ± 6.9 de	21.7 ± 6.0 cd	23.3 ± 5.6 bc	42.5 ± 4.8 d	73.4 ± 4.0 c
S-(+)-Carvone	8.7 ± 4.0 a	8.3 ± 3.1 ab	18.3 ± 4.0 bc	12.0 ± 8.0 ab	57.5 ± 8.5 bc
Cuminaldehyde	19.4 ± 7.2 ab	8.0 ± 3.7 ab	23.3 ± 9.5 bcd	64.0 ± 8.1 e	92.5 ± 4.8 de
Estragole	70.8 ± 9.2 f	3.3 ± 3.3 a	3.3 ± 3.3 a	70.0 ± 4.7 ef	96.7 ± 2.1 ef
(+)-Fenchone	48.1 ± 8.4 cd	5.0 ± 3.4 a	16.0 ± 5.1 bc	3.3 ± 2.6 ab	3.3 ± 3.3 a
Myristicin	13.6 ± 3.9 a	5.0 ± 3.4 a	8.3 ± 4.0 a	37.5 ± 4.8 cd	67.5 ± 2.5 c
(+)-α-Pinene	16.0 ± 10.3 a	4.0 ± 2.4 a	10.0 ± 0.0 ab	22.5 ± 5.6 bc	68.0 ± 5.4 c
(-)-α-Pinene	9.1 ± 3.8 a	6.0 ± 2.4 a	8.0 ± 2.0 a	16.3 ± 5.6 b	53.3 ± 4.6 b
(-)-β-Pinene	0.7 ± 0.6 a	4.0 ± 2.4 a	6.0 ± 2.4 a	24.0 ± 4.0 bc	90.0 ± 6.3 de
γ-Terpinene	94.8 ± 2.6 g	5.0 ± 2.9 a	7.5 ± 4.8 a	42.5 ± 4.1 d	78.3 ± 3.1 c
F	51.947	94.125	42.624	60.326	42.318
df	132	99	102	102	97
p	0.000	0.000	0.000	0.000	0.000

[a] Hatching and mortality means (% ± SEM) within the same column followed by the same letters are not significantly different based on one-way ANOVA followed by the LSD test ($P < 0.05$).

[b] Ethanol final concentrations: 0.02 mL L^{-1} for juveniles; 0.1 mL L^{-1} for eggs and adults.

[c] Positive control tested at 50 mg L^{-1} against eggs and adults, and at 10 mg L^{-1} against juveniles.

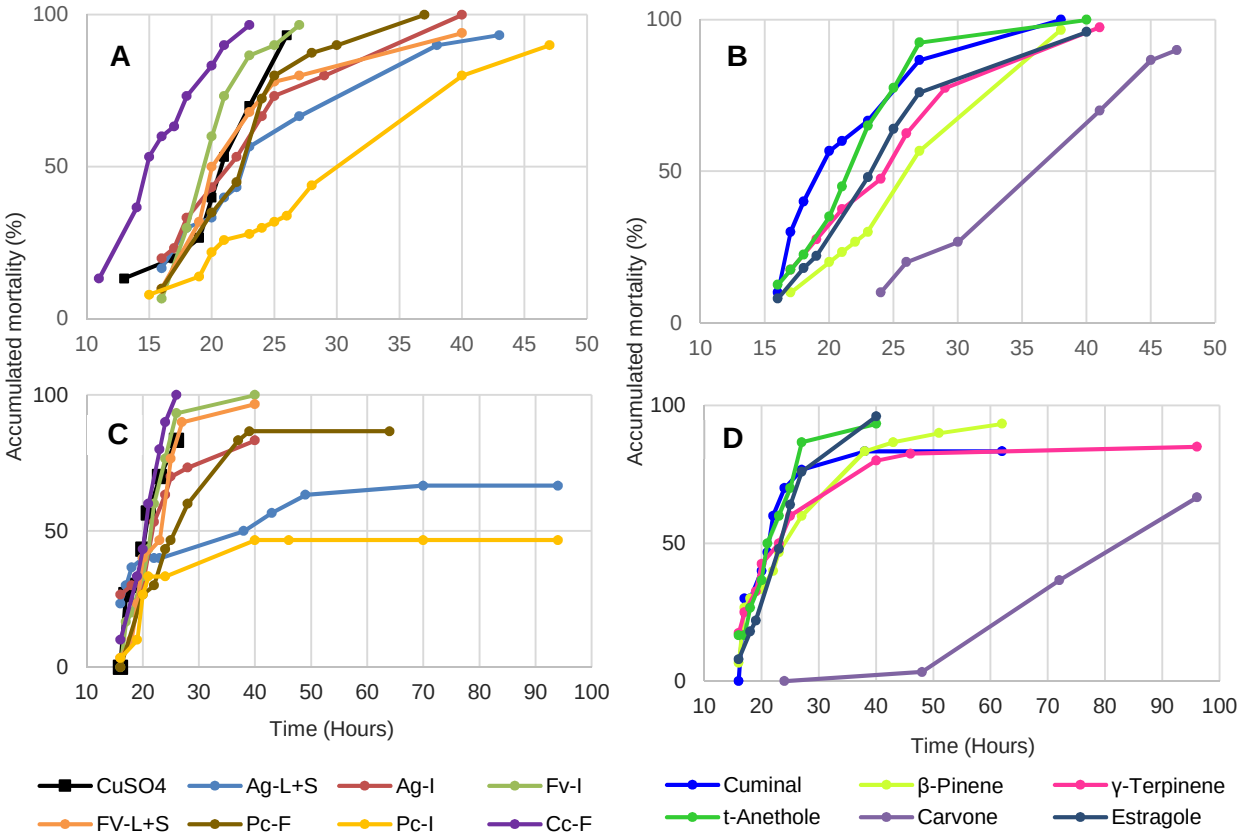


Figure IV.4 (A-D). Accumulated mortality of *Radix peregra* adults throughout time when exposed continuously (**A** and **B**) or during a shorter period of 16 h (**C** and **D**) to seven Apiaceae EOs (**A** and **C**) and six volatile compounds (**B** and **D**). Note: Dots represent times mean values obtained from 4 replicates. Standard error of means varied from 0 to ±15 h. Ag: *Anethum graveolens*, Cc: *Cuminum cyminum*, Fv: *Foeniculum vulgare* var. *vulgare*, Pc: *Petroselinum crispum*, -I: Infrutescences, L:Lleaves more stems, -F: Fruits.

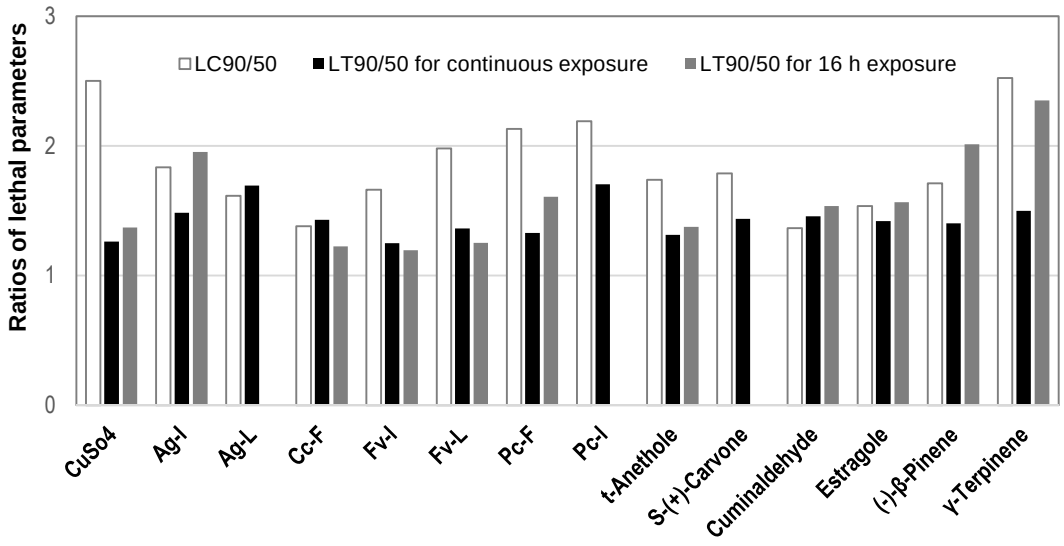


Figure IV.5. Relation between the 90% and 50% lethality values (lethal concentrations and lethal times) on *Radix peregra* adults and comparison of the lethal parameter ratios for seven Apiaceae essential oils and six volatile compounds.Ag: *Anethum graveolens*, Cc: *Cuminum cyminum*, Fv: *Foeniculum vulgare* var. *vulgare*, Pc: *Petroselinum crispum*, -I: Infrutescences, -L: Leaves more stems, -F: Fruits.

CHAPTER V

Apiaceae EOs and volatile compounds activities against armyworm, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae)

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1. Abstract

The potential of four well-known Apiaceae species as natural sources of biochemicals with anti-insect properties was assessed in the present work. Essential oils (EOs) from *Anethum graveolens, Cuminum cyminum, Foeniculum vulgare* var. *vulgare* and *Petroselinum crispum* in addition to 11 pure compounds were evaluated for their antifeedant, growth inhibitory and insecticidal activities against a highly damaging phytophagous, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae).

Anti-insect activity varied according to plant species/composition, type and exposure period. EOs from *P. crispum* (parsley) and *A. graveolens* (dill) fruits, *trans*-anethole and cuminaldehyde displayed acute effects on larvae feeding and growth (FDI and GI >70%). *Vis a vis* to the acute toxicity, *A. graveolens*, *C. cyminum* (cumin) and *F. vulgare* (bitter fennel) EOs and some of their constituents were effective by fumigation (≥80%). Satisfactory contact toxicities (>70%) were observed for five compounds and all EOs (except *F. vulgare* EOs) when tested by the filter paper impregnation method. For the most active EOs/compounds, dose-dependent and time-dependent toxicities were determined and inverse relationships of LC_{50} with time were established.

Dill, cumin and parsley EOs as well as the compound (S)-carvone, cuminaldehyde and myristicin were effective against *P. unipuncta* fourth-instar larvae. The application of these EOs or their major compounds as deterrents and/or insecticides for the control of this lepidopteran could be advantageous.

Keywords: Armyworm, bitter fennel, dill, parsley, cumin, volatile compounds, feeding deterrence, contact and fumigant toxicity.

2. Introduction

The true armyworm, *Pseudaletia* (sin *Mythimna*) *unipuncta* (Haworth) (Lepidoptera: Noctuidae), is a seasonal migrant and a polyphagous pest of grasslands and mixed pastures. It is one of the most important pests of agricultural crops in North America (McNeil et al., 2000) and Europe (Bues et al., 1986). In the Azores Islands (Portugal), high infestations by *P. unipuncta* during summer and early autumn (Tavares et al., 1992) can cause considerable plant damage, corresponding to approximately 8% of the annual production and estimated at EUR 5 million (Tavares, 1992). The hazards of excessive pesticides use for insect pest control have increased the need for effective and biodegradable pesticides. In the last two decades, this growing awareness has motivated the research for safer and environmental friendly alternatives. Biological control with entomopathogenic nematodes (Rosa & Simões, 2004) and application of bioactive natural compounds from plants with phagodeterrent/insecticide properties have been proposed for *P. unipuncta* control (Akhtar et al., 2008; Rosa et al., 2010).

In the context of research for new biopesticides, extracts from plants have received special attention and volatile constituents of EOs are among the candidates with highest potential (Shaaya & Rafaeli, 2007). The relative chemical simplicity and familiarization of the market for aromatic constituents in conjunction with the less toxic nature of these compounds are elements that contribute to the receptiveness to EOs applications. According to a literature survey on biopesticidal potential of EOs from the year 2000 onward (Tripathi et al., 2009) plants of Myrtaceae, Lamiaceae, Asteraceae, Apiaceae, and Rutaceae families are highly targeted for insecticidal activities against specific insect orders like lepidoptera, coleoptera, diptera, isopteran, and hemiptera. EOs have been explored for repellent, fumigant, larvicidal, and adulticidal activities against many species and their potential has been the subject of several reviews (Regnault-Roger, 1997; Isman, 2000; Shaaya & Rafaeli, 2007; Isman, 2006; Tripathi et al., 2009). The insecticidal activity of Apiaceae EOs has been evaluated against a number of arthropods, from stored product and agricultural pests to diseases vectors (Tunç et al., 2000; Kim & Ahn, 2001; Kim et al., 2002; Chaubey, 2008; Lopez et al., 2008; Işik & Görür, 2009; Seo et al., 2009).

On the based on the high bioactive potential of plants belonging to the Apiaceae family and their relatively safe utilization as herbs and spices, in traditional medicine and food, EOs extracted from *Foeniculum vulgare* var. *vulgare*, *Anethum graveolens*, *Petroselinum crispum* and *Cuminum cyminum*, as well as 11 pure compounds, were assayed against *P. unipuncta* for an evaluation of their potential, as natural control agents of this insect. The results of this research are here presented and discussed.

3. Materials and methods

3.1. EOs

EOs that were used in the several bioassays against armyworm are summarized in Table V.1.

Table V.1. Apiaceae essential oils used in anti-insect activity assays against *Pseudaletia unipuncta* larvae.

Scientific name	Common name	Plant parts	Abbreviation
Anethum graveolens L.	Dill	Green infrutescences	Ag-I
		Dried mature fruits	Ag-F
Foeniculum vulgare Mill. subsp. *vulgare* var. *vulgare*	Bitter fennel	Green infrutescences	FV-I
		Stems with leaves	Fv-L
Cumin cyminum L.	Cumin	Fruits [a]	Cc-F
Petroselinum crispum var. *neapolitanum* Danert	Italian parsley	Green infrutescences	Pc-I
Petroselinum crispum (Mill.) Nyman ex A.W. Hill	Parsley	Fruits [a]	Pc-F

[a] *Petroselinum crispum* and *Cuminum cyminum* fruits essential oils were purchase from Sigma-Aldrich, Co.

3.2. Chemicals

Eleven pure compounds were included in anti-insect activity assessments on the base of their relative abundance in the studied EOs. *trans*-Anethole [*trans*-1-methoxy-4-(1-propenyl) benzene, 99% purity, MW = 148.2], (*S*)-(+)-carvone (96% purity, MW = 150.22), cuminaldehyde (4-isopropylbenzaldehyde, 98% purity, MW = 148.2), estragole (1-allyl-4-methoxybenzene, 98% purity, MW = 148.2), (+)-fenchone (99.5% purity, MW = 152.2), γ-terpinene (≥97% purity, MW = 136.23), (-)-β-pinene (99% purity, MW = 136.23), (-)-α-pinene and (+)-α-pinene (98%, MW = 136.23) were purchased from Sigma-Aldrich and Fluka (Aldrich chemical Co., St. Louis, MO, USA). The compounds myristicin (≥95%) and apiole (≥90%) were isolated from *P. crispum* EO by column chromatography as described in section 3.4 of Chapter II.

For feeding deterrence/growth inhibition and paper disk assays, azadirachtin (~95% purity, MW = 720.71, from Sigma) and lambda-cyhalothrin (Judo ® commercial formulation with 100 g L^{-1} of compound from Sapec Agro S.A, Portugal) were used as positive controls, respectively. Solvents, *n*-hexane ethyl acetate and ethanol, were of reagent grade (≥99.9%).

3.3. Bioassays

3.3.1. Insects

Pseudaletia unipuncta fourth-instar larvae were obtained from an established laboratory colony maintained for more than six generations. Larvae were reared on an artificial diet (Poitout & Bues, 1974) and adults were supplied with 10% honey solution in water (Fig. V.1). Insect colonies were maintained in laboratory at 23 ± 1 ºC and 70 ± 5% relative humidity, under a L16:D8 photoperiod.

Fourth-instar larvae of *P. unipuncta,* with the same age and weighing 30-50 mg, were used in this study. All experiments were carried out in an incubator under the same conditions described above.

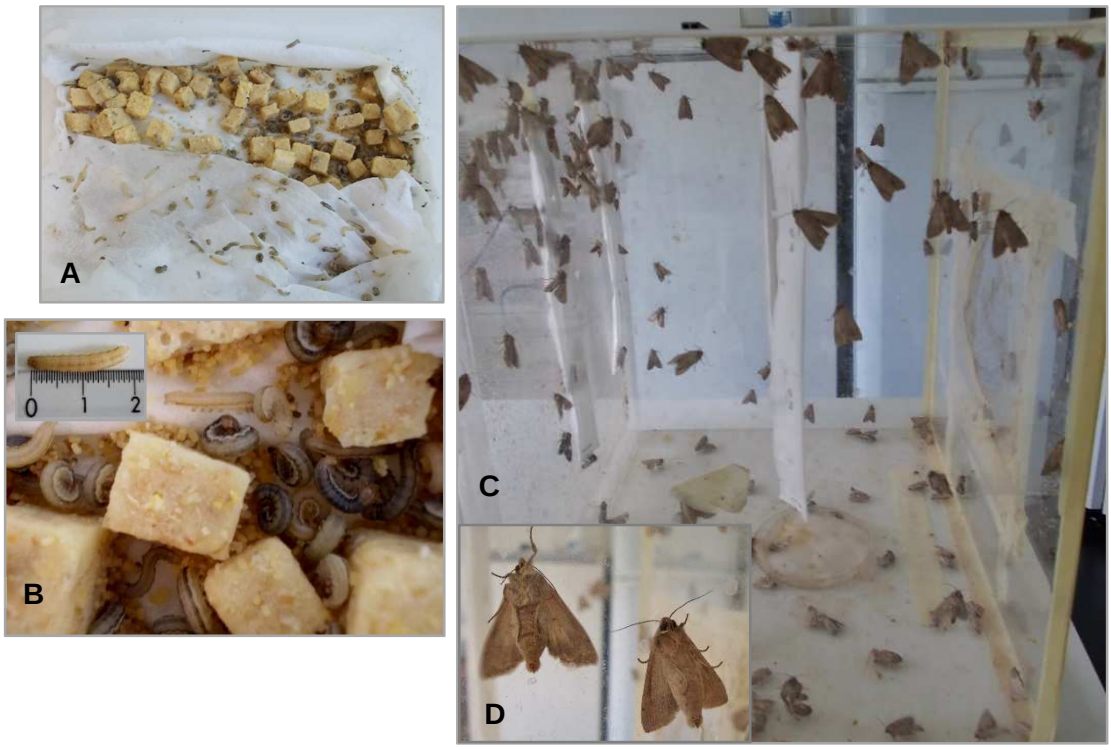


Figure V.1(A-D). *Pseudaletia unipuncta* colony maintained at the Biology Department of the Azores University, in Ponta Delgada, Portugal.

(A) Third-instar larvae are reared inside aerated box with artificial diet cubes. **(B)** Fourth-instar larvae with similar sizes and weights chosen for biological assays (around 2 cm long and 30 to 50 mg each). **(C)** Adults are kept inside an acrylic box for mating. Every day the parchment strips for females' oviposition are replaced and insects are feed with 10% honey solution soaked cotton placed in a Petri dish. **(D)** Adult males (40 mm of wingspan).

3.3.2. Feeding deterrence and growth inhibition

Antifeedant and growth inhibitory activities of EO and pure compounds against *P. unipuncta* were evaluated by the diet-no-choice method (adapted from El-Aswad et al., 2003) using corn (*Zea mays* L.) leaves as food (Fig. V.2). Three independent assays with 10 fourth-instar larvae per treatment (n= 30 individuals), including controls, were carried out on different days. Freshly collected leaves were previously disinfected in 5% formaldehyde solution for 20 min, rinsed with deionized water for 10 min, and dried over absorbent paper before cutting in 4 cm^2 equal pieces. Diet treatment consisted of a unique dosage by covering each preweighted leaf section with 17.5 µL of EOs/constituents (175 µg cm^{-2} or 0.7 mg/leaf from 40 mg mL^{-1} stock solutions in ethanol), pure ethanol (negative control), or azadirachtin (positive control at 10 µg cm^{-2} from a 2.3 mg mL^{-1} stock solution in ethanol), which was left for 15 min in order to evaporate the solvent before larvae feeding.

Bioassays were conducted in sterile Petri dishes (diameter = 4.5 cm) with a thin agar layer (2% w/v) covering the bottom, to prevent food and larvae dehydration. After a 6 h starvation period, larvae were preweighted, distributed individually in Petri dishes and allowed to feed continuously on a treated piece of diet. Food replacements, with single pieces of leaves freshly prepared, were made every 24 h or whenever necessary.

Larvae and diet (provided diet and/or nonconsumed diet) weights (mg) were recorded initially and after 48 and 72 h. After each assay, the final dry weight of larvae was estimated by drying at 50 ºC for 4 days (dry contents varied from 9.8 to 14.2%). Larvae and leaves initial dry weights were extrapolated on the base of mean dry contents determined for representative samples. Thirty individuals and pieces of corn leaves pieces were used for this purpose, and mean dry weights contents were 12.0 (±1.04)% and 28.3 (±2.97)%, respectively.

The antifeedant effects were evaluated by the estimation of the FDI (Feeding Deterrence Index) and the GI (Growth inhibition)[1], which were calculated according to Huang et al. (1997) and El-Aswad et al. (2003), respectively. All calculations were performed on a dry weight basis.

[1] The formulae used to determine the FDI (4) and GI (5) are described in the Appendix C.

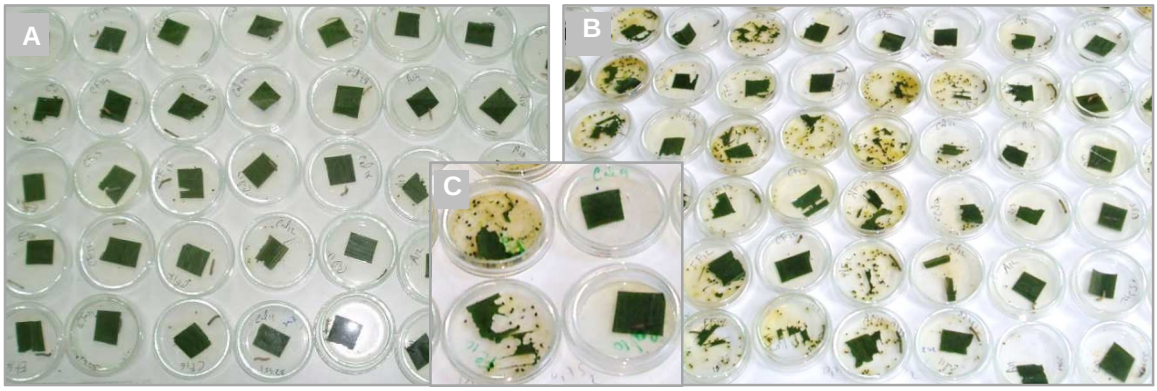


Figure V.2. The no-choice diet assay performed during 3 days in glass Petri dishes, each one containing a *Pseudaletia unipuncta* fourth-instar larva provided with a 4 cm^2 piece of corn leave treated with an EO/compound solution (175 µg cm^{-2}).

(A) Starting of the experiment; **(B)** Food consumption after 48 h of experiment; **(C)** Detail of the effect of bitter fennel EO (in the left) and cuminaldehyde (in the right) over larvae feeding after 48 h.

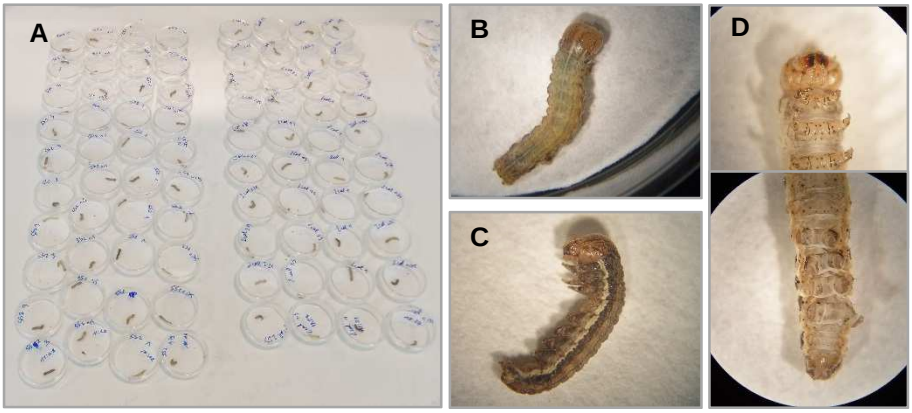


Figure V.3 (A-D). Evaluation of essential oils toxicity to *Pseudaletia unipuncta* 4th instar larvae through the paper disk assay.

(A) Impregnated paper disk bioassay arrangement. Each Petri dishes (3.2 cm inner diameter) contains an EO impregnated filter paper and a 4th instar larvae. **(B)** Living larva in ecdysis, after 72 h of exposure to lower concentration of EO, exhibiting an abnormal development of the body but no signs of intoxication nor involuntary movements(observation with stereoscopic microscope and a x12 magnification). **(C)** Dead larva in a lateral prostrated position after exposure to EO impregnated filter paper (observation with stereoscopic microscope and a x12 magnification). **(D)** Dead larvae presenting extreme dehydration of the lower body parts as a result of exposure to EO (observation with stereoscopic microscope and a x25 magnification).

3.3.3. Contact toxicity through paper impregnation

The filter paper impregnation method was used to examine contact toxicity. In a first experiment, all EOs and pure compounds activities were screened at a single concentration (250 µg cm^{-2}, equivalent to 2 mg per filter paper). Bioassays were conducted in sterile Petri dishes (3.2 cm inner diameter and 1.0 cm high) containing one filter paper disk (8.0 cm^2) impregnated with 50 µL of EO/compound stock solutions (40 mg mL^{-1} of pure ethanol), ethanol (as solvent control) or positive control (lambda-cyhalothrin emulsion in distilled water at 40 mg mL^{-1}). The filter papers were air-dried for 15 min and a single fourth-instar larva was placed into each Petri dish, without food. About 12 h later, one piece of diet (corn leaves) was given to all living larvae. For each treatment three to six replicates of 10 larvae each were used.

Mortality was assessed at 9, 24 and 48 h after treatment onset by observation of larvae with a stereoscopic microscope. Individuals were considered dead when no movement was detected after prodding with a dissecting pin.

3.3.3.1. Dose–response (LCs) and time–response (LTs) assays

An evaluation of the dose-related lethality was conducted afterwards with the most active EOs/compounds following the procedure aforementioned (3.3.3).

For each EO/compound a set of five to seven optimized doses were chosen among the following concentrations: 31, 63, 94, 125, 187, 250, 281, and 375 µg cm^{-2}. Assays were performed four times with 10 replicates per concentration including control groups with ethanol (Fig. V.3, A). Mortality was assessed after 24, 36, 48 and 72 h to determine lethal times (LTs) and evaluate lethal concentrations (LCs) variation over time.

3.3.4. Toxicity through fumigation

The evaluation of the fumigant activity of EOs and pure volatile compounds was conducted using sealed plastic jars with a total inner volume of 0.043 dm^3 (4.5 cm of diameter and 2.7 cm high). For treatment, filter paper disks (diameter = 3 cm) were impregnated with 50 µL of concentrated solution in ethanol containing 10.7 mg of EO or compound. Treated disk were disposed at the top of each jar, and the solvent was left to evaporate during 15 min. Ten fourth-instar larvae were placed inside each jar, and food was provided to prevent cannibalism. Contact of larvae with impregnated filter paper was avoided by disposing a thin net between. The tested concentration was chosen in an attempt to reproduce similar concentration of volatiles per volume of air (250 mg dm^{-3}) with those probably occurred inside the 8.0 cm^3 Petri dishes used for contact toxicity screening (250 µg cm^{-2}). Three to six replicates per treatment and solvent control were done.

Mortality was assessed at 12, 24 and 48 h by observation of larvae through jar with stereoscopic microscope. At 12 and 24 h, individuals were considered dead when no movement was detected, and in the end of experiment the mortality was confirmed by normal procedure, by prodding with a dissecting pin.

3.4. Statistical analyses

The data obtained for antifeedant, growth-inhibition and insecticidal activity of EOs/compounds were submitted to a one-way analysis of variance (one-way ANOVA) procedure. Prior to analysis, antifeedant and growth inhibition data were transformed by $\log_{10}(x+1)$, while mortality rate in both fumigation and contact insecticidal assays were submitted to arcsine transformation. Means with significant variance and *F*-statistic were separated at 5% significance level by Duncan's test available on *IBM SPSS statistics* package 20.0 (IBM Corp.).

Dose-response data obtained by contact toxicity assay were submitted to probit analysis (*IBM SPSS statistics*) to calculate lethal concentrations (LC_{50} and LC_{90}) required to kill 50 and 90% of insects, at 24, 36, 48 and 72 h. The same procedure was used to determine lethal times (LT_{50} and LT_{90}), at a single concentration. Additionally, variations of LC_{50} over time were evaluated by submitting transformed data (LC_{50}^{-1}) to linear regression model on *Graph Pad Prism 4*) (Prism 4.00 for Windows, Graph Pad software Inc., USA).

4. Results and discussion

4.1. EOs composition

The qualitative and quantitative characterization of EOs used in the present study were previously characterized in the Chapter II. The major EO compounds, representing more than 1%, are exhibited in figure II.6.

4.2. Feeding deterrence and growth inhibition

The Bioactivities of six different EOs and nine pure compounds on *P. unipuncta* fourth-instar larvae feeding and growth after 48 and 72 h of experiment are presented in Table V.2 and V.3, respectively. The FDI determined for EOs and compounds were significantly different [*F*-test = 46.31 (48 h), 46.42 (72 h); df = 47; $p < 0.001$] and varied from -26.2 to 99.7% and -20.3 to 99.7% after 48 and 72 h, respectively. Significant differences among treatments were also observed for GI results [*F*-test = 46.82 (48 h), 59.93 (72 h); df = 46; $p = 0.000$], as values ranged from -21.0 to 136.3% at 48 h and -1.1 to 127.4% at 72 h.

Table V.2. Effects of *Foeniculum vulgare* var. *vulgare* (bitter fennel), *Anethum graveolens* (dill), *Petroselinum crispum* (parsley), *Cuminum cyminum* (cumin) essential oils and pure compounds on *Pseudaletia unipuncta* 4th instar larvae feeding on fresh corn leaves treated with 175 µg cm^{-2}, assessed after 48 and 72 hours of treatment.

Treatments	Mort. 72h (%)	Relative mean consumed diet [a] (% ± SEM) [b]		Feeding Deterrence Index (% ± SEM) [b]	
		48 h	72 h	48 h	72 h
Ethanol	0.0	43.7 ± 4.8 ghi	57.5 ± 5.3 e	....	....
Azadirachtin [c]	0.0	10.5 ± 1.5 bc	8.6 ± 1.1 ab	85.1 ± 1.1 g	93.3 ± 0.7 h
EOs					
Fv-I	0.0	33.6 ± 4.7 efg	56.4 ± 1.8 e	8.0 ± 1.7 b	11.5 ± 3.0 c
Fv-L	0.0	42.4 ± 1.1 ghi	65.4 ± 1.9 f	- 26.2 ± 8.5 a	- 20.3 ± 3.6 a
Ag-F	6.7	6.8 ± 2.6 abc	26.3 ± 8.6 c	84.7 ± 5.2 g	75.6 ± 6.4 g
Ag-I	3.3	28.7 ± 3.5 ef	39.9 ± 1.4 d	17.7 ± 6.5 bcd	31.5 ± 8.5 de
Pc-F	32.4	0.1 ± 0.1 a	0.2 ± 0.2 a	99.7 ± 0.3 g	99.7 ± 0.3 h
Cc-F	3.3	22.5 ± 3.6 de	47.1 ± 3.8 de	44.6 ± 2.3 ef	32.9 ± 4.0 de
Compounds					
trans-Anethole	12.9	4.6 ± 0.7 ab	17.1 ± 3.9 bc	92.4 ± 1.7 g	84.6 ± 4.6 gh
Estragole	3.3	15.9 ± 1.8 cd	42.1 ± 4.8 de	59.5 ± 4.0 f	49.8 ± 5.8 f
(+) -Fenchone	0.0	48.6 ± 2.6 hi	52.9 ± 5.4 def	19.7 ± 2.8 bcd	25.5 ± 6.9 cd
(*S*)-(+)-Carvone	0.0	34.4 ± 1.1 efg	55.5 ± 6.3 def	46.3 ± 3.4 ef	45.9 ± 2.4 g
Myristicin	100 [d]	-	-	-	-
Apiole	60.0 [d]	-	-	-	-
γ- Terpinene	0.0	43.3 ± 5.1 ghi	53.8 ± 3.0 def	25.8 ± 1.8 cd	20.1 ± 4.9 cd
Cuminaldehyde	12.9	8.4 ± 4.5 abc	22.3 ± 6.3 c	84.7 ± 4.4 g	74.0 ± 6.7 g
(-)-α-Pinene	0.0	41.4 ± 7.9 fgh	53.3 ± 1.3 def	32.5 ± 3.7 de	29.8 ± 5.5 d
(+)-α-Pinene	0.0	47.9 ± 2.9 hi	54.1 ± 2.2 def	20.3 ± 4.1 bcd	21.0 ± 3.7 cd
(-)-β-Pinene	0.0	53.2 ± 6.3 i	66.6 ± 4.3 f	13.1 ± 6.2 bc	-6.0 ± 4.7 b

[a] Mean consumed diet per larvae (mg of dry leaf) relatively to the initial mean dry weight of leaves. [b] Means values for three replicates with ten fourth-instar larvae per replicate. Means (± SEM) in the same column followed by the same letters are not significantly different based on one-way ANOVA followed by Duncan´s Multiple range test ($p < 0.05$). [c] Positive control with 95% pure azadirachtin tested at 10 µg cm^{-2}. [d] High mortality was recorded in all experiments for the tested concentration.

Table V.3. Effects of *Foeniculum vulgare* var. *vulgare*, *Anethum graveolens, Petroselinum crispum*, *Cuminum cyminum* essential oils and pure compounds (at 175 µg cm^{-2}) on fourth-instar larvae of *Pseudaletia unipuncta* growth, assessed after 48 and 72 hours of feeding on treated corn leaves.

Treatments	Relative mean weight gain [a] (% ± SEM)[b]		Growth inhibition (% ± SEM) [b]	
	48 h	72 h	48 h	72 h
Ethanol	74.0 ± 6.3 fg	133.3 ± 6.6 h	....	....
Azadirachtin [c]	-7.7 ± 3.2 b	-9.7 ± 1.1 b	112.2 ± 5.4 efg	127.4 ± 9.2 gh
EOs				
Fv-I	52.5 ± 6.7 e	81.0 ± 4.9 fg	34.0 ± 8.1 bc	43.2 ± 2.5 cd
Fv-L	84.7 ± 7.4 g	126.2 ± 4.1 h	- 21.0 ± 6.7 a	- 1.1 ± 2.3 a
Ag-F	7.2 ± 1.2 c	25.0 ± 4.3 c	91.2 ± 2.0 e	83.2 ± 3.2 f
Ag-I	53.5 ± 2.7 e	88.9 ± 3.5 fg	32.5 ± 7.2 bc	37.2 ± 6.5 cd
Pc-F	- 22.0 ± 2.5 a	- 25.3 ± 1.2 a	136.3 ± 6.4 g	122.8 ± 1.4 h
Cc-F	31.1 ± 3.1 d	69.3 ± 0.7 e	49.6 ± 3.2 cd	38.9 ± 6.0 cd
Compounds				
trans-Anethole	- 9.8 ± 4.2 b	- 8.1 ± 5.5 b	132.2 ± 11.2 fg	117.4 ± 4.0 gh
Estragole	30.4 ± 4.2 d	53.2 ± 2.7 d	62.3 ± 1.5 d	62.6 ± 1.2 e
(+)-Fenchone	55.6 ± 4.8 ef	93.5 ± 6.0 g	23.6 ± 4.3 b	30.8 ± 3.0 c
(*S*)-(+)-Carvone	39.2 ± 2.0 de	74.3 ± 4.0 ef	46.4 ± 0.7 cd	46.8 ± 2.3 d
Myristicin [d]	-	-	-	-
Apiole [d]	-	-	-	-
γ-Terpinene	43.7 ± 7.2 de	81.0 ± 4.2 efg	39.1 ± 6.4 bc	38.0 ± 5.2 cd
Cuminaldehyde	- 4.0 ± 1.0 bc	- 2.0 ± 1.0 b	106.4 ± 1.4 ef	101.7 ± 0.8 g
(-)-α-Pinene	40.9 ± 5.4 de	78.9 ± 8.8 efg	42.6 ± 4.8 c	39.8 ± 6.7 cd
(+)-α-Pinene	49.1 ± 7.4 e	88.7 ± 7.8 fg	40.0 ± 7.5 bc	35.3 ± 6.7 cd
(-)-β-Pinene	57.6 ± 7.0 ef	114.5 ± 6.7 h	32.8 ± 3.1 bc	12.2 ± 5.3 b

[a] Mean weight gain of larvae (mg of dry larvae) relatively to their initial mean dry weight. [b] Means values for three replicates with ten fourth-instar larvae per replicate. Means (± SEM) in the same column followed by the same letters are not significantly different based on one-way ANOVA followed by Duncan´s Multiple range test ($p < 0.05$). [c] Positive control with 95% pure azadirachtin tested at 10 µg cm^{-2}. [d] High mortality was recorded in all experiments for the tested concentration.

Only *P. crispum*, *A. graveolens* fruits EOs (Pc-F and Ag-F) and the pure constituents *trans*-anethole and cuminaldehyde showed effective feeding deterrence (FDI >70%). Avoidance of food treated with Pc-F, *trans*-anethole or cuminaldehyde by larvae was pronounced, especially in the first 48 h, which caused substantial weight loss (negative values in Table V.3) and GI above 100%.

Despite their higher concentration (175 µg $cm^{-2)}$, the inhibitory effects of these treatments were statistically similar to those exhibited by the antifeedant compound azadirachtin at 10 µg cm^{-2} (17 times less concentrated). Additionally, low to moderate activity was attributed to Cc-F, (*S*)-(+)-carvone and estragole (FDI ≤ 45% and GI ≤ 60%, after 48 h). Nonetheless, the low feeding deterrence activity of Cc-F described herein against *P. unipuncta* (45% at 175 µg cm^{-2}) was far greater than the activity recorded on *S. litura* (antifeedant Index = 40% for 2 mg cm^{-2}) (Elumalai et al., 2010). Approximated feeding deterrence (50%) has been attributed to other EOs. For example, a DC_{50} value of 206 µg cm^{-2} was reported for clove oil against the same noctuid specie (Akhtar et al., 2008). Relatively to the remaining EOs/compounds, these were considered ineffective for feeding dissuasion and even stimulant, as it is the case of Fv-L and the compound (-)-β-pinene after 72 h (Table V.2). For all treatments, excepting Pc-F and the positive control, an increase in the consumption percentage was observed in the last 24 h, revealing some habituation to treated food. According to Usher and co-workers (1988), deprivation might allow acceptance of less preferred plants after repeated contacts and such behavioral flexibility would have adaptive value. Induction of detoxification enzymes and alteration of compounds toxicity are possible adaptive responses.

Our findings also suggest that *trans*-anethole was not only phagorepellent but it may also have interfered in the process of assimilation and conversion of food to biomass. Post-ingestion effects, such as digestive disorders or physiologic stress, could be the primary cause for the larvae weight loss and death observed in 13% of individuals. Evidence of *trans*-anethole metabolization to a hydroxylated form was found in feces of the tobacco cutworm (*Spodoptera litura*) after topical and oral administration of the compound (Passreiter et al., 2004). However, the impact on growth and larvae fitness was not referred. An effective feeding deterrence of *trans*-anethole on fifth-instar larvae of *S. litura* was already reported and 50% deterrence (DC_{50}) was observed at a concentration of 103 µg cm^{-2} (Hummelbrunner & Isman, 2001). Regarding the results obtained for EOs and their major compounds tested individually, a few relations may be established. In the particular case of *A. graveolens*, the Ag-I was significantly less active than Ag-F. These findings suggest that the less significant deterrent effect might be a consequence of the (*S*)-(+)-carvone lower content in green infrutescences (64%), relatively to fruits EO (84%), and/or the occurrence of antagonistic effects with other compounds (Fig. II.6, B). However, when tested pure and at a slightly higher dosage, a significant decrease in the feeding deterrence of (*S*)-(+)-carvone was recorded revealing the contribution of other compounds (β-phellandrene + limonene), coexisting in the dill fruits EO in lower amount (14%), to enhance the antifeedant property of carvone in this natural mixture. For Cc-F, we confirmed that its activity was closely related with the major constituent content, cuminaldehyde (39%) (Fig. II.6, D), which was more active (GI and FDI values 2-2.6 time superior) when tested pure at a higher concentration (2.6 times). The results obtained for γ-terpinene and β-pinene showed that both compounds have negligible effects in this context when tested individually and at much higher dosage (respectively, 6.2 and 8 times more concentrated than in the EO). Nonetheless, the occurrence of synergism between cuminaldehyde and further compounds is not discarded. Fennel EOs showed irrelevant activities in this experiment, and the results obtained for both natural mixtures

could not be related to any individual activity of the major compounds included in the study [*t*-anethole, estragole, fenchone, (+) and (-)-α-pinene and (-)-β-pinene]. The stimulant effects observed with fennel stems and leaves EO is attributed to the specificity of the mixture presenting a considerable increase in the pinenes and phellandrenes proportion relative to (+)-fenchone and estragole, in comparison with the infrutescences EO (Fig. II.6, A).

Pc-F was the most effective among the EOs, monoterpenes and phenylpropane derivative compounds tested in the present work, as virtually no food intake was registered. Severe weight loss and, consequently, starvation was the major cause of the high mortality (32%). The phagorepellent property of this EO was evident (DC_{50} and DC_{90} should be inferior to 175 µg cm^{-2}) and certainly more related to the myristicin (32%) and apiole (16%) than pinenes contents (30%) (Fig. II.6, C), even though some synergisms may occur. The strong toxicity observed for both tested phenylpropanoids (no consumption and 100% and 50% of death for myristicin and apiole, respectively, after 72 h) did not allowed to complete the evaluation of their effects at the unique dose tested, and further assays should be envisaged. As far as we know, less attention has been given to the anti-insect activities of EOs from this specie. We consider that the EO from parsley fruits has some potential, and a DC_{50} value close to the one estimated for rotenone on *P. unipuncta* third-instar larvae (62 µg cm^{-2}) (Akhtar et al., 2008) is plausible.

In the domain of antifeedant/deterrent biopesticides, a tremendous effectiveness has been attributed to Meliaceae species and their limonoid triterpenes compounds (El-Aswad et al., 2003; Isman, 2005; Isman & Akhtar, 2007). Feeding deterrent activity of *Azadirachta indica, Melia volkensii* and *A. excelsa* extracts against *P. unipuncta* third-instar larvae were remarkable (DC_{50} = 0.6, 10.8, and 46.9 µg cm^{-2}, respectively) (Akhtar et al., 2008). The high effectiveness of the limonoid compound azadirachtin, against the same phytophagous specie, was confirmed in our study.

4.3. Evaluations of acute toxicity: paper disk assay and fumigation

The toxicity of seven EOs and nine compounds evaluated by fumigation and paper disk assay with *P. unipuncta* larvae are described in the Tables V.4 and V.5, respectively. Differences recorded among treatments by fumigation [*F*-test = 27.55 (12 h), 31.59 (24 h), 52.57 (48 h); df = 62; p <0.001] and filter paper impregnation [*F*-test = 16.72 (9 h), 22.87 (24 h), 62.57 (48 h); df = 84; p <0.001] were significant.

In general, some activity was observed in fumigant and contact toxicity assays performed at the unique screening dosages of 250 µg cm^{-3} and 250 µg cm^{-2}, respectively. *A. graveolens* EOs exhibit larvicidal properties in both assays, causing mortalities above 50% in the first exposure periods (9 and 12 h). Similar results were observed for Cc-F, although its activity was stronger through contact (93%), and *F. vulgare* var. *vulgare* and *P. crispum* EOs gave ambiguous results when comparing the two assays. Fumigant effectiveness was registered with fennel EOs (100 and

96%, after 48 h treatments), while negligible toxicity was recorded in the disk contact assay. On the contrary, Pc-F and PC-I did not show significant effects when larvae were exposed to its vapors, despite the contact toxicities of these EOs evaluated evidenced in paper impregnation assay (94 and 68% mortality at 48 h).

Table V.4. Toxicity of *Foeniculum vulgare* var. *vulgare*, *Anethum graveolens, Petroselinum crispum*, *Cuminum cyminum* essential oils and pure compounds against *Pseudaletia unipuncta* fourth-instar larvae evaluated by fumigation (250 µg cm^{-3}) and assessed after 12, 24 and 48 hours of treatment.

Treatments		**Mortality** (% ± SEM) [a]		
	n [b]	**12 h**	**24 h**	**48 h**
Ethanol	300	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
EOs				
Fv-I	40	40.0 ± 5.8 b	97.5 ± 2.5 g	100.0 ± 0.0 e
Fv-L	50	6.0 ± 4.0 a	58.0 ± 5.8 cd	96.0 ± 2.4 de
Ag-F	30	96.7 ± 3.3 d	100.0 ± 0.0 g	100.0 ± 0.0 e
Ag-I	60	65.8 ± 9.4 c	90.0 ± 6.3 fg	100.0 ± 0.0 e
Pc-F	40	5.0 ± 2.9 a	5.0 ± 2.9 ab	5.0 ± 2.9 a
Pc-I [d]	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Cc-F	50	14.0 ± 4.0 ab	58.0 ± 8.0 cd	80.0 ± 8.4 bc
Compounds				
trans- Anethole	40	0.0 ± 0.0 a	34.3 ± 3.3 bc	68.9 ± 8.2 b
Estragole	40	0.0 ± 0.0 a	72.5 ± 2.5 de	97.5 ± 2.5 de
(+)- Fenchone	40	2.5 ± 2.5 a	12.5 ± 6.3 ab	22.5 ± 7.0 a
(*S*)-(+)- Carvone	40	95.0 ± 2.9 d	100.0 ± 0.0 g	100.0 ± 0.0 e
Myristicin	- [d]	-	-	-
Apiole	- [d]	-	-	-
γ- Terpinene	40	82.5 ± 4.8 c	92.5 ± 3.9 fg	100.0 ± 0.0 e
Cuminaldehyde	40	28.0 ± 9.2 ab	80.0 ± 7.1 ef	88.0 ± 4.9 cd
(-)-α-Pinene	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
(+)-α-Pinene	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
(-)-β-Pinene	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a

[a] Means (± SEM) of mortality in the same column followed by the same letters are not significantly different based on one-way ANOVA followed by Duncan´s Multiple range test ($p < 0.05$). [b] Number of tested insects. [c] EO extracted from the *Petroselinum crispum* var. *neapolitanum* Danert [d] These compounds were not included in the fumigation assessment as they are less volatile at room temperature.

Table V. 5. Toxicity of *Foeniculum vulgare* var. *vulgare*, *Anethum graveolens, Petroselinum crispum*, *Cuminum cyminum* essential oils and pure compounds against *Pseudaletia unipuncta* fourth-instar larvae evaluated by contact disk assay at 250 µg cm^{-2} and assessed after 9, 24 and 48 hours of treatment.

Treatments	*n* [b]	Mortality (% ± SEM) [a]		
		9 h	24 h	48 h
Ethanol	60	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
lambda-Cyhalothrin [c]	40	0.0 ± 0.0 a	12.5 ± 4.8 ab	62.5 ± 6.3 b
EOs				
Fv-I	30	3.7 ± 3.3 ab	7.4 ± 3.3 a	7.4 ± 3.3 a
Fv-L	30	3.6 ± 3.3 ab	7.1 ± 3.3 a	7.1 ± 3.3 a
Ag-F	40	83.3 ± 3.3 f	100.0 ± 0.0 g	100.0 ± 0.0 f
Ag-I	60	50.0 ± 7.1 e	88.3 ± 5.4 ef	95.0 ± 3.4 f
Pc-F	60	10.0 ± 7.7 ab	60.0 ± 6.3 cd	93.3 ± 3.3 ef
Pc-I [d]	40	0.0 ± 0.0 a	40.0 ± 9.1 bc	67.5 ± 4.8 bc
Cc-F	40	32.5 ± 7.5 cd	80.0 ± 3.2 de	92.0 ± 2.0 ef
Compounds				
trans-Anethole	60	20.0 ± 10.0 bc	73.3 ± 8.0 de	95.0 ± 2.2 f
Estragole	40	2.5 ± 2.5 ab	5.0 ± 5.0 a	15.0 ± 6.4 a
(+)-Fenchone	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
(*S*)-(+)-Carvone	40	37.5 ± 4.8 cde	97.5 ± 2.5 fg	97.5 ± 2.5 f
Myristicin	50	2.0 ± 2.0 ab	72.0 ± 4.9 d	100.0 ± 0.0 f
Apiole	40	0.0 ± 0.0 a	20.0 ± 7.1 ab	72.5 ± 8.5 cd
γ- Terpinene	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Cuminaldehyde	50	44.0 ± 9.3 de	75.0 ± 9.9 de	80.0 ± 8.2 de
(-)-α-Pinene	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0,0 ± 0.0 a
(+)-α-Pinene	40	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
(-)-β-Pinene	40	2.5 ± 2.5 ab	2.5 ± 2.5 a	2.5 ± 2.5 a

[a] Means (± SEM) of mortality in the same column followed by the same letters are not significantly different based on one-way ANOVA followed by Duncan´s Multiple range test ($p < 0.05$). [b] Number of tested insects- [c] Pyrethroid insecticide. [d] EO extracted from the *Petroselinum crispum* var. *neapolitanum* Danert

Concerning the tested compounds, estragole, γ-terpinene, and (+)-fenchone were more active by fumigation than by contact with larvae body. The toxicity of *trans*-anethole though contact was higher (96%) relatively to its isomer estragole (15%), despite its lower fumigation effectiveness (69%). Similar observations were described by Bhardwaj et al. (2010) against the tobacco armyworm. Cuminaldehyde larvicidal activity was approximately the same in both assays (88 and 80% of mortality after 48 h of fumigation and contact with impregnated paper, respectively). Even though the results obtained for some treatments were slightly different 24 and 48 h, both toxicity

assays demonstrated that some EOs and pure compounds might have broader activity (Fig. V.4) and probably several modes of action (dill EOs, cumin EO, (*S*)-(+)-carvone, cuminaldehyde and *t*-anethole), whereas others can be more active by a specific way of entry/contact (bitter fennel and parsley EOs, estragole and γ-terpinene) (Fig. V.4). Based on the differences of effectiveness determined for several EO constituents against the larvae of *S. littoralis*, Pavela (2010) stated that the fumigation effects were directly related with the capability of substances to move in space.

Anethum graveolens EOs have been widely recognized for their insecticidal properties namely against *Aedes aegypti* larvae (Lichtenstein et al., 1974; Promsiri et al., 2006), the coleopterans *Acanthoscelides obtectus* (Regnault-Roger et al., 1993)*, Callosobruchus maculatus* (Tripathi et al., 2001) and *Callosobruchus chinensis* (Chaubey, 2008), and the Japanese termite (*Reticulitermes speratus*) (Seo et al., 2009). Likewise, a strong activity of *C. cyminum* EOs on insects and other arthropods has been reported. Cumin EO was effective as fumigant against *A. obtectus* (Regnault-Roger et al., 1993), the cotton aphid (*Aphis gossypii*) (Tunç & Şahinkaya, 1998), the pulse beetle (*C. chinensis*) (Chaubey, 2008), the larvae of *Lycoriella ingenua* (Park et al., 2008), and as contact larvicidal agent against *S. litura* (Elumalai et al., 2010). In some of these studies, and as we observed, the toxicity of dill and cumin EOs were closely related with the high contents of carvone and cuminaldehyde, respectively. Carvone and cuminaldehyde were 100% and 98% active by fumigation at 5×10^{-3} mg mL^{-1} air against *L. ingenua* (Park et al., 2008). Cumin and dill EOs, characterized by 43% of cuminaldehyde and 35% of carvone, respectively, were among the most fumigant EOs against *A. obtectus* (Regnault-Roger et al., 1993).

Bitter fennel EOs and its major constituent, estragole, were much more active without skin contact. A plausible hypothesis is that the delivery of these oils to target tissues may occur more effectively through a vapor phase, preferentially through penetration via the respiratory system. There are several evidence of the fumigant potential of *F. vulgare* EOs and/or its major constituents against stored product coleopterans (Kim & Ahn, 2001), mosquito species (Kim et al., 2002; Traboulsi et al., 2005) aphid (Işik & Görür, 2009) and lepidopteran (Abdelgaleil, 2010). On the basis of the differences observed on the fumigation screening for bitter fennel EOs and their major compounds (fruits EO ≥ stems and leaves EO ≥ estragole > *t*-anethole > (+)-fenchone >> α- and β-pinenes), we consider that the fumigation activity of both EOs is certainly related with the estragole content. Nevertheless, synergist/antagonist activities between major and minor constituents are probable. It has been demonstrated that some ketone monoterpenes, namely (+)-fenchone, were more effective as fumigants than alcohols (Abdelgaleil, 2010) and that compounds with a phenol, alcohol, aldehyde, and ketone functional group are generally more toxic than hydrocarbons (Seo et al., 2009; Park et al., 2008). Kim & Ahn (2001) observed a higher activity of estragole and (+)-fenchone against the two pests *Sitophilus oryzae* and *C. chinensis* relative to *trans*-anethole. In our study, (+)-fenchone was found to be less active as fumigant relatively to both phenols, the aldehyde and the monoterpenoid hydrocarbon tested.

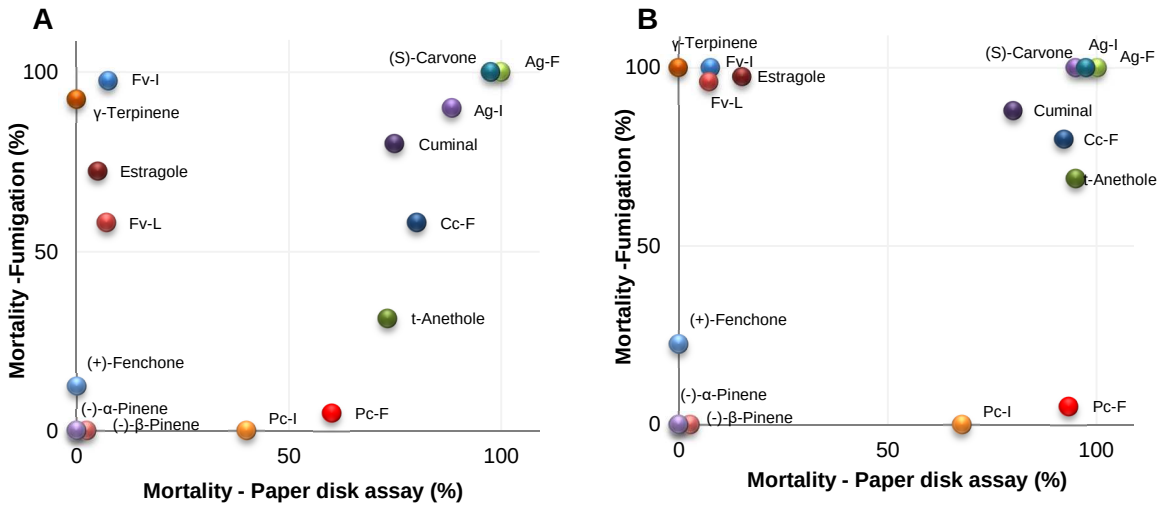


Figure V.4 (A and B). Plotting of lethal effects observed by fumigation *versus* contact (paper impregnation) treatments against *Pseudaletia unipuncta* 4th instar larvae after: **(A)** 24 h and **(B)** 48 h. *Foeniculum vulgare* var. *vulgare*, *Anethum graveolens, Petroselinum crispum* and *Cuminum cyminum* essential oils and pure compounds lethality after 24 and 48 h are represented by the same colored dots.

Note: We used proportional amounts of EO/compound in both experiments considering the calculated inner volumes of the fumigation jar and the Petri dishes. However, it is predictable that, after some hours of experiment, the concentrations of volatiles in the vapor phase may have been superior in the fumigated environment as consequence of a minor gas exchange comparatively to Petri dishes (ventilated).

Regarding parsley fruits EOs, chemical profiles had an important role in bioactivities reported herein. A low fumigant activity of fruits EO was already reported by Regnault-Roger et al. (1993) for a EO constituted by 43% of apiole and 10% of thymol (LC_{50} > 100 mg dm^{-3}). Likewise, Tripathi et al. (2001) noted that *Anethum sowa* EO fraction, which was rich in dillapiole, had no fumigant toxicity against *C. maculatus* despite its ovicidal activity. The absence of toxicity through the fumigation method can be explained, to some extent, by the predictable reduced concentrations of phenylpropane derivatives in the vapor phase at room temperature, as a consequence of their high boiling points (276.5 and 294 ºC for myristicin and apiole, respectively) and low vapor pressures (0.008 and 0.003 mm/Hg for myristicin and apiole, respectively)[2]. Thus, on the basis of the observed results, toxicity exerted at the tissue level via penetration through the insect cuticle or digestive apparatus (during grooming) is more plausible. In fact, some insecticidal potential has been attributed to phenylpropene derivative compounds, but their activity is greatly influenced by the variations in the isomeric forms and/or functionalities attached to either the aryl ring or the alkyl side chain (Seo et al., 2009). Lichtenstein et al. (1974) reported that apiole, dillapiole, and myristicin were much more toxic to fruit flies than S-carvone and that apiole, having two methoxy groups, appeared to be more toxic than myristicin, which has just one. Conversely, in the present study, the contact

[2]Supplementary information concerning the physical properties of some standards are provided in the Appendix A (Table A.4).

toxicity of myristicin was significantly superior to the apiole insecticidal activity recorded at 24 and 48 h and (*S*)-(+)-carvone was much more effective than both phenylpropenes, in the first 24 h of exposure. Both compounds, apiole and myristicin, are methylenedioxyphenyl-containing phenylpropene constituents commonly found in Apiaceae and Piperaceae species, which have been documented for their significant enhancing effect on the efficacy of synthetic or naturally occurring insecticides, acting as natural synergists (Lichtenstein & Casida, 1963; Lichtenstein et al., 1974; Berenbaum & Neal, 1985).

Briefly, our results are in accordance with a previous study as we observed that the efficiency of some EOs and constituents were different depending on the type of application and that their activity could be related, in some extent, with the physical properties of volatile constituents (Pavela, 2010).

Furthermore, the larvae' behavior in response to acute doses of treatments is another important aspect to be mentioned. The observable effects that were recorded during assays following the contact of larvae with the most active EOs and/or compounds (paper disk assay) are consistent with the set of symptoms associated to the neurotoxic action typically reported (Fig. I.8). A notable hyperactivity with incoordination of movements was evident only few minutes following the application. Then, death was preceded by a general state of paralysis as well as sporadic spasms. Simultaneously, we observed other physiological effects during the appraisal of acute toxicity. Subsequently to the contact with paper disks impregnated with bioactive products, several moribund larvae exhibited extreme dehydration (Fig. V.3). In opposite, in some fumigation assays we generally witnessed the swelling of larvae body.

4.3.1. Determination of lethal concentrations (LCs) and toxicity over time of exposure (LTs)

Paper impregnation was the chosen method to probe toxicity caused by contact exposure of *P. unipuncta* fourth-instar larvae to EOs and pure compounds. Lethal parameters (LCs and LTs for 50 and 90% of mortality) were determined for treatments that showed higher effectiveness in the single-dose screening assay: the EOs Ag-I, Pc-F, Cc-F, and constituents *trans*-anethole, (*S*)-(+)-carvone, myristicin and cuminaldehyde. After 24 h of treatment LC_{50} of EOs were considered significantly different and (*S*)-(+)-carvone LC_{50} value was found to be statistically distinct from that of other compounds (Table V.6). Results also indicate that for the evaluation of insecticidal properties the LC_{50} parameter has a better discrimination power, since less significant differences were detected among treatments based on LC_{90}. Thus, for a 24 h evaluation, Ag-I and (*S*)-carvone were considered the most active (108 and 109 µg cm^{-2}) and Cc-F was the least effective of all treatments (197 µg cm^{-2}). On the basis of the accumulated mortalities recorded over time of exposure, we were able to compare the treatments toxicity for an intermediate single-dose, with larvicidal effects above 50% (Table V.7).

The 50% killing performance achieved with Ag-I, and its major compound carvone, was significantly higher than Pc-F and myristicin, *trans*-anethole or cuminaldehyde. After 13 and 18 h of treatment at a medium concentration, 50% of individuals were killed by (*S*)-(+)-carvone and dill EO, respectively, while more than twice that time was needed for *trans*-anethole and cuminaldehyde. Linear regressions between the inverse of OE/constituents activities (LCs^{-1}) and time of exposure (h) were established, except for myristicin, and they are presented in Table V.8.

All slopes were significantly different from zero with the exception of that of *P. crispum* (p = 0.062, r = 0.94). The comparison of the different slopes gives useful information about the potency of treatments. A higher effectiveness and toxicity were recorded for EOs or constituents with highest slopes, as was the case for Ag-I and its major compound, (*S*)-(+)-carvone, followed by Pc-F and *trans*-anethole.

Table V.6. LC_{50} and LC_{90} (µg cm^{-2}) estimated by the disk assay method for the most active essential oils and constituents against fourth-instar larvae of *Pseudaletia unipuncta* after 24 hours of treatment.

Treatments [a]	**LC_{50}** [b, c] (95% CL)	**LC_{90}** [b, c] (95% CL)	**Slope** [b, c] (± SEM)	***H*** [b, d]
EOs				
Ag-I	107.7 a (95.0 – 121.3)	343.6 b (268.5 – 509.3)	2.5 ± 0.32 a	2.60
Pc-F	156.0 b (137.4 – 181.6)	641.4 b (451.6 – 1159.1)	2.1 ± 0.28 a	0.68
Cc-F	196.8 c (181.9 – 215.7)	404.1 b (344.0 – 509.8)	4.1 ± 0.42 b	1.38
Compounds				
trans- Anethole	178.7 bc (161.4 – 202.6)	504.7 b (396.2 – 726.0)	2.8 ± 0.31 ab	1.89
(*S*)-(+)-Carvone	108.6 a (102.4 – 113.9)	160.0 a (150.8 – 153.0)	7.6 ± 0.75 c	1.46
Myristicin	157.5 b (143.1– 175.8)	443.5 b (355.4 – 615.6)	2.9 ± 0.30 ab	0.73
Cuminaldehyde	151.4 b (133.2 – 176.3)	661.6 b (482.6 – 1052.3)	2.0 ± 0.21 a	0.51

[a] The number of insects used for determination of LCs within each treatment varied between 200 and 280, excluding the ones used in controls. [b] Values were determined by probit analysis based on mortalities recorded by contact toxicity assay performed at different optimized concentrations (five to six concentrations between 31 and 375 µg cm^{-2}). [c] LC_{50}, LC_{90} values and 95% confidence limits (CL) expressed in µg cm^{-2} of essential oil or compound required to kill insects. LC values and slopes within a column followed by the same letter are not significantly different based on nonoverlapping of 95% CL. [d] *H*, heterogeneity factor (χ^2/ degree of freedom).

Table V.7. LT_{50} and LT_{90} (hours) for the most active essential oils and four pure compounds estimated by the disk assay method against fourth-instar larvae of *Pseudaletia unipuncta*, at 125 µg cm^{-2}.

Treatments [a]	**Mortality** 72 h (%)	**LT_{50}** [b, c] (95% CL)	**LT_{90}** [b, c] (95% CL)	**Slope** [b, c] (± SEM)	***H*** [b, d]
EOs					
Ag-I	85.0	18.1 a (14.5 – 21.3)	83.2 b (65.7 – 119.4)	1.9 ± 0.23 a	1.35
Pc-F	78.0	29.7 (23.5 – 34.5) b	118.0 bc (87.1 – 216.3)	2.1 ± 0.38 a	0.35
Cc-F	40.0	…[e]	…[e]	…	…
Compounds					
trans-Anethole	70.0	40.1 c (34.8 – 46.0)	151.4 bc (107.4 – 294.3)	2.2 ± 0.38 a	1.43
(*S*)-(+)-Carvone	95.0	13.4 a (10.5 – 16.0)	48.4 a (41.1– 60.7)	2.3 ± 0.26 a	2.57
Myristicin	97.5	25.2 b (22.4 – 27.5)	45.8 a (41.9 – 51.8)	4.9 ± 0.57 b	1.56
Cuminaldehyde	62.5	44.0 c (37.1 – 53.5)	238.2 c (155.6 – 494.7)	1.8 ± 0.25 a	0.40

[a] The number of insects used for determination of LTs within each treatment varied between 160 and 200, excluding the ones used in controls. [b] Values were determined by probit analysis based on mortalities recorded on contact toxicity assay performed over time (24, 36, 48 and 72 hours) at 125 µg cm^{-2}. [c] LT_{50}, LT_{90} values and 95% confidence limits (CL) are expressed in hours of treatment required to kill insects. LT values and slopes within a column followed by the same letter are not significantly different based on nonoverlapping of 95% CL. [d] *H*, heterogeneity factor (χ^2/ degree of freedom). [e] Not determined for the chosen concentration of 125 µg cm^{-2}. Plotting of probit versus $\log_{10}$ (concentration) for linear regression was not possible due to low mortalities recorded over time.

Table V.8. Linear variation of the inverse LC_{50} with time of exposure assessed for a three-day period.

Treatments		**Equations** [a]	SEM		***p***	***r***
			Slope	y- Intercept		
OEs	Ag-I	$LC_{50}^{-1} = 2.7 \times 10^{-4}\, t + 2.3 \times 10^{-3}$	2.3×10^{-5}	1.1×10^{-3}	0.007	0.992
	Pc-F	$LC_{50}^{-1} = 1.4 \times 10^{-4}\, t + 4.1 \times 10^{-3}$	3.8×10^{-6}	1.8×10^{-3}	0.061[b]	0.939
	Cc-F	$LC_{50}^{-1} = 0.49 \times 10^{-4}\, t + 4.1 \times 10^{-3}$	4.5×10^{-6}	0.22×10^{-3}	0.008	0.992
Compds	*trans*-Anethole	$LC_{50}^{-1} = 1.3 \times 10^{-4}\, t + 2.6 \times 10^{-3}$	2.4×10^{-5}	1.1×10^{-3}	0.031	0.969
	(*S*)-(+)-Carvone	$LC_{50}^{-1} = 2.3 \times 10^{-4}\, t + 6.3 \times 10^{-3}$	1.6×10^{-5}	7.9×10^{-4}	0.005	0.995
	Myristicin	...[c]	…	…	…	...
	Cuminaldehyde	$LC_{50}^{-1} = 0.62 \times 10^{-4}\, t + 5.2 \times 10^{-3}$	5.6×10^{-6}	0.27×10^{-3}	0.008	0.992

[a] LC_{50}^{-1} (μg^{-1} cm^2) was correlated with time of exposure - *t* (hours). [b] Slope was not significantly different from zero ($p > 0.05$). [c] The variation of myristicin LC_{50}^{-1} for the studied period do not fit into the linear regression model.

Concentrations needed to obtain 50% of activity reduced substantially over time, including for myristicin (concentrations were 2.4-2.0 smaller between 24 and 72 h). Lower decreases of LCs_{50} were observed for Cc-F and its principal constituent cuminaldehyde (1.48 and 1.45 less, respectively). Furthermore, similar patterns of variation (approximated slopes) were founds for dill and cumin EO and their major compounds (*S*)-(+)-carvone and cuminaldehyde, respectively, evidencing the direct relation existing between these compounds and the performances of EOs where these are found in abundance.

Regardless of the fact that some retarded/cumulative effects may have occurred in individuals exposed to a certain dose during the specific studied period, we consider that this evaluation gives a strong indication of the performance and insecticidal potency of tested EOs/compounds. Time-series approaches help to differentiate, among candidates with considerable activity, which have acute/faster activity, for a reduce amount of toxicant, from others. Effectiveness for short time exposure may contribute to reduce costs and environmental impact. From toxicological, economic, and environmental viewpoints, these are desirable attributes for novel biopesticides, especially for rare or less abundant natural compounds from botanical origin.

5. Conclusion

The present work revealed the anti-insect potential of EOs/compounds from four well-known Apiaceae species on a major agricultural pest. Activities varied according to plant species/composition and/or type of exposure. Additionally, results indicated that the same mixture/compound may have different fates and consequences in one specie and that several or distinct mechanisms may be involved in fumigant and contact toxicant action. Sublethal toxicity through feeding might have been the cause of growth and consumption inhibitions observed for some treatments. According to Akhtar and Isman (2004), growth inhibition and feeding deterrence can increase the time of developmental stages and the search for viable food, exposing herbivores to increased mortality as a result of biotic and abiotic factors. Therefore, in the field context, a reduction in food consumption, by debilitated and less developed larvae, might help to mitigate some of the damage caused by this pest.

In this study, the fumigant activities of dill and fennel EOs, estragole, (*S*)-(+)-carvone and γ-terpinene were demonstrated, as well as a promising contact toxicity of parsley EO and *trans*-anethole. Insecticidal activities of dill and cumin EOs and their major compounds (*S*)-(+)-carvone and cuminaldehyde were recorded in all evaluations perform against *P. unipuncta* caterpillar, suggesting that these may have multiple toxic effects. The acute/strong toxicity of Ag-I was noteworthy and directly related to the extremely high content of (*S*)-(+)-carvone (67 and 84%), a compound with recognized insecticidal activity and displaying the most acute symptoms of neurotoxicity in this study. Cuminaldehyde, the major compound of Cc-F (39%), was responsible, to some extent, for the EO anti-insect properties but it did not entirely justify the results (additive effects

or synergisms with other constituents). The promising activities recorded for parsley EOs seems to be related with specific activities of the phenylpropene derivatives. In general, the individual evaluation made for all major compounds of the studied OE was not sufficient to explain the observed properties of respective EOs. However, it is important to emphasize that a more complete evaluation of several constituents individually may not necessarily lead to a better understanding of the toxicity of such complex mixtures (the whole is probably not the sum of its parts). As an alternative, an efficient approach has been proposed by Jiang et al. (2009) using artificial blends with omitted components to discriminate the role of each compounds on the overall toxicity of a reproduced EO.

Our findings suggest that EOs of *A. graveolens*, *C. cyminum* and *P. crispum* characterized herein were effective against *P. unipuncta.* The application of these EOs as deterrents and/or insecticides for the control of this lepidopteran could be advantageous considering the great productivity of these annual/biannual species and the high availability, low cost, and satisfactory efficiency of their EOs.

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CHAPTER VI

Activities of Apiaceae EOs and volatile compounds on hatchability, development, reproduction and nutrition of *Pseudaletia unipuncta* (Lepidoptera: Noctuidae)

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1. Abstract

The inhibitory activities of *Anethum graveolens*, *Cuminum cyminum*, *Foeniculum vulgare* and *Petroselinum crispum* essential oils (EOs), plus eleven volatile compounds, on embryonic development of a phytophagous pest, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) were evaluated. Lethal and sub-lethal effects of some treatments during the postembryonic development were also monitored. The higher effectiveness to inhibit the eggs hatching was achieved with *P. crispum* fruit EO and its two main compounds (apiole and myristicin). *F. vulgare* EOs, as well as the bioactive anti-insect compound azadirachtin (1 mg mL^{-1}) and the volatiles *trans*-anethole, estragole, (+)-fenchone, α-(+)- and α-(-)-pinene, (-)-β-pinene and γ-terpinene (15 mg mL^{-1}), had negligible inhibitory effects on larvae emergence, although, some of them induced post-exposure mortality during larval phase. *A. graveolens* EO from leaves and stems, and six individually tested compounds, including azadirachtin, extended significantly the duration of larval development from 1.9 to 6.5 days. (+)-Fenchone and (-)-β-pinene reduced significantly the oviposition potential of females, by 46% and 33%, respectively.

Additionally, a quantitative nutritional approach was conducted in order to assess adverse effects of EOs and compounds on 4^{th} instar larvae growth and metabolism. After 72 h of feeding with treated corn leaves (0.7 mg/leaf), *P. crispum* fruit EO (Pc-F) and azadirachtin (0.04 mg/leaf) caused significant adverse effects on larva nutrition (lower consumption and Relative Consumption Rate) and growth (weight loss and negative Relative Growth Rate), followed by *trans*-anethole and cuminaldehyde. As verified for the positive control azadirachtin, *trans*-anethole and cuminaldehyde had negative and significant influences on the Relative Metabolic Rate (RMR) and on the efficiency of conversion of food to biomass (measured as ECI and ECD), with consequent higher Metabolic Costs (MC) to the larvae.

P. crispum EO from fruits revealed great potential as a controlling agent of *P. unipuncta* in early life stages, acting as a hatching inhibitor, larvicide and phagodeterrent.

Keywords: Armyworm, ovicidal, sub-lethal effects, developmental stages, nutritional indices, dill, parsley, cumin, bitter fennel

2. Introduction

Over the past three decades, the development of biorational insecticides has been the central goal of numerous investigations. Plant-based pesticides have become a popular alternative, among other complementary strategies integrating pest management programs. Volatile constituents of EOs represent one of the most reported classes of compounds from botanical origin with proved toxicity to a great variety of arthropods. Their potential applications as safer alternatives to conventional insecticides have been extensively discussed in several reviews (Regnault-Roger, 1997; Isman, 2000; Koul et al., 2008; Tripathi et al., 2009; Regnault-Roger et al., 2012). In contrast to conventional insecticides, EOs and their constituents are non-persistent in the environment, and no bioaccumulation or biomagnification has been reported to date (Regnault-Roger et al., 2012). From a phytochemical viewpoint, EOs are heterogeneous mixtures characterized not only by a great diversity (abundance of biosynthetically distinct compounds) but also by a high redundancy (several analogs of one class and enantiomers) (Bakkali et al., 2008; Regnault-Roger et al., 2012).

In the context of the screening research for plant-based pesticides, plants belonging to the Apiaceae family have been identified as a valuable source of several bioactive compounds (Lichtenstein et al., 1974; Berenbaum & Neal, 1985; Hadacek et al., 1994; Momin & Nair, 2002; Evergetis et al., 2009; Evergetis et al., 2013; Pavela & Vrchotová, 2013). Activities of Apiaceae EOs as antifeedants, insecticides, insect repellents and growth regulators were recently reviewed (Ebadollahi, 2013 and references therein). *F. vulgare* Mill., *A. graveolens* L., *P. crispum* (Mill.) Nyman ex A.W. Hill and *C. cyminum* L., are four well-known aromatic plants abundantly produced in several countries and regularly used in culinary as herbs and condiments. The EOs extracted from these Apiaceae species have shown ovicidal, larvicidal, adulticidal, deterrent and/or repellent activities against several species of the Arthropoda taxa; from diseases vectors (Lee, 2006; Evergetis et al., 2012), agricultural pests (Işik & Görür, 2009) and household parasites (Seo et al., 2009; Yeom et al., 2012), to stored-product insects (Tunç et al., 2000; Kim & Ahn, 2001; Chaubey, 2008; Ebadollahi, 2011; Kim et al., 2013). The insecticidal activities of their EOs and/or respective major compounds against *Pseudaletia unipuncta*, one of the most important pests of agricultural crops in North America (McNeil et al., 2000) and Europe (Bues et al., 1986), were recently screened (Sousa et al. 2013). *Pseudaletia* (sin. *Mythimna*) *unipuncta*, Haworth 1809 (Lepidoptera: Noctuidae), also known as the true armyworm, is a seasonal and migrant noctuid which cause considerable plant damage in small grain crops (namely barley, oats, rice, rye and wheat), sugarcane, maize and forage grasses (Silva et al., 2003). A variety of extracts and allelochemicals from several botanical origins have been previously tested for their antifeedant and crop protection potential against *P. unipuncta* and other important phytophagous insects (Akhtar & Isman, 2004; Akhtar et al., 2008; Isman et al., 2008; Rosa et al., 2010). In general, the evaluation toxic activity of plant extracts against many lepidopterans are centered on acute toxicity tests or feeding-deterrence assays, for estimation of the respective lethal and deterrent doses. Besides the direct induced mortality, sub-lethal effects of insecticides on

arthropod physiology and behavior must be considered for a complete analysis of their impact (Desneux et al., 2007). In fact, compounds with lower toxicity may still confer protection to crops by reducing fitness of insect herbivores via inhibition of larval growth and/or by disruption of insects' development (Hummelbrunner & Isman, 2001), compromising the adults' emergence and progeny. In that way, several studies have examined the deleterious effects of plant extracts and botanicals on growth, development, life-span, reproduction and/or nutrition of lepidopterans (Koul et al., 1990, Huang et al., 1997; Akhtar & Isman, 2003; Akhtar & Isman, 2004; Yazdani et al., 2013).

In the previous published work (Sousa et al., 2013), and corresponding chapter V of this thesis, the screening performed on the activities of the essential oils of the target Apiaceae species, proved their toxicity against the lepidopteran species, *Pseudaletia unipuncta*. In this work, new assays were performed with the same kind of the EOs and compounds, with the objective to determine the respective effects on the eggs hatchability as well as the sub-lethal toxicity (development, reproduction and nutrition) towards this insect. EOs and constituents were assessed for their eggs hatching inhibitory activity and the ensuing effects of the treatments, with lower toxicity to the embryos, were monitored through time by following mortality, as well as post-exposure sub-lethal effects over insects' development and reproduction. Furthermore, the post-ingestive effects of EOs/compounds on 4th instar larvae were evaluated in a short-time study by means of nutritional indices calculation, in order to evaluate in what extent EOs/compounds can disturb larvae nutrition, metabolism and capacity of conversion of food into body mass.

3. Materials and methods

3.1. EOs and chemical composition

F. vulgare subsp. *vulgare* var. *vulgare* (bitter fennel), *A. graveolens* (dill) and *P. crispum* var. *neapolitanum* (flat leaf parsley) EOs were obtained from established cultures, while EOs from fruits of *P. crispum* (parsley) and *C. cyminum* (cumin) were purchased from Sigma-Aldrich, Co. The EOs quantitative and qualitative detailed profiles were presented and discussed in the Chapter II. Having in view to make easier the interpretation of the results discussed in the current chapter, the summarized composition of the EOs assayed in this work is presented in table VI.1.

3.2. Chemicals

Eleven high purity standard volatile compounds were included in all assays based on their relative abundance in the studied EOs (Table 1). *trans*-Anethole (99%), (S)-(+)-carvone (96%), cuminaldehyde (98%), estragole (98%), (+)-fenchone (99.5%), γ-terpinene (≥ 97%), (-)-β-pinene (99%), (-)-α-pinene and (+)-α-pinene (98%) were purchased from Sigma-Aldrich and Fluka (Aldrich

chemical Co., St. Louis, MO, USA). The compounds myristicin (≥ 93%) and apiole (≥ 85%) were purified from *P. crispum* EO by column chromatography on silica gel as previously described in Chapter II).

Table VI.1. Studied essential oils (EOs) from Apiaceae species and their respective major compounds

Species	Plant organs (EO abbreviation)	Compounds (%) [a, b]
Anethum graveolens	Green infrutescences (Ag-I)	(S)-Carvone (66.6), β- phellandrene + limonene (24.7)
	Green leaves more stems (Ag-L)	α-Phellandrene (60.0), dill ether (16.4), *o*-cymene (6.9), β-phellandrene + limonene (6.8)
Cumin cyminum	Fruits [c] (Cc-F)	Cuminaldehyde (39.4), γ-terpinene (15.8), β-pinene (12.4), *p*-cymene (10.4), *p*-mentha-1,4-dien-7-al (9.7)
Foeniculum vulgare subsp. *vulgare* var. *vulgare*	Green infrutescences (Fv-I)	Estragole (64.9), fenchone (15.7), β-phellandrene (5.5), α-phellandrene (4.9)
	Green leaves more stems (Fv-L)	Estragole (28.4), α-phellandrene (24.3), α-pinene (18.0), β-phellandrene (10.1), fenchone (6.2)
Petroselinum crispum	Fruits [c] (Pc-F)	Myristicin (31.5), α-pinene (16.2), apiole (15.9), β-pinene (13.6), allyl-tetramethoxybenzene (8.6), β-phellandrene (6.6)
	Green infrutescences (Pc-I)	Apiole (24.3), α-pinene (23.4), β-pinene (20.8), β-phellandrene (10.6), 1,3,8-*p*-menthatriene (10.6)

[a] Summarized characterization of EOs considering only major compounds with relative contents ≥ 5% (adapted from chapter II). [b] Compounds were identified by mass spectra comparison and retention index calculation. The quantifications were performed by GC-FID analysis; [c] Essential oils were purchased from Sigma-Aldrich Co.

3.3. Bioassays

3.3.1. Insects

For this study, we used eggs and larvae from the *P. unipuncta* laboratory colony maintained at the Biology Department of Azores University (Ponta Delgada, S. Miguel, Azores archipelago, Portugal).

Eggs of *P. unipuncta* recently laid (0–24 h) on strips of vegetal parchment paper placed inside the oviposition cages, were disinfected in 5% formaldehyde solution for 20 min and rinsed with distilled water for 5 min before assays. Larvae were reared on an artificial diet (Poitout and Bues, 1974) and adults were supplied with 10% sucrose solution in water. Insects were maintained in laboratory at 22-24 ºC and 70 ± 5% of relative humidity, under a L16:D8 photoperiod. All experiments were carried out in these conditions.

3.3.2. Hatching inhibition and dose–response assay

Vegetal parchment strips, containing recently laid eggs of the previous 24h, were cut in small section and treated by dipping for 5 seconds in freshly prepared solutions of EOs/compounds in pure ethanol. A general screening was first performed at 15 mg mL^{-1}, based on a preliminary assay, and additionally at 10, 7.5, 5, 2.5 and 1 mg mL^{-1} for the most active treatments. Along with the negative ethanol control, a single dose of azadirachtin (1 mg mL^{-1}) was included in this assay as positive control. Five to 7 strips pieces (replicates) were used, corresponding to an average number of 387 ± 117 eggs per concentration and treatment/control. Following the complete evaporation of the solvent (air dried, 15 min) the replicates were placed inside individual aerated boxes and kept in incubation at 22-24 ºC.

Artificial diet was supplied 4 days later and before the onset of hatching, to prevent cannibalism. Observations were made daily, until the 10th day following the treatment, to register the day of the hatching in each replicate (if more than 50% of eggs were hatched). The percentage of unhatched eggs was calculated after 6-7 days, time required for larvae emergence in the ethanol control to be completed. The hatching inhibition caused by the treatments was corrected relatively to the ethanol control whenever it was superior to 5%, using Abbott's formula[1](Abbott, 1925).

3.3.3. Post-exposure effects

3.3.3.1. Larvae, pupae and adults' development and early death

We considered that an extended study about possible delayed effects during post-embryonic development of armyworm (Fig. VI.1) could be relevant for treatments (2 EOs and 7compounds) which did not show pronounced hatching inhibitory or ovicidal activity at 15 mg mL^{-1}.

The newly emergent larvae of the foregoing hatching inhibition assay were reared with abundance of food and maintained grouped during the 1st and 2nd instar to avoid causing trauma or stress. Handling procedures were limited and performed only twice a week for substitution of boxes and diet.

Afterward, 30-40 single 3rd instar larvae were randomly selected and distributed in individual boxes to examine subsequent post-exposure effects of EOs/compounds on each individual. Insects were monitored daily during their lifespan to register the duration (days) of larval, pupal and adults' stages (considering the hatching date as day 1), the occurrence of early death in each one, pupae' weights and adults' sex (Fig. VI.1). In order to calculate larvae, pupae, adults' mortality (%) as well as the accumulated death, individuals within treatments were randomly grouped for data processing (9-12 replicates with 3-4 insects per replicate).

The mortality of neonates, 1st and 2nd instars was not included in the evaluation of the post-exposure lethality because larvae are very small and easily traumatized by handling. The replicates

[1] Formula (3) provided in the Appendix C.

were used for calculation of mean percentage values and for statistical analysis. Larvae rearing, adults' feeding and room conditions were as describe in 3.3.1.

3.3.3.2. Fertility and female oviposition potential

After the emergence of adults, males and females were paired and distributed in small cages (12 cm x 10 cm x 9 cm) and allowed to mate. Ten to fifty couples were used per treatment. Vegetal parchment strips were placed in cages for oviposition and replaced every 4 to 5 days. Eggs laid by each female during all the reproductive period were counted and the number of unfertile couples was also registered. The feeding of moths and room conditions were as describe in 3.3.1.

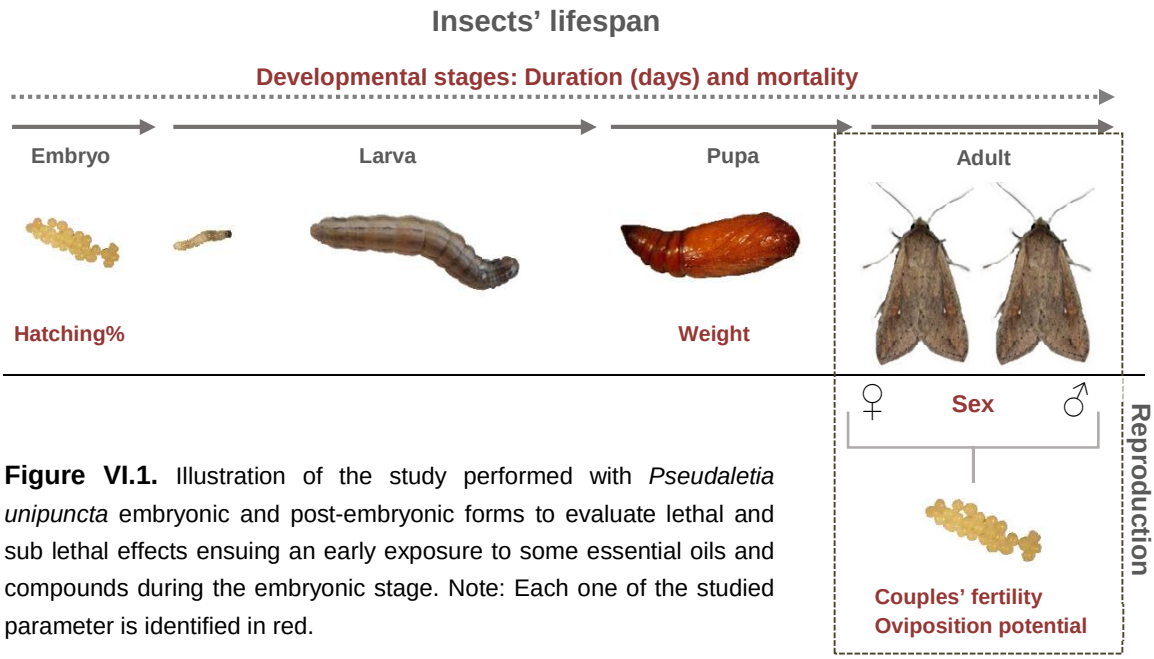


Figure VI.1. Illustration of the study performed with *Pseudaletia unipuncta* embryonic and post-embryonic forms to evaluate lethal and sub lethal effects ensuing an early exposure to some essential oils and compounds during the embryonic stage. Note: Each one of the studied parameter is identified in red.

3.3.4. Effects on nutritional physiology

The effects of EO and pure compounds over *P. unipuncta* 4th instar larvae growth, food consumption, food conversion efficiency and other metabolic parameters were evaluated for a 72 h period experiment according to Koul et al. (1990) and Huang et al. (1997). The bioassay was performed with newly molted 4th instar larvae (30 to 50 mg of average weight), never exposed to any of the studied allelochemicals. At least three independent assays with ten larvae per treatment/control were done (n = 30).

- Pre-weighed larvae were individually distributed in sterile Petri dishes (diameter = 4.5 cm) with a thin agar layer (2% w/v) in the bottom to prevent dehydration.
- Fresh corn leaves (*Zea mays* L.), disinfected in 5% formaldehyde solution for 20 min and rinsed with deionized water for 10 min and dried over absorbent paper, were cut in 4 cm^2 pieces.
- Each pre-weighed leaf section was treated with EOs/compounds (175 µg cm^{-2}), pure ethanol (negative control) or azadirachtin (positive control at 10 µg cm^{-2}) on the top of the leaves, and left for 15 min in order to evaporate the solvent.
- After a 6 h starvation period, larvae were fed with single leaf pieces freshly prepared.
- Every 24 h, larvae were supplied with fresh leaves while feces and non-consumed diet (always some food leftover in all treatments) were removed, weighed and dried.
- At the end of the experiment (72 h), the weights of the feces and non-consumed diet of the last 24 h, as well as larvae' final weight were recorded.

The amount of total ingested food (mg) was estimated by subtracting the sum of the remaining diet at 24, 48, and 72 h (dry weight equivalent) from the total dry weight of the supplied diet. Larvae and leaves initial dry weights were extrapolated based on mean dry contents determined for representative samples (n=30) in the beginning of the experiments (12.0 ± 1.04% and 28.3 ± 2.97%, respectively). Final dry weights of larvae, feces and non-consumed diet (mg) were estimated by drying at 50 ºC for 4 days (dry contents varied from 9.8 to 14.2%). All weight measurements were made on a Mettler Toledo AB204-S/FACT balance (accuracy of 0.1 mg).

3.3.4.1. Nutritional parameters calculation

To evaluate treatment effects on the amount of food that was consumed, digested, assimilated, excreted, and converted into body mass during the experimental period, seven nutritional indices were determined according to Waldbauer (1968) and modified by Scriber & Slansky Jr. (1981)[2]:

(6) AD, Approximated Digestibility (%); **(7)** ECI, Efficiency Conversion Index of Ingested food (%); **(8)** ECD, Efficiency Conversion Index of Digested food (%), **(9)** MC, Metabolic Cost (%); **(10)** RMR, Relative Metabolic Rate (mg mg^{-1} day^{-1}); **(11)** RGR, Relative Growth Rate (mg mg^{-1} day^{-1}); **(12)** RCR, Relative Consumption Rate (mg mg^{-1} day^{-1}).

The calculation on a dry weight basis was used as described by other authors (Koul et al., 1990; Senthil-Nathan et al., 2005a; Yazdani et al., 2013) in an attempt to minimize the influence of water

[2] Respective formulae are described in the Appendix C.

content variations in plant material and excreta, and/or possible dehydration of larvae caused by some treatments.

3.4. Statistical analyses

Data from the hatching inhibition assay, developmental (duration and mortality observed in larvae, pupae and adults, and pupae' weight) and reproduction (number of eggs deposited per each female) studies were transformed by the $(x+ ½)^{½}$ function prior to the one-way ANOVA analysis. Means of treatments were separated at 5% significance level by LSD post-hoct test (SPSS software version 20.0 for Windows, SPSS Inc.).

The concentration causing 50% of hatching inhibition (IC_{50}) was determined for some of the most active EOs/compounds by performing a Probit analysis (SPSS software) (Litchfield & Wilcoxon, 1949). Log-Probit linear regressions were obtained from dose-response results, and generally five or four data-sets were selected to ensure a good adjustment of data to the model. The goodness-of-fit was evaluated by the qui-square (χ^2) test and was found satisfactory whenever values were lower than the critical χ^2 value (7.815 and 5.991) for 3 and 2 *df*, respectively, at 5% significance level.

For the nutritional indices, data were submitted to one-way ANOVA test without previous transformation. For each index, mean multiple comparisons were performed using the Duncan´s multiple range test (p = 0.05) (SPSS software version 20.0 for Windows, SPSS Inc.). Correlation between the food consumption and larvae growth were determined by the Pearson test (p=0.05).

4. Results

4.1. Ovicidal activity and dose-related hatching inhibitory activity

The results of inhibitory effects on hatching of *P. unipuncta*, obtained for 7 EOs from Apiaceae species and 11 volatile compounds in a first screening assay (at 15 mg mL^{-1}), are presented in figure VI.2. EOs isolated from dill, parsley and cumin inhibited satisfactory the emergence of larvae (> 70% inhibition). Likewise, the main constituents (S)-carvone (from dill infrutescences), myristicin and apiole (found in parsley EOs), and cuminaldehyde (from cumin EO) were as effective as the respective EOs, when tested individually. No significant activity was observed to other major compounds, γ-terpinene and pinenes, despite their considerable amounts in the cumin and parsley EOs, respectively. By contrast, bitter fennel EOs and their corresponding compound (+)-fenchone, estragole, *trans*-anethole, and pinenes were found to have very limited (≤ 10%) or no activity, even at the highest tested dose.

The results of the dose-related activity obtained for the most active EOs/compounds are presented in table VI.2 and figure VI.3. With the exception of the dill EO Ag-L, all treatment showed inhibitory hatching activity (> 50%) at doses equal or superior to 5 mg mL^{-1}. For the lowest dose, only the parsley EO Pc-F, and the respective major compounds myristicin and apiole, still exhibited high effectiveness over eggs hatching, much superior to azadirachin tested at 1 mg mL^{-1} (see level of activity of azadirachtin in figure VI.2 with those of EOs and compounds at the same concentration of 1 mg mL^{-1} in table VI.2). The probit analyses allowed determining the IC_{50} of six treatments (Table VI.2). Among the treatments for which an estimate of this parameter was possible, myristicin presented the lowest IC_{50} (below 1 mg mL^{-1}), followed by cuminaldehyde, Ag-I, Cc-F, (S)-(+)-carvone and Ag-L. This last EO was significantly less active (10.64 mg mL^{-1}) against *P. unipuncta* eggs than the one isolated from infrutescences of the same plant. However, Ag-I, and its major compound (S)-(+)-carvone, presented similar effectiveness (IC_{50} = 3.60 and 3.74 mg mL^{-1}, respectively).

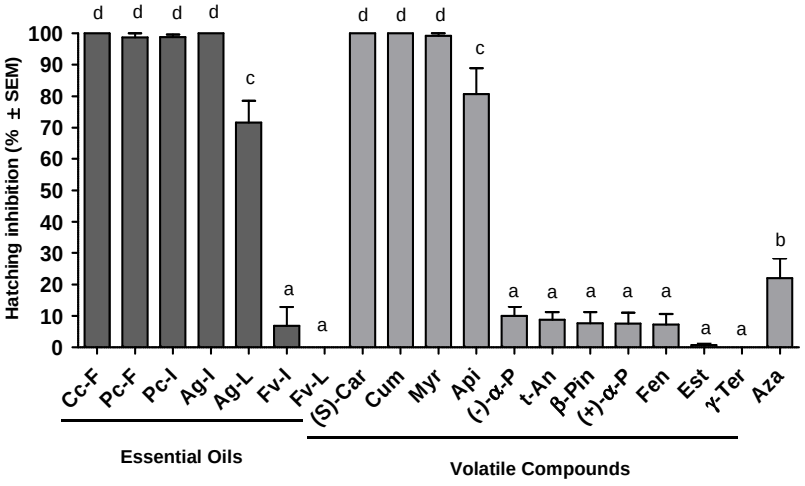


Figure VI.2. Inhibition of *Pseudaletia unipuncta* hatching after single dose treatments (15 mg mL^{-1}) with essential oils and comounds. *Anethum graveolens* (**Ag**)*, Cuminum cyminum* (**Cc**)*, Foeniculum vulgare* var. *vulgare* (**Fv**) and *Petroselinum crispum* (**Pc**) essential oils from: fruits (**-F**), Infrutescences (**-I**), Leaves more stems (-**L**), and compounds: **Api**: Apiole, **t-An**: *trans*-Anethole, **Aza**: Azadirachtin[a]*,* **Car**: (S)-(+)-Carvone, **Cum**: Cuminaldehyde, **Est**: Estragole, **Fen**: (+)-Fenchone, **Myr**: Myristicin, **(+)-α-P**: (+)-α-Pinene, **(-)-α-P**: (-)-α-Pinene, **β-Pin**: (-)-β-Pinene, **γ- Ter**: γ-Terpinene.

Mean values (± standard error) were determined for 5-7 replicates and corrected with Abbott´s formula relatively to the control with ethanol, 6-7 days after treatment when non-hatched eggs percentages ≤5% were recorded. Means with the same letters do not differ significantly, based on one-way ANOVA followed by LSD test (p= 0.05). [a] Azadirachtin (95%) was tested against eggs at a sub lethal dose of 1 mg mL^{-1}, to follow insects' development in the subsequent study.

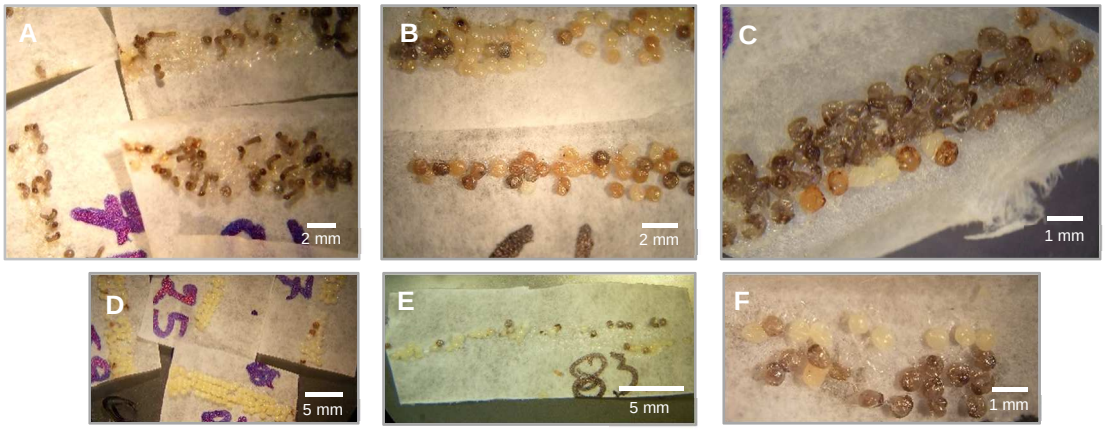


Figure VI.3 (A-F). Effects of essential oils and compounds on *Pseudaletia unipuncta* eggs development and hatching after 6-7 days of exposure. **(A)** Developed and hatched eggs exposed to cumin fruits essential oil. Although hatching percentage was high, numerous neonates were moribund and unable to search for food; **(B)** Retarded/interrupted development of eggs treated with dill infrutescences essential oil (10 mg mL^{-1}); **(C)** Most of the developed embryos remained unhatched as a results of apiole exposure (5 mg mL^{-1}); **(D)** Eggs exposed to cuminaldehyde (5 mg mL^{-1}) showing a disruption of the embryonic development; **(E)** Only a few eggs remained undeveloped and unhatched after treatment with (S)-carvone (5 mg mL^{-1}); **(F)** Developed and undeveloped embryos exposed to myristicin (5 mg mL^{-1}).

Note: Freshly laid eggs are white to pale-yellow and, as development progresses, their color darken to a metallic grey just before hatching.

Table VI.2. Dose-related hatching inhibitory activity of *Anethum graveolens, Petroselinum crispum*, *Cuminum cyminum* essential oils (EOs) and their major compounds on *Pseudaletia unipuncta*, 6-7 days after eggs treatment.

Dose (mg mL^{-1})	Hatching inhibition [a, b] (% ± SEM)						Hatching inhibitory concentration			
	15	**10**	**7.5**	**5**	**2.5**	**1**	**IC_{50}** (mg mL^{-1}) (95% CL) [c]	**Slope** (± SEM)	χ^2	**Sig.**
EOs										
Ag-I	100 ± 0.0 b	93.2 ± 5.4 b	94.1 ± 4.2 c	75.6 ± 13.1 bc	22.0 ± 3.3 a	15.2 ± 1.7 ab	3.60 (3.28 – 3.91) b	4.74 ± 0.44	0.95	0.62
Ag-L	71.2 ± 7.1 a	50.7 ± 7.9 a	22.3 ± 4.4 a	9.9 ± 3.3 a	-	-	10.64 (9.84 – 11.64) c	4.09 ± 0.43	2.24	0.33
Cc-F	100 ± 0.0 b	92.5 ± 3.5 b	97.7 ± 1.8 c	67.7 ± 10.9 bc	28.3 ± 6.0 a	14.8 ± 5.4 ab	3.68 (3.25 – 4.10) b	3.47 ± 0.32	0.34	0.84
Pc-F	98.6 ± 1.4 b	97.7 ± 1.8 b	75.8 ± 6.6 b	73.8 ± 8.6 bc	-	72.9 ± 10.8 d	-	-	-	-
Pc-I	98.7 ± 0.7 b	100 ± 0.0 b	100 ± 0.0 c	94.2 ± 4.3 cd	93.2 ± 5.1 c	18.4 ± 6.6 ab	-	-	-	-
Compounds										
Api	80.4 ± 8.4 a	100.0 ± 0.0 b	100.0 ± 0.0 c	100.0 ± 0. 0 d	99.2 ± 0.8 c	83.7 ± 7.0 d	-	-	-	-
Car	100 ± 0.0 b	100.0 ± 0.0 b	78.7 ± 6.1 b	58.6 ± 6.5 b	36.5 ± 3.0 ab	6.8 ± 2.2 a	3.74 (3.29 – 4.27) b	2.49 ± 0.24	1.30	0.52
Cum	100 ± 0.0 b	95.5 ± 2.9 b	94.3 ± 3.6 c	-	55.3 ± 14.7 b	25.6 ± 7.9 b	1.97 (1.68 – 2.26) a	2.57 ± 0.20	3.36	0.34
Myr	99.3 ± 0.7 b	100.0 ± 0.0 b	99.0 ± 1.0 c	88.7 ± 4.0 cd	-	55.8 ± 6.6 c	0.87 (0.03 – 1.83) a	1.92 ± 0.22	4.51	0.11
df	46	47	48	69	41	49	-	-	-	-
F	8.506	21.246	47.121	21.412	26.602	27.533	-	-	-	-
***p* ≤**	0.001	0.001	0.001	0.001	0.001	0.001	-	-	-	-

Api: Apiole, **Car**: (S)-(+)-Carvone, **Cum**: Cuminaldehyde, **Myr**: Myristicin. *df* = n - 2.

[a] Mean values (± standard error of the mean - SEM) were determined for 5-7 replicates and corrected with Abbott´s formula relatively to the control with ethanol, six days after treatment when non-hatched eggs percentages ≤ 5% were recorded; [b] Within columns, means with the same letters do not differ significantly based on one-way ANOVA, followed by LSD test (p= 0.05) (statistical analysis was computed with transformed data); [c] IC_{50} were considered significantly different when the 95% confidence limits (CL) fail to overlap.

4.2. Post-exposure effects on *P. unipuncta* development and reproduction

This study focused on post-exposure effects of treatments that showed lower ovicidal and/or hatching inhibition activities. As such, only the surviving larvae emerged from the eggs treated with Ag-L, Fv-I, Fv-L, *trans*-anethole, estragole, (+)-fenchone, pinenes, γ-terpinene, azadirachtin were reared until the completion of their life-cycle. Parsley EOs (Pc-F and Pc-I), myristicin and apiole treatment were not included since a scarce number of larvae emerged, and few L_1 survived after emergence. The early death of the insects during their entire lifespan (larval, pupal and adult stage), as consequence of the exposure to low ovicidal doses of EOs/compounds during embryonic development, is shown in the Table VI.3.

When compared to mortality percentages recorded in negative controls, estragole and γ-terpinene were the treatments displaying the most significant post-exposure lethality on larvae (from L_3 to pupa), and more markedly on pupae (19 and 31%) ($p=0.05$). Among the remaining treatments which had some influence on survival, *trans*-anethole induced some lethal effects during larval development, while γ-terpinene exhibited significant effects during pupal stage (19%). None of the treatments decreased significantly the percentage of living adults when compared to water or ethanol ($p=0.05$). The overall mortality observed in groups of individuals formerly exposed to (-)-α-pinene, Fv-L, (+)-fenchone and (-)-β-pinene, Azadirachtin, Ag-L, Fv-I, *trans*-anethole, γ-terpinene, estragole and (+)-α-pinene (by increasing order) was 1.6 to 4.2 times superior to the registered natural death. However, only estragole, (+)-α-pinene and γ-terpinene showed statistically significant difference from negative controls regarding the post-exposure mortality ($p \leq 0.05$) (Table VI.3). Moreover, the previous exposure to azadirachtin during embryonic development did not caused significant death during the studies period (from L_3 until maturity).

The results regarding the sub-lethal effects of EOs/compounds on *P. unipuncta* developmental stages, sex ratio and reproduction, are presented in Table 4. Almost all treatments had significant effects on larval stages duration, but only *trans*-anethole extended the duration of embryonic development (1 day approximately) ($p=0.05$). The exposure of eggs to γ-terpinene, estragole, α-pinenes, β-pinene, Ag-L, azadirachtin and (+)-fenchone (by increasing order) delayed significantly the duration of larval stage from 1.9 to 6.5 days, when compared to the negative controls ($p=0.05$). Afterwards, no noteworthy influence on the duration of the succeeding developmental stages could be undoubtedly attributed to treatments.

Table VI.3. Lethality recorded for *Pseudaletia unipuncta* larval (from L_3 to pupa), pupal and adult stages, after exposure during the embryonic development to Apiaceae essential oils (EOs) and pure compounds at a low ovicidal concentration (15 mg mL^{-1}).

Treatments	**Mortality** (% ± SEM)			
	Larval stage [a, b, c]	**Pupal stage** [a, b]	**Adult stage** [a, b]	**Total** [a, b]
Controls				
Water	0.0 ± 0.0 a	2.8 ± 2.8 a	5.6 ± 3.7 ab	8.3 ± 4.2 a
Ethanol	0.0 ± 0.0 a	4.2 ± 2.8 a	4.9 ± 3.3 ab	8.3 ± 4.7 a
Azadirachtin [d]	0.0 ± 0.0 a	9.1 ± 6.1 ab	9.1 ± 6.1 ab	18.2 ± 7.6 abc
EOs				
Ag-L	0.0 ± 0.0 a	13.3 ± 4.2 ab	5.6 ± 3.7 ab	18.9 ± 3.6 abc
Fv-I	2.8 ± 2.8 a	13.9 ± 4.4 ab	2.8 ± 2.8 ab	19.4 ± 5.6 abc
FV-L	0.0 ± 0.0 a	13.9 ± 6.2 ab	0.0 ± 0.0 a	13.9 ± 6.2 ab
Compounds				
trans-Anethole	5.6 ± 3.6 b	5.6 ± 2.8 ab	11.1 ± 4.3 b	22.2 ± 3.2 abc
Estragole	13.9 ± 4.4 c	18.5 ± 4.5 bc	3.7 ± 3.7 ab	33.3 ± 5.1 c
(+)-Fenchone	0.0 ± 0.0 a	7.6 ± 5.2 ab	9.1 ± 6.1 ab	16.7 ± 7.1 ab
(+)-α-Pinene	5.6 ± 3.3 b	31.0 ± 3.0 c	0.0 ± 0.0 a	35.0 ± 3.7 c
(-)-α-Pinene	0.0 ± 0.0 a	13.2 ± 3.8 ab	0.0 ± 0.0 a	13.2 ± 3.8 ab
(-)-β-Pinene	0.0 ± 0.0 a	16.7 ± 8.3 ab	0.0 ± 0.0 a	16.7 ± 8.3 ab
γ- Terpinene	0.0 ± 0.0 a	18.5 ± 5.9 bc	14.8 ± 7.6 b	29.6 ± 8.7 bc
df	125	125	125	125
F	5.512	2.426	1.298	2.165
***p* ≤**	0.001	0.01	0.5	0.05

[a] Mean values and standard error of the mean (SEM) were determined for groups of 3-4 individuals for a total of 9-12 replicates; [b] Mean values with the same letters do not differ significantly, based on one-way ANOVA followed by LSD test ($p = 0.05$) (statistical analysis was computed with transformed data); [c] The percentage of death during the 1st and 2nd instar larvae were not included in the study; [d] Lethality during post-embryonic development after eggs exposure to azadirachtin at 1 mg mL^{-1}.

Table VI.4. Sub-lethal effects on *Pseudaletia unipuncta* (development duration and reproduction), as consequence of a previous exposure during embryonic development to Apiaceae essential oils (EOs) and compounds at low ovicidal concentration (15 mg mL^{-1}).

Treatments	Duration (Days)					Pupae´ weight (mg) [a, b]	Sex ratio (♀%)	Eggs/ ♀ (No.) [c]	Sterility (%) [d]
	Hatching [a]	Larval stage [a,b]	Pupal stage [a,b]	Adult stage [a,b]	Total Lifespan [a,b]				
Controls									
Water	5.3 ± 0.2 a	32.5 ± 0.7 b	14.1 ± 0.3 bcd	10.0 ± 0.7 a	56.2 ± 1.0 a	271.9 ± 8.4 c	42.9	699 ± 112.4 c	20.0
Ethanol	5.6 ± 0.2 ab	32.2 ± 0.4 b	14.0 ± 0.2 abc	12.5 ± 0.5 bcd	58.4 ± 0.9 ab	272.0 ± 6.0 c	55.6	771 ± 104.6 c	25.0
Azadirachtin [e]	5.8 ± 0.2 ab	38.2 ± 0.9 g	14.9 ± 0.2 d	13.8 ± 1.5 c d	66.4 ± 1.3 d	249.2 ± 8.3 b	40.0	729 ± 81.0 c	0.0
EOs									
Ag-L	-	37.0 ± 0.5 fg	14.6 ± 0.2 d	13.0 ± 0.8 bcd	64.2 ± 1.1 cd	230.2 ± 5.9 a	48.4	902 ± 74.9 c	0.0
Fv-I	6.0 ± 0.0 bc	29.8 ± 0.4 a	13.4 ± 0.2 ab	12.9 ± 1.1 bcd	56.4 ± 1.2 ab	290.5 ± 5.0 d	48.3	929 ± 139.1 c	18.2
FV-L	6.0 ± 0.0 bc	33.1 ± 0.2 bc	13.4 ± 0.2 a	11.8 ± 0.9 abcd	58.4 ± 1.0 ab	301.3 ± 4.1 d	51.6	931 ± 102.5 c	33.3
Compounds									
trans-Anethole	6.4 ± 0.2 c	33.0 ± 0.5 bc	13.6 ± 0.2 ab	9.9 ± 0.6 a	56.7 ± 0.8 ab	289.4 ± 5.9 d	48.4	784 ± 157.9 c	10.0
Estragole	6.0 ± 0.0 bc	34.5 ± 0.5 cde	14.2 ± 0.4 bcd	11.1 ± 0.7 ab	59.2 ± 0.9 b	287.9 ± 5.4 cd	66.7	717 ± 99.6 c	14.3
(+)-Fenchone	5.8 ± 0.3 ab	38.7 ± 1.1 g	14.5 ± 0.4 cd	13.4 ± 1.3 cd	66.4 ± 1.7 d	230.7 ± 7.6 ab	71.4	465 ± 70.7 ab	11.8
(+)-α-Pinene	5.3 ± 0.2 a	35.8 ± 0.6 ef	14.0 ± 0.3 abc	13.4 ± 1.1 bcd	62.9 ± 1.1 c	244.5 ± 6.4 ab	52.0	766 ± 52.3 c	0.0
(-)-α-Pinene	5.6 ± 0.2 ab	35.3 ± 0.5 de	14.4 ± 0.2 cd	13.7 ± 0.6 d	63.2 ± 0.7 c	245.8 ± 4.3 b	56.5	575 ± 63.2 bc	0.0
(-)-β-Pinene	5.9 ± 0.2 b	35.7 ± 0.9 def	13.9 ± 0.4 abc	14.1 ± 1.4 cd	64.0 ± 1.2 cd	238.7 ± 10.7 ab	68.8	378 ± 79.4 a	28.6
γ- Terpinene	5.8 ± 0.2 ab	34.1 ± 0.4 cd	13.7 ± 0.4 abc	11.2 ± 1.1 abc	59.6 ± 1.5 b	253.7 ± 5.7 bc	65.2	601 ± 118.6 bc	0.0
df	74	418	373	347	356	429	-	120	-
F	3.138	19.790	2.739	2.515	11.073	15.103	-	3.133	-
p ≤	0.005	0.001	0.001	0.005	0.001	0.001	-	0.001	-

[a] Mean values and respective SEM were determined for variable numbers of individuals (20 ≤n≤ 40); [b] Mean values (± standard error of the mean - SEM) with the same letters do not differ significantly, based on one-way ANOVA followed by LSD test (p = 0.05) (statistical analysis was computed with transformed data); [c] Mean number of eggs deposited by each female until its death; [d] Percentage of non-reproductive couples, for a number varying between 10 and 15 couples per treatment; [e] Post exposure effects on the insect development after eggs treatment with azadirachtin at 1 mg mL^{-1}.

Regarding pupae' weight evaluation, lighter pupae were developed from eggs exposed to azadirachtin, Ag-L, (+)-fenchone, β-pinene, α-pinenes and γ-terpinene, whereas heavier ones were grown in the groups treated with Fv-I, Fv-L, and their two compounds *trans*-anethole and estragole.

Concerning the effects on adults' sex ratio, when compared to the ethanol treatment (56% females), the group of individuals exposed to γ-terpinene, estragole, (-)-β-pinene and (+)-fenchone showed higher percentage of females (65 to 71%). Among compounds and EOs, only (+)-fenchone and (-)-β-pinene treatments have changed significantly the oviposition potential (p=0.05), reducing the number of eggs/female, respectively, to 60% and 49% of the controls (Table VI.4). In the whole experiment, the absence of reproductive ability did not exceed 33% of couples.

4.3. Post-ingestive effects on fourth-instar larvae nutrition and metabolism

The nutritional indices determined for *P. unipuncta* 4th instar larvae, after 72 h of feeding with corn leave treated with 175 µg cm^{-2} (equivalent to 0.7 mg) of EOs (n = 5) or pure compounds (n = 10), are presented in figure VI.5. In the case of Pc-F EO the RMR, ECI, ECD, AD and MC metabolic parameter were not applicable due to the insignificant intake of food recorded in two individual experiments (2/3 of the total number of larvae). The nutritional indices values determined for each treatment along with the statistical analysis are available as supplementary data in the end of this chapter (Table VI.5).

For all parameters, treatments showed significant difference ($0.001 \leq p \leq 0.005$). When compared with the ethanol group, a significant decrease in food consumption was observed for almost all treatments with the exception of bitter fennel EOs and (-)-β-pinene, Fv-L being the only treatment that have induced a significant increase (Fig. VI.5, A). With the exception of this last EO, all treatments caused a diminution in the larvae mean weight gain relatively to the negative control. Furthermore, the larvae feed with *P. crispum* EO*, trans*-anethole and cuminaldehyde treated corn leaves, showed the most significant decrease of feeding behavior ($p \leq 0.05$), with consequent weight loss (negative values in Fig. VI.5, C), similarly to the response to azadirachtin. Despite the distinct effect observed among treatments, the variation in the diet consumption was proportional to the variation of larvae weight gain and a significant correlation ($p < 0.0001$) of these variables was found at 48 h (r = 0.958) and 72 h (r = 0.978) (Fig. VI.4). From this data we can deduce that, in a survival situation, early 4th instar larvae needed as a minimum food an equivalent of 4.767 (± 0.787) mg of dry corn leaves to maintain their metabolic function without losing weight.

It is worth mentioning, that *trans*-anethole and fennel EOs showed higher deviations from this trend at 72 h and that the recorded larvae absolute weight gains were lower than those predicted by the linear function (Fig. VI.4).

Pc-F and azadirachtin, followed by *trans*-anethole and cuminaldehyde, were the treatments causing the most significant adverse effects on larva nutrition, seen both in consumption (Fig. VI.5,

A) and RCR (Fig. VI.5, B), and growth, with reductions in weight gain (Fig. VI.5, C) and RGR (Fig. VI.5, D). In addition, (S)-carvone and estragole seem to have had some effect over these parameters. Regarding the impact of treatments over larvae metabolism (RMR, ECD, ECI and MC), azadirachtin, *trans*-anethole and cuminaldehyde had a negative and significant influence ($p \leq 0.05$) on the RMR, ECD and ECI indices (Fig. VI.5, F; VI.5, G; and VI.5, H; respectively), causing higher costs (MC) to the insect organism (Fig. VI.5, E) (3.8 to 1.5 times superior). (S)-(+)-carvone and (+)-fenchone had also negative impacts on RMR, while fennel EOs influenced positively the RMR (Fig. VI.5, F). However, none caused a significant increase of the MC, as observed with cuminaldehyde and *trans*-anethole treatments (Fig. VI.5, E).

The digestion of food treated with EOs and volatile compounds, revealed by the AD index, ranged between 49.6 and 67.1% but it was not statistically different from the ethanol control group (58.9%) (Supplementary data). Only azadirachtin reduced significantly ($p \leq 0.05$) the digestibility of food (AD_{aza} =17%) when compared to the remaining treatments.

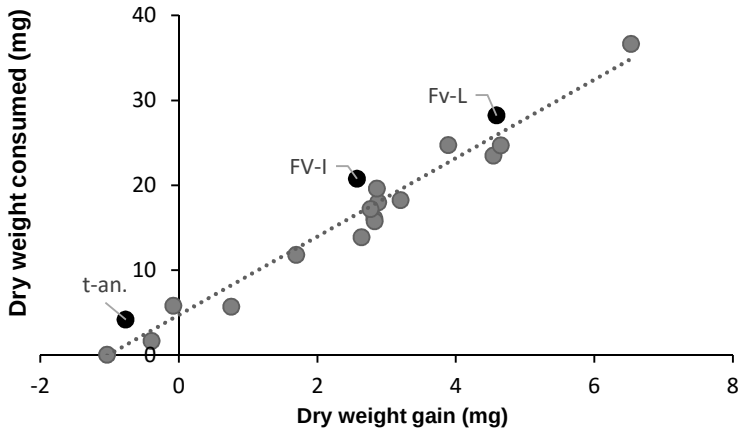


Figure VI.4. Correlation of *Pseudaletia unipuncta* 4th instar larvae growth with diet consumption (dry weight basis) after 72 h of feeding on corn leaves pieces.

y = 4.646 (± 0.247) x + 4.767 (± 0.787), R^2 = 0.957, $d.f$ =16, F =354.5, p < 0.0001.

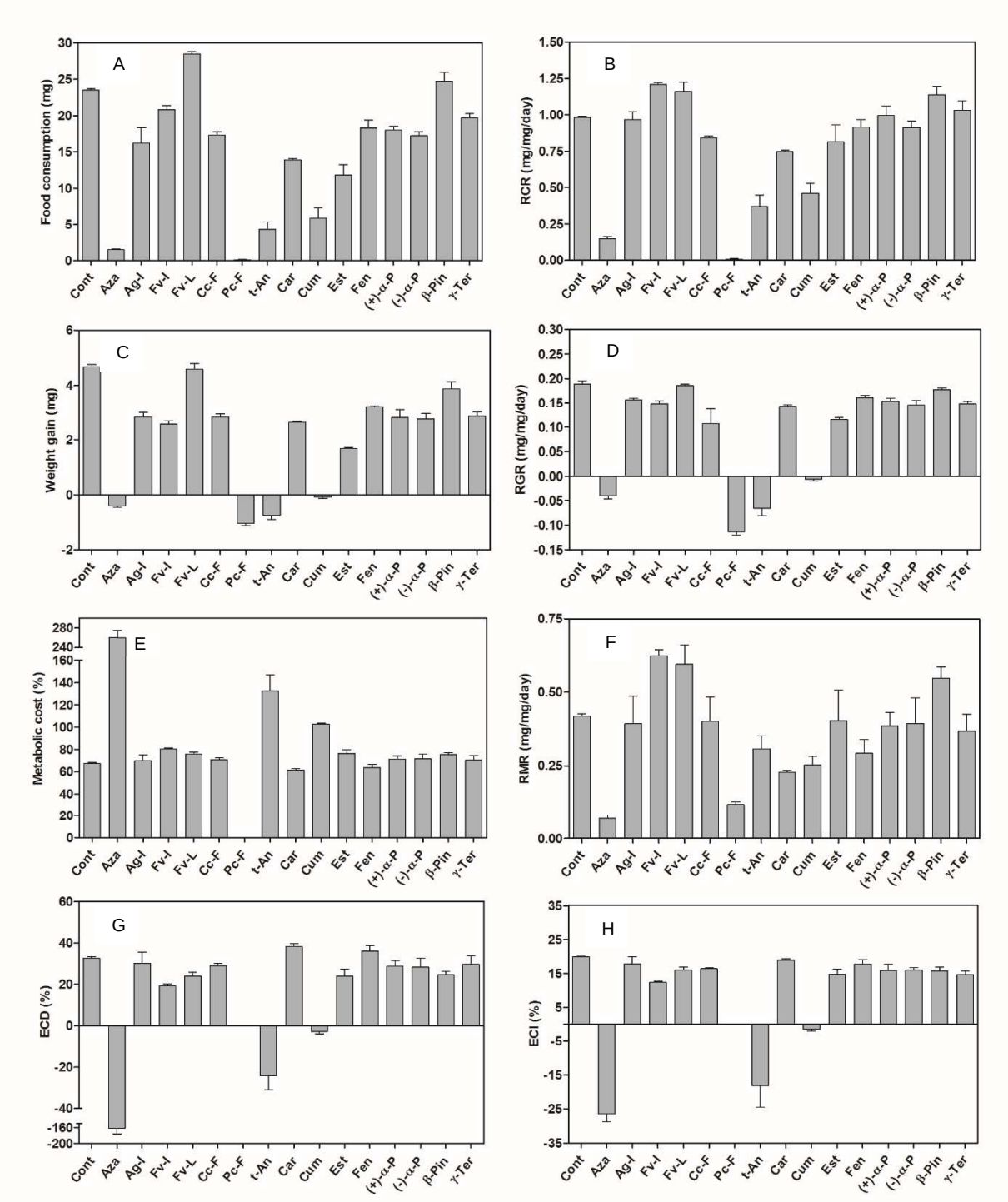


Figure VI.5 (A-H). Effects of Apiaceae essential oils (EOs) and respective major compounds on *Pseudaletia unipuncta* 4th instar larvae nutrition and metabolism after 72h of feeding with treated corn leaves at 175 µg cm^{-2}. **(A)** Food consumption, **(B)** Relative Consumption Rate, **(C)** Larvae growth, **(D)** Relative Growth Rate, **(E)** Metabolic cost, **(F)** Relative Metabolic Rate, (G) Efficiency of Conversion of Digested food, **(H)** Efficiency of Conversion of Ingested food.

Cont: Ethanol control, **Aza**: Azadirachtin, positive control at 10 µg cm^{-2}, **Ag-I**: *Anethum graveolens* infrutescence EO*,* **Fv-I**: *Foeniculum vulgare* var. *vulgare* infrutescence EO, **Fv-L:** *Foeniculum vulgare* var. *vulgare* leave more stems EO, **Cc-F**: *Cuminum cyminum* fruit EO*,* **Pc-F**: *Petroselinum crispum* fruit EO, ***t*-An**: *trans*-Anethole, **Car**: (S)-(+)-Carvone, **Cum**: Cuminaldehyde, **Est**: Estragole, **Fen**: (+)-Fenchone, **(+)-α-P**: (+)-α-Pinene, **(-)-α-P**: (-)-α-Pinene, **β-Pin**: (-)-β-Pinene, **γ-Ter**: γ-Terpinene. Mean values (± standard error) were determined for 3 experiments with 10 replicate each. Note: Calculations of ECD, ECI and MC indices for PC-F were not possible due to absence of food consumption in some experiments.

5. Discussion

Plant-based metabolites, including volatile compounds, display several functions in plant defense. These semiochemicals may influence insects' feeding and reproductive behaviors by deterring feeding and oviposition (Isman, 2002). Moreover, lethal activities as ovicidal, larvicidal, and adulticidal have been reported for several compounds from aromatic plants (Koul et al., 2008; Tripathi et al., 2009; Ntalli & Menkissoglu-Spiroudi, 2011).

In the present paper, the issue related with the activities of EOs and compounds on egg hatching and postembryonic development, along with the effect on the caterpillar feeding behavior and its nutrition, were analyzed in detailed experiments. In the first assay we demonstrated the potentialities of four EOs (Pc-F, Pc-I, Cc-F and Ag-I), and their respective major compounds [myristicin and apiole, cuminaldehyde and (S)-carvone], as alternative plant products with effectiveness against the true armyworm eggs and larvae emergence. Notwithstanding the higher activity of compounds to inhibited eggs hatching (≤ IC_{50}), relatively to the EOs where they were identified, EOs presented linear regressions with higher slopes. This fact indicates that for a given increase in EO concentration a larger effect will be induced when compared to that of individual compounds. Because of their great number of constituents, EOs can interact with several targets, affecting different functions and decreasing the resistance of organisms (Ntalli & Menkissoglu-Spiroudi, 2011). From a toxicological viewpoint, when we look for an acute effect, the synergism occurring in unique, natural and complex mixtures is an aspect as relevant as the inhibitory or lethal concentration criterion. Myristicin was found to be the most active treatment, and so it was expected a low IC_{50} for the Pc-F EO, where this compound is abundant. However, it was not possible to have a good fit of the data, and hence to estimate the IC_{50} for this treatment, due to the high inhibition values obtained for all the concentrations tested (Table VI.2). The same fact explains the lack in IC_{50} for apiole and Pc-I, where apiole is the major compound. Regarding the compound cuminaldehyde, it was found to be significantly more active than the EO Cc-F (2 times superior), where it was identified as major compound (39%). Based on both results, we consider that most of the hatching inhibitory activity exhibited by the Cc-F EO may be attributed to this major compound, although other identified constituent not included in assessments (i.e. *p*-cymene, *p*-mentha-1,4-dien-7-al) could have some contribution in the overall activity. Furthermore, the effectiveness of (S)-(+)-carvone (IC_{50} = 3.74 mg mL^{-1}) was lower than the two other compound, but not statistically different from that of Ag-I EO (3.60 mg mL^{-1}). Their bioactivity is consistent with the fact that this compound accounts for 67% of the Ag-I EO.

In previous studies, the vapors of cumin EO were found to be 100% active against embryos of *Ephestia kuehniella* (Lepidoptera) and the confused flour beetle, *Tribolium confusum* (Tunç et al., 2000). Furthermore, *C. cyminum* and *A. graveolens* EOs, were found to reduce the hatching rate of *Callosobruchus chinensis* when fumigated (Chaubey, 2008). EOs extracted from parsley seed

showed hatching inhibitory activity on the eggs of the Indian meal moth, *Plodia interpunctella* (74% inhibition at 24 µL L^{-1} air) (Rafiei-Karahroodi et al., 2011).

Beside the number of non-hatched eggs evaluated herein, distinct effects of EOs/compounds could be observed (Fig. VI.3). Freshly laid eggs (0-24 h post-oviposition) of *P. unipuncta* are white to pale-yellow (0.6-0.7 mm spheres) and, as development progresses, their color darken to a metallic grey just before hatching. However, we have observed that eggs treated with concentrations above 5 mg mL^{-1} of Ag-I and Cc-F EOs, as well as with their respective main compounds, (S)-(+)-carvone and cuminaldehyde, showed no maturation and some signs of desiccation. It this situation, it was assumed that EOs and compounds have penetrated the egg shell, probably thought micropyles and/or aeropyles, and reached the embryo, displaying a clear ovicidal activity. A time-series study performed by Berger-Twelbeck et al. (2003), with the ovicide 4-methoxy-benzoic acid propargylester against eggs of the lepidopteran *Manduca sexta,* suggest that the degradation of the embryo is a consequence of the rupture of the serosa. When the cohesion of the serosal membrane is compromised in an early phase of egg development, the embryo dies prematurely. In addition to its physiological and metabolic functions, the serosa often secretes a cuticle that helps to prevent egg' desiccation (Strand, 2013). Thus, more than the chorion, the serosal membrane provides a barrier to ovicides penetration into the embryo (Berger-Twelbeck et al., 2003) and water loss (Strand, 2013).

In contrast, a different effect was observed on eggs exposed to Pc-F, Pc-I, myristicin and apiole. These treatments significantly compromise the hatching event, however, apparently, embryogenesis was not disrupted and almost all embryos seemed to be developing. At lower concentrations, whenever emergence occurred, the few neonate larvae were morbid, being unable to move and eat. It is known that mature embryos of Lepidoptera species have to consume the egg-shell, partially or completely, to emerge (Fehrenbach, 2003). Therefore, it is plausible that in this process larvae may have simultaneously ingested residual amounts of compounds, namely myristicin and apiole, which were retained in the egg-shell, causing toxicity through ingestion. Myristicin and other methylenedioxyphenyl-containing phenylpropene (MDP), like apiole and dillapiole, have been frequently described as natural synergists (Lichtenstein & Casida, 1963; Lichtenstein et al., 1974; Berenbaum & Neal, 1985). After been ingested by insects, myristicin can interfere with mixed-function oxidases and induce susceptibility to other secondary metabolites, enhancing their toxicity at much lower concentrations (Berenbaum & Neal, 1985).

For these EOs and compounds aforementioned the post-embryonic developmental study was not achieved due to the high rate of mortality or the impaired fitness of *P. unipuncta* neonates, while for EOs and compounds which did not have pronounced ovicidal activity we have monitored the post-exposure effects during insect lifespan. Concerning the one-dose hatching inhibition assay, the much lower activity exhibited by individual compound, (+)-fenchone, estragole, *trans*-anethole, and pinenes (Fig. VI.1), corroborates the ineffectiveness observed for the respective natural mixture (Fv-I and Fv-L) where these compounds co-occur in variable amounts (Table VI.1).

It is also interesting to note that two of the three most active volatiles in causing post-exposure premature death, γ-terpinene and estragole (Table VI.3), were by far the least active regarding

hatching inhibition (Fig. VI.2). After the larvae emergence, the most persistent effects throughout insects' life were observed for (+)-fenchone and (-)-β-pinene, which have induced significant effects in total lifespan. Interestingly, these two compounds were the only ones that caused the most dramatic change in sex ratio (more females), plus significant reductions in pupae weight and females oviposition potential (Table VI.4). For the remaining treatments, which includes azadiracthin at a lower concentration (1 mg mL^{-1}), extension of lifespan (2 to 6.5 more days), decrease/increase of pupal weight, and/or increase of mortality, were the typical post-exposure effects to be recorded. According to Hummelbrunner and Isman, 2001, a longer larval developmental stage represents a significant disadvantage for insect herbivores, increasing the probability of death in a field situation.

In our analysis we also investigated possible relations between some parameters. A tight positive relationship between pupae' weight and the number of eggs laid by each female was found ($n = 12$, $r = 0.809$, $p = 0.0014$) (data not shown). Additionally, the average number of eggs per female observed in treatments was inversely related with the percentage of females ($n = 11$, $r = -0.847$, $p = 0.001$) (data not shown). This last finding suggest that some EOs/compounds may induce a changes in sex differentiation during development and that compounds causing an increase in the female ratio could influence negatively the abundance of eggs deposited by each females.

The developmental inhibitory activity of some Apiaceae EOs was previously assessed against the larval and adults forms of several insects, by mean of fumigation. Sub-lethal doses of EOs from *A. graveolens* and *Trachyspermum ammi*, caused aberrations in the different developmental stages and inhibition of pupation and adults' emergence of *Tribolium castaneum* (Chaubey, 2007). Similar effects, in addition to a decrease in the number of laid eggs, were reported against another stored-product insect, *Callosobruchus chinensis,* as consequence of larvae fumigation with *T. ammi, A. graveolens* and *C. cyminum* EOs (Chaubey, 2008). *A. graveolens* EO have also showed inhibitory activity on *T. castaneum* reproduction by preventing females to oviposit (Chaubey, 2007). Essential oils isolated from plants belonging to the Labiatae family have been reported for their effects on insects' development and reproduction. When fumigated over *Acanthoscelides obtectus* inseminated females (at 77 µL L^{-1} of air), *Rosmarinus officinalis, Lavandula hybrida, Mentha viridis* and *M. microphylla* EOs exhibited oviposition deterrence, affected fecundity, and unfavorably influenced offspring emergence (decreased hatchability and increased neonate larval mortality) (Papachristos & Stamopoulos, 2002). Great emphasis has been given to the EO from *Acorus calamus* (Araceae), which strongly influenced the male fertility acting as a chemosterilant agent in the red cotton bug (*Dysdercus koenigii*) (Koul et al., 1977).

Due to their ability to retard or accelerate insect development, and disrupt ecdysis, some plant secondary metabolites have been identified as IGR. Azadirachtin is a remarkable example of a natural chemical from plant origin with capacity to modify insects' behavior and several physiological functions. This triterpenoid has been described for its exceptional growth regulatory action, being able to disrupt molting and metamorphosis and block reproduction in several species (Dorn et al., 1986; Isman & Akhtar, 2007). Besides, it can affect vitellogenesis (Sayah et al., 1996) and ovarian development, by causing degenerative changes in follicles (Sayah et al., 1996; Senthil Nathan et

al., 2008). In the present study, we found no significant effects of azadirachtin on the fertility or the oviposition potential of *P. unipuncta* females, as a result of eggs exposure to 1 mg mL^{-1} of this compound.

For a more comprehensive study of EOs/compounds sub-lethal activities, we presented herein an evaluation of their effects on the true armyworm nutritional physiology. The nutritional indices analysis is considered a useful approach to appraise whether a secondary metabolites have antifeedant and/or toxic activity after feeding (Waldbauer, 1968).

Our findings (reduction in food consumption, ECD and ECI values) indicates that azadirachtin, *trans*-anethole and cuminaldehyde caused feeding deterrence and interfered severely in the process of assimilation and conversion of food to body mass. Based on the weight loss observed, we consider that the digested food was used in metabolic processes, namely detoxification activity, which increased the energetic cost (high MC). Eventually the maintenance of homeostasis could have been at the expense of the organism's reserves. *trans*-Anethole and, in a lesser extent cuminaldehyde, seem to have induced the same anorectic effect frequently described with azadirachtin (Isman & Akhtar, 2007) and as observed herein.

Furthermore, digestive disorder, longer persistence of food in digestive tract and physiological stress are plausible event that might have been caused by these three compounds. Evidences of *trans*-anethole metabolization to a hydroxylated form were found in feces of the tobacco cutworm (*Spodoptera litura*) after topical and oral administration (Passreiter et al., 2004). Senthil Nathan et al. (2005b), have described the significant inhibition of gut enzymes activity (acid phosphatases, alkaline phosphatases and adenosine triphosphatases) and weight loss caused by azadirachtin and other limonoid compounds on the rice leaf folder, *Cnaphalocrocis medinalis*.

The EO from *A. calamus* rhizome, and its phenylpropenoid *cis*- and *trans*-asarone, showed feeding deterrence on the noctuid species, *Peridroma saucia* (variegated cutworm) (Koul et al., 1990). Moreover, a strong growth inhibition and toxicity by ingestion or topical application, with consequent reduction of ECD and ECI, was proved to be induced exclusively by the *cis*-asarone isomer (Koul et al., 1990). Contrarily, *trans*-asarone, from *Daucus carota* EO, showed inhibition of the larval growth (> 96%) in other Lepidoptera species (*Helicovarpa zea*, *Heliothis virescens* and *Manduca sexta*), when used at 100 µg mL^{-1} in artificial diet (Momin & Nair, 2002). Likewise, Huang et al. (1997) reported the influence of *Myristica fragrans* (Myristicaceae) EO on the maize weevil (*Sitophilus zeamais*) behavior (reduced food consumption) and weight loss, as well as possible deleterious effects. Post-ingestive toxicity, revealed by a decrease in the ECI index, was also recorded for *L. angustifolia* EOs against the lesser mulberry pyralid, *Glyphodes pyloalis* (Yazdani et al., 2013).

With our experiment it was not possible to investigate post-ingestive effects of Pc-F EO and its compounds on *P. unipuncta* larvae since the consumption of food was virtually null, and high morbidity was observed in the case of both compounds after the larvae first contact with treated leaves (60% and 100% of death for apiole and myristicin, respectively, after 72 h). We have

previously showed that the EO from parsley fruits and both compounds, exhibit important larvicidal activity when assessed by the contact disk assay (Chapter V). Even though, the toxicity after ingestion of little amounts of Pc-F, apiole or myristicin is not discarded taking into account the following aspects. First, the final amount of chemicals existing in the pieces of corn leaves (0.7 mg per leaf, which was equivalent to 0.7-1% of the food fresh weight) is much lower than those used in the previous larvicidal assay (3 times less) and, secondly, the larvae' symptoms perceived in both situations were distinct. The EOs isolated from parsley fruits and green infrutescences have proved to be of great efficacy not only as ovicide but also as larvicide, as discussed in the previous chapter. Furthermore, Pc-F exhibited feeding and growth inhibitory effects as well. This wide spectrum of activities is advantageous and seems adjusted to a field situation, where several stages of *P. unipuncta* are co-habiting and parasitizing plants. In this context, if applied during oviposition period, after larvae emergence, or during the first stages of larval development, it could contribute to the control *P. unipuncta* population, reducing the number of emerged and developing larvae and decreasing the food consumption and growth rate of the most resistant. Additionally, the increase in search time for viable food, and consequent delays on development, could expose the debilitated and less developed larvae to increased mortality, as a result of biotic and abiotic factors (Akhtar & Isman, 2004).

6. Conclusion

In this study we have shown the biological effects displayed by EOs from four Apiaceae species and their major compounds against armyworm, at the developmental and nutritional levels. EOs from *P. crispum* and its two phenylpropanoids, myristicin and apiole, reduced efficiently the eggs hatchability in the later stages of embryonic development, while, *A. graveolens* and *C. cyminum* EOs, and their respective major compounds, (S)-(+)-carvone and cuminaldehyde, compromised the eggs viability shortly after treatment.

Despite their lack of ovicidal or inhibitory activity over hatching, the effects of some EOs and compounds were revealed *a posteriori*, throughout insects' postembryonic development, such as, delay of the pupation, debility and early death of larvae. Generally, longer larval stage and life-span were associated with the occurrence of lighter/smaller pupae, but not necessarily with a decrease in the emergence of adults. Furthermore, we found that the impairment the insects' development, represented by the pupae' weight parameter, was strongly related with a decrease of the offspring. It was demonstrated that exposure to (+)-fenchone and (-)-β-pinene during embryonic development can significantly influence the ability of *P. unipuncta* to originate progeny. Although the aforementioned compounds were not among those presenting noteworthy ovicidal properties, results suggest that they may have interfere in the sex differentiation and reproductive function of the insect.

Regarding the impact of treatments over larvae metabolism for the 3-day-period of experiment, our findings indicates that some EOs and compounds may impair feeding behavior and, consequently, larvae growth, without necessarily causing digestive disorder or interfering in the efficiency of conversion of food to biomass. In contrast, *trans*-anethole and cuminaldehyde exhibited strong post-ingestive toxicity, similarly to the observed with azadirachtin, and appear as reasonable alternatives to limonoids compounds.

The biological activity of some Apiaceae EO and compounds against the damaging noctuid, *P. unipuncta*, were evidenced in the present work. As discussed herein and in the previous study the activities of *P. crispum* EOs, is strongly related with the activity of myristicin and apiole, and their improved toxicity is performed through contact with integument. Regardless the fact that the nutritional and metabolic impacts of these two compounds could not be further explored due to the high mortality rates recorded, the results suggest that the EO and its compounds may also exert noxious activity on larvae through ingestion. Hence, further researches concerning some molecular targets are foreseen to identify their modes of action.

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8. Supplementary data

Table VI.5. Effects of Apiaceae essential oils and respective major compounds on *Pseudaletia unipuncta* 4th instar larvae growth, consumption and metabolic parameters after 72 h of feeding on treated food.

Treatments	Mort. [a]	weight gain [b]	Food consumption [b]	RGR [b]	RCR [b]	RMR [b]	ECI [b]	ECD [b]	MC [b]	AD [b]
		(mg of dry weigh)		(mg mg^{-1} day^{-1})			(%)			
Ethanol	0.0	4.6 ± 0.1 h	23.5 ± 0.2 hi	0.188 ± 0.007 h	0.981 ± 0.008 def	0.419 ± 0.008 cd	19.9 ± 0.17 e	32.5 ± 0.7 de	67.5 ± 0.7 ab	58.9 ± 3.2 bcde
Azadirachtin [c]	0.0	-0.4 ± 0.1 bc	1.5 ± 0.1 a	- 0.040 ± 0.006 b	0.149 ± 0.013 a	0.068 ± 0.012 a	-23.4 ± 2.4 a	-160.8 ± 14.2 a	260.8 ± 14.2 e	17.4 ± 2.2 a
Ag-I	3.3	2.8 ± 0.2 ef	16.1 ± 2.1de	0.157 ± 0.003 fgh	0.966 ± 0.054 def	0.393 ± 0.093 cd	17.8 ± 2.0 de	30.1 ± 5.4 de	69.9 ± 5.4 ab	60.8 ± 5.4 bcde
Fv-I	0.0	2.6 ± 0.1 e	20.8 ± 0.6 gh	0.149 ± 0.005 fg	1.208 ± 0.014 h	0.625 ± 0.020 e	12.3 ± 0.4 d	19.2 ± 0.8 d	80.8 ± 0.8 b	64.0 ± 1.2 de
Fv-L	0.0	4.6 ± 0.2 h	28.2 ± 0.3 j	0.186 ± 0.005 h	1.160 ± 0.064 gh	0.595 ± 0.065 e	16.2 ± 0.9 de	24.4 ± 2.0 de	75.6 ± 2.0 ab	67.1 ± 2.1 e
Cc-F	3.3	2.8 ± 0.1 ef	17.3 ± 0.5 ef	0.107 ± 0.030 d	0.840 ± 0.015 cd	0.402 ± 0.081 cd	16.4 ± 0.4 de	28.9 ± 1.2 de	71.1 ± 1.2 ab	62.4 ± 1.5 bcde
Pc- F	32.4	-1.0 ± 0.1 a	0.1 ± 0.1 a	-0.113 ± 0.007 a	0.007 ± 0.007 a	0.118 ± 0.010 ab	nd	nd	nd	nd
trans-Anethole	12.9	-0.8 ± 0.2 ab	4.3 ± 1.1 b	-0.065 ± 0.015 b	0.372 ± 0.077 b	0.307 ± 0.046 bc	-18.0 ± 6.4 b	- 24.2 ± 6.8 b	132.5 ± 14.3 d	66.5 ± 3.8 de
Apiole	60.0	-	-	-	-	-	-	-	-	-
(S)-(+)-Carvone	3.0	2.6 ± 0.04 e	13.9 ± 0.2 cd	0.142 ± 0.004 ef	0.748 ± 0.009 c	0.229 ± 0.006 abc	19.0 ± 0.4 de	38.3 ± 1.27 de	61.7 ± 1.3 a	49.6 ± 0.7 b
Cuminaldehyde	12.9	-0.1 ± 0.05 c	5.8 ± 1.5 b	-0.006 ± 0.004 c	0.461 ± 0.068 b	0.253 ± 0.030 bc	-1.4 ± 0.6 c	-2.7 ± 1.2 c	102.7 ± 1.2 c	54.1 ± 2.4 bcd
Estragole	3.3	1.7 ± 0.03 d	11.8 ± 1.4 c	0.116 ± 0.004 de	0.814 ± 0.117 cd	0.404 ± 0.104 cd	14.8 ± 1.5 de	23.9 ± 3.6 de	76.1 ± 3.6 ab	63.0 ± 3.8 cde
(+)-Fenchone	0.0	3.2 ± 0.1 f	18.2 ± 1.1 efg	0.161 ± 0.005 fgh	0.915 ± 0.050 cde	0.293 ± 0.046 bc	17.7 ± 1.3 de	36.0 ± 2.8 de	64.0 ± 2.8 a	50.0 ± 6.8 b
Myristicin	100	-	-	-	-	-	-	-	-	-
(+)-α-Pinene	0.0	2.8 ± 0.3 ef	18.0 ± 0.5 efg	0.156 ± 0.006 fg	0.994 ± 0.067 defg	0.385 ± 0.046 cd	15.9 ± 1.7 de	28.8 ± 2.8 de	71.2 ± 2.8 ab	58.8 ± 7.7 bcde
(-)-α-Pinene	0.0	2.8 ± 0.2 ef	17.2 ± 0.6 ef	0.146 ± 0.009 fg	0.912 ± 0.044 cde	0.394 ± 0.087 cd	16.0 ± 0.6 de	28.4 ± 4.2 de	71.6 ± 4.2 ab	53.9 ± 1.4 bcd
(-)-β-Pinene	0.0	3.9 ± 0.3 g	24.7 ± 1.2 i	0.177 ± 0.005 gh	1.135 ± 0.061 fgh	0.547 ± 0.040 de	15.7 ± 1.2 de	24.6 ± 1.6 de	75.4 ± 1.6 ab	60.3 ± 1.4 bcde
γ- Terpinene	0.0	2.9 ± 0.2 ef	19.6 ± 0.6 fg	0.149 ± 0.004 fg	1.029 ± 0.067 efg	0.369 ± 0.058 cd	14.6 ± 1.2 de	29.6 ± 4.2 de	70.4 ± 4.2 ab	50.3 ± 4.6 bc
df		47	47	47	47	47	44	44	44	44
F		141.403	79.041	98.237	41.985	7.361	48.055	106.025	100.184	10.076
p≤		0.001	0.001	0.001	0.001	0.001	0.001	0.005	0.001	0.001

RGR: Relative growth rate; **RCR**: Relative consumption rate; **RMR**: Relative metabolic rate; **ECI**: Efficiency of conversion of ingested food; **ECD**: Efficiency of conversion of digested food; **MC**: Metabolic cost. nd: Not determined, the calculation was not possible to due to absence of food consumption in some experiments.

[a] Accumulated mortality recorded at 72h; [b] Mean values (± standard error) were determined from 3 independent experiments with 10 replicates each. Within columns, means followed by the same letters do not differ significantly, based on one-way ANOVA followed by Duncan´s post test ($p = 0.05$); [c] Azadirachtin (95%) was included in the study as a positive control at a lower concentration (10 µg cm^{-2}).

CHAPTER VII

Final considerations

1. Concluding remarks

For the past 30 years, investigations carried out with a wide spectrum of plant secondary metabolites have highlighted their advantages in the protection of human beings, domestic animals and plant crops (Akhtar & Isman, 2004; Dubey et al., 2010). Despite this growing academic interest on the pesticidal properties of plant metabolites and the recognition of its huge potential by the general public, their commercialization is still scant and limited to some parts of the world (Koul, 2004; Isman & Grieneisen, 2014). Indeed, only a handful of botanical products are applied in crop protection as active ingredients against insects (Koul, 2004). Thus, the present state of the art situation points towards a clear disconnection between both the scientific-academic and the productive-commercial fields (Isman & Grieneisen, 2014). Therefore, a concerted effort of research institutes, universities, nongovernment organizations (NGO), and government organizations is essential to elevate the stature of biopesticides (Mishra et al., 2015).

As a complementary approach or alternative to synthetic pesticides, EOs and volatile compounds of numerous plant species are currently under consideration as components of crop protection products as well as in repellent formulations to prevent disease transmission (Isman, 2000; Shaaya & Rafaeli, 2007; Regnault-Roger et al., 2012; Suwansirisilp et al., 2013). The efficacy of EOs as mosquito larvicides and repellents has been a very active field of investigation in recent years, and the treatment of standing waters with EOs has been suggested as a suitable option to control the density of mosquitoes' populations at their larval stage (Isman et al., 2011; Pavela, 2015). Undoubtedly, the best market opportunities for EO-based pesticides are for certified organic production of high-value crops where there are fewer competing products, and in benefit of human and animal safety (management of structural pests, indoor pests and mosquitoes) (Isman & Machial, 2006; Isman, 2008; Isman et al., 2011).

In this perspective, the present work constituted an attempt to valorize EOs from 4 well-known aromatic Apiaceae species contributing also to the development of environmentally friendly means for the control of vector borne-diseases and agricultural pests.

In order to achieve this goal, we firstly characterized the chemical composition of EOs originated from bitter fennel, cumin, dill and parsley plants and study their variation during cultivation (with the exception of cumin) and between plant parts. Subsequently, we tested the EOs from the most suitable plant parts (leaves plus stems and fruits) taking into consideration yield, commercial availability (raw materials or EOs) and worldwide production.

In the current study we have established, in accordance with the literature, that accumulation and composition of EOs were species-specific, and that the type of organ, its developmental stage and the origin of the plant source material have a significant influence on EO content. Moreover, we identified the occurrence of intra-individual variability within plants of bitter fennel, with some of them presenting a *trans*-anethole dominant profile while other had a estragole-dominant profile.

This knowledge of the ontogenetic and or intraspecific variability is of great practical importance, allowing to determine the harvesting time that could provide maximum yields, or to select the raw material with a specific chemical composition (Góra et al., 2002). As a result, we determined that umbels-bearing fruits of bitter fennel, dill and parsley delivered the highest yield. The fruit-ripening stage seems an appropriate time for harvesting dill, parsley and bitter fennel. In the last case, the fruits fully grown but still green accumulated more EO (used in bioassays as Fv-I), suggesting that the extraction of EO from bitter fennel infrutescences is more profitable if done before fruits are totally ripen, as previously shown by Stefanini and coworkers (2006).

Furthermore, a better understanding of EOs production pattern during plant development opens the possibility to select the plant part/material that could provide a certain chemical profile in view of a specific bioactivity. In this case, we have concluded that the collection of dill infrutescences with ripened fruits is advantageous, both in term of EO amount and isolation of a product enriched in (*S*)-carvone (Ag-I), which have herein shown remarkable activity against *P. unipuncta* (eggs and larvae) and *R. peregra* (eggs and adults).

As a whole, the results discussed in chapter III, IV, V and VI demonstrated the potential of EOs from the four studied species (under de abbreviations Ag-I, Ag-L, Cc-F, Fv-I, Fv-L, Pc-F, and Pc-I), and of some of their respective major compounds, as alternative botanical pesticides. EOs and compounds showed distinct performances depending on the type of assay, the tested organism, and/or its developmental stage. As observed in chapter IV and V, EOs from parsley and cumin and the compounds myristicin, (*S*)-(+)-carvone and cuminaldehyde were also highly active towards eggs of both animal species *P. unipuncta* and *R. peregra.* Moreover, as far as we know, this is the first report of the molluscicidal activity of parsley, dill and cumin EOs against a host freshwater snail.

The results showed that EOs and the tested allelochemicals, exhibit toxicity at differing dosages. Not only the lethal concentrations (LCs) determined herein and in many other reports appear to be high when compared to synthetic pesticides (organophosphates, organochlorines or carbamates), as EOs and their constituents are, in the vast majority of cases, more environmentally benign and less toxic to vertebrates (LD_{50} values ranging from 0.6 to 5 g kg^{-1} in rats when ingested orally) (Koul et al., 2008). Nonetheless, additional toxicological studies against non-target organisms are required. Comparison of lethal doses (acute toxicity) is frequently used to evaluate the biological efficiency of EOs. Nevertheless, EOs can exhibit other activities at the developmental and nutritional levels (as we have shown and discussed in Chapter VI). In this study, we established that the studied EOs exhibit some activities other than acute lethality against the true armyworm. It was demonstrated that an exposure to (+)-fenchone and (-)-β-pinene during the embryonic development could influence the ability of *P. unipuncta* to originate progeny (as observed in chapter VI). Moreover, it was showed that, when performing a non-choice-diet assay, Pc-F, *trans*-anethole and cuminaldehyde have a significant negative repercussion on larvae feeding growth and metabolic rate.

In addition, we have delivered a scientific basis to some traditional applications of *F. vulgare* in the control of insects. Our study demonstrates that bitter fennel could be a productive source of

biological agents to control particular host species, namely freshwater snails and mosquitoes (Chapter III). Besides, *trans*-anethole revealed acute insecticidal, nematicidal and molluscicidal activities, being more active than its isomer (estragole) against *A. atroparvus*, *P. unipuncta*, *M. javanica* and *R. peregra*. Notwithstanding some interesting results obtained with Fv-I and Fv-L, we expect that the use of fennel EO extracted from plants with *trans*-anethole dominant profiles (existing in the studied population) could present more interesting from the biopesticidal standpoint. Alternatively, the *dulce* variety could eventually represent a good option owing to its larger percentage of *trans*-anethole (Pitasawat et al., 2007; Ntalli et al., 2011). In general, *trans*-anethole is used as a natural flavoring ingredient in the food industry, and it could be also a good alternative to synthetic chemicals for the control of freshwater snails, mosquitoes and plant-parasitic nematodes, based on our findings and of other several authors (Chantraine et al., 1998; Oka et al., 2000). From our survey we have also determined that the EO from cumin fruits (Cc-F) which comprise a high fraction of cuminaldehyde, or cuminaldehyde *per se*, could be promising biochemical agents that could be incorporated into both insecticidal and molluscicidal formulations of botanical origin.

Interestingly, we also found evidences of a wide range of pesticidal activity of *P. crispum* EOs. As we concluded from a review of the literature, this species has been much less explored in what concerns its biopesticidal activities. Although its lower EOs productivity, especially of vegetative parts, may justify a lack of interest in investigating its applications, this plant species should not be undervalued since it offers a variety of compounds with great potential as pesticidal or synergizing agents. The study accomplished shows that EOs from fruits or infrutescences, composed by a dominant fraction of phenylpropene derivatives, are highly active against the freshwater snail, the lepidopteran, and the mosquito herein used as target-species (results concerning their mosquitocidal activity is shown in the additional/ unpublished work section of the Appendices). Furthermore, both the phenylpropene-derivatives extracted from parsley EOs, myristicin and apiole, displayed promising activities. From these findings we concluded that parsley EOs, which contains high percentages of myristicin and apiole, could be of great efficacy not only as ovicide, but also and larvicide, phagodeterrent and growth inhibitor. It is probable that additional biopesticidal activities could by be attributed to this EO in the near future.

In this study, we aimed also to understand and compare the level of effectiveness of the tested products by making an effort to analyze more than one parameter/criteria. As we could verify in lethality assays performed with *P. unipuncta* larvae and adults of *R. peregra,* the treatments displaying the most acute dose-related toxicity were not necessarily the most time-effective. So, the time-dependent study approach has given valuable contributes for interpreting results. Furthermore, the comparison of the different mortality rates (slopes) also gives useful information about the potency of the treatments. A higher effectiveness and toxicity were recorded for EOs or constituents with highest slopes. The progressive accumulation of the active compound(s) in the organisms and/or its metabolization into more toxic forms by the organism enzymatic system, have been suggested as possible events responsible for these time-related or elapsed toxicity (Kumar & Singh,

2006). By consequence, the intake of EOs constituents, the kinetic of the process and their bioavailability in the tissue might conceivably be different for each chemical and natural mixture. Besides, the stability of EOs compounds (related to degradation or volatilization rates) (Seo et al., 2015), their vapor pressure and octanol–water partition coefficients (Lucia et al., 2013), and thermolability are extremely relevant aspects influencing EOs' efficacy (Pavela, 2014b). Even though many aspects remained to be solved as regards to the physiological phenomena triggered by EOs and other toxicological aspects, it could be concluded from our study that time-series approaches were helpful to differentiate, among candidates with considerable activity, which have more acute/faster activity.

Another challenge of this work was to establish composition *vs* activity relationships. For some EOs, the bioactivity measured was sometimes related with the compounds included in the study. The biocidal effects observed for EOs in each assay were relatively consistent with the activity determined for the compounds tested in terms of LCs, dose-mortality rates (slopes), or other evaluated activities. The effectiveness of Cc-F was ascribed to its major compound cuminaldehyde (found at 39%) and Ag-I was related to the individual toxicity of (S)-(+)-carvone which represent the major part of this EO (2/3). It was clearly established that the presence of (S)-carvone as a major volatile constituent of dill fruits is fundamental to obtain the enhanced activity of this particular EO (Ag-I), since the EO of others dill parts (Ag-L), which do not contain (S)-carvone, was significantly less effective in all assays. Nevertheless, since we could not complete the evaluation of all the compounds identified in EOs, and some of them have not been included in all assessments due to their limited availability or others constrains, many relationships remained to be established. Even so, the contribution of non-tested compounds existing in the EOs was well patent in several occasions.

Nevertheless, contrasting results were also recorded when testing a particular EO/compound on different developmental forms of the tested-organisms or when comparing distinct assays. Besides, in some rare cases (namely with bitter fennel EOs), differences on the qualitative or quantitative composition of EOs from the same plant species caused no considerable impact regarding their biological activity. All these ambiguous results evidence that, despite being an important facet of toxicology, the composition of extracts is just one piece of the puzzle. Comprehensive evaluations including combinations of identified major and minor constituents have proved to be helpful to researchers (Ntalli et al., 2010; Koul et al., 2013; Pavela, 2014a). Therefore, we find indispensable to orientate our efforts towards the elucidation of the toxicity of such complex mixtures, by determining the nature and degree of their interactions in some situations (additive, synergistic or antagonistic).

The knowledge of chemical profiles variation in organs of the three cultivated species, combined with the results of the toxicological assessments, allows us to suggest that umbels-bearing fruits in the pre-ripening stage (eventually ripen fruits) is an optimum source of biologically active EOs against two insect species and one mollusk. Dill, bitter fennel and parsley fruits (or dry fruits)

accumulated the highest amounts of volatile compounds per g of dry biomass, in their maturing umbels, which substantiated the role of these allelochemicals in the protection of this organ in particular, and the seeds it contains.

The findings of this study, along with the review of the literature concerning EOs of some Apiaceae species, are a contribution to proceeding works namely with the purpose to: (i) implement protocols and optimize the production of EO with specific profiles/constituents from the four species and to (ii) consider alternative sources (species and chemotypes) enriched in some interesting bioactive compounds.

2. Outcomes of the work and future perspectives

The research required for the development of botanical pesticides comprises a multi-step process. Although our studies are still in an early phase of this multi-step process, they provide the necessary framework and working hypotheses to drive future studies. In our opinion, the outcomes of the present work can be taken as a starting point for several new research topics, more precisely concerning the following issues:

(i) the effectiveness ranges of the tested EOs;

(ii) the undesirable effects on non-target organisms and plant crops - toxicity and selectivity issues;

(iii) the better knowledge of compounds interactions and their efficacy when combined;

(iv) the understanding of the mechanism of action of some constituents as regards to their acute toxicity;

(v) the stability of phytochemicals in formulations and their controlled release.

(i and ii) In continuation of the present screening approach, it is our interest to identify and reveal further activities of effective and environmentally friendly products from botanical origin. We shall address efforts to provide evidences of their wide-range effectiveness, by conducting complementary studies with other pest species and intermediate hosts. Such efforts should be directed to additional biological assessments at the laboratory and field scales. Whether or not these treatments are effective at the field-scale or in glasshouse environment is certainly an indispensable topic of future investigations.

The fact that some EOs may have a negative impact on small non-target organisms such as zooplankton have been discussed (Pavela, 2014b). Therefore, the evaluation of possible detrimental effects on natural enemies of pests, non-target animals in soil and aquatic systems is an essential research topic. The determination of critical thresholds for the release/ application of these compounds/ mixtures on specific cultures in order to warrant plant protection but with negligible impact on their physiology and other organisms is indispensable. Furthermore, in virtue of eventual

strong phytotoxic effects of EOs and their compounds to plant species and crops, we shall consider to assess the anti-germinative/ herbicidal of the products tested that showed the most promising potential. After obtaining such information, we would be able to present suitable recommendations not only for their use against specific pests but also in a particular crop, if applicable.

(iii) In addition, our results open the possibility for further investigations concerning interactions between EOs constituents. As we have outlined in the preceding chapters, the composition and interactions occurring in unique, natural and complex mixtures is an aspect as relevant as the inhibitory or lethal concentration criterion. The assumption that the whole extract/mixture is not the sum of its parts was established at several occasions. Nevertheless, to a certain extent, the individual assessment of the various constituents was not completely suitable to deliver a full interpretation of the activity of such complex mixtures. It is our opinion that a better knowledge of the combined action of constituents in those mixtures is a central point. So, as a step forward towards the elucidation of the bioactivity herein described, we have initiated a study with some artificial binary mixtures considering constituents in different ratios against *P. unipuncta* larvae, in order to find combinations with higher toxicity. This part of the work, which is in progress, is also of paramount importance when envisaging the development of standardized products.

(iv) Once a promising extract has been discovered, the next step is to find out how it is affecting the organism, i.e. what is its mode of action? This kind of information is also needed to safeguard non-target organisms (Akhtar & Isman, 2004). Because of their great number of constituents, EOs can interact with several targets, affecting different functions (Ntalli & Menkissoglu-Spiroudi, 2011). The focus of recent researches has now shifted towards understanding of the mechanism of action of biopesticides and molecular events. Several authors have demonstrated the anticholinesterasic activity of monoterpenes *in vitro* and discussed their role as AChE inhibitors (Miyazawa et al, 1997; Picollo et al, 2008; López & Pascual-Villalobos, 2010). In this context, we also recorded useful indications of the neurotoxic MOA of EOs and constituents. Other than the observation of symptoms caused in organisms, we have obtained some preliminary results about the possible effects of some of the tested products on the cholinergic nervous systems, through the screening of their inhibitory action on AChE enzymatic activity (as revealed in the appendix D concerning additional/unpublished work). The *in vitro* assays performed with purified AChE have evidenced the distinctive levels of inhibition for some EOs and individual compounds. Nonetheless, several major compounds, namely those found in parsley EO (myristicin, apiole), still remain to be evaluated. For a more accurate assessment these results should be also demonstrated and validated by appropriate *in vivo* assays. Hence, compounds displaying the highest inhibitory effect on AChE activity should be further investigated in order to ascertain if the inhibition is reversible and to distinguish the type of inhibitory action they exert (competitive, noncompetitive or uncompetitive) through the estimation of kinetic parameters. The implementation of further biological assessments namely as regards to other enzymatic systems would be an important step towards the elucidation of some MOA.

(v) As we progress in the nanotechnology era, a new generation of formulations is becoming available. The incorporation of EOs in micro and nano-sized delivery devices would be a useful strategy to increase the dispersity, wettability and/or penetration properties of EOs, enhancing their stability and biological efficacy (Koul, 2008). Several controlled-release systems, including nanotechnological devices, are been studied for pesticides applications (Lai et al., 2006; Chen et al., 2009; Yang et al. 2009; Seo et al., 20015) and we consider that in this context there are excellent opportunities for technological-based entrepreneurship linked to this knowledge area (startup companies developing a patented technology, such as Botanocap, are already in the market). Some efforts in the design and study of delivery systems at the micro and nanoscale are also underway with the aim to promote their gradual release and mitigated some of the limitations intrinsic to the use of EOs, such as their high volatility, degradation and lack of solubility in water (a brief overview of a part of the work is provided in appendix D).

The finding that some EOs may offer a range of applications rises some expectation. However, a great deal of fascinating biochemistry and applications remain to be discovered as regard to the study of Apiaceae species. At this stage, the attention may be focused on new biological assessments and on the improvement of products stability and release through the design and evaluation of micro- and nanoformulations. Both approaches will be decisive to consolidate the current results, conferring a more innovative and commercial dimension to this research project.

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APPENDICES

Supplementary information **| A**

Field condition and EOs characterization **| B**

Formulae **| C**

Additional/unpublished works **| D**

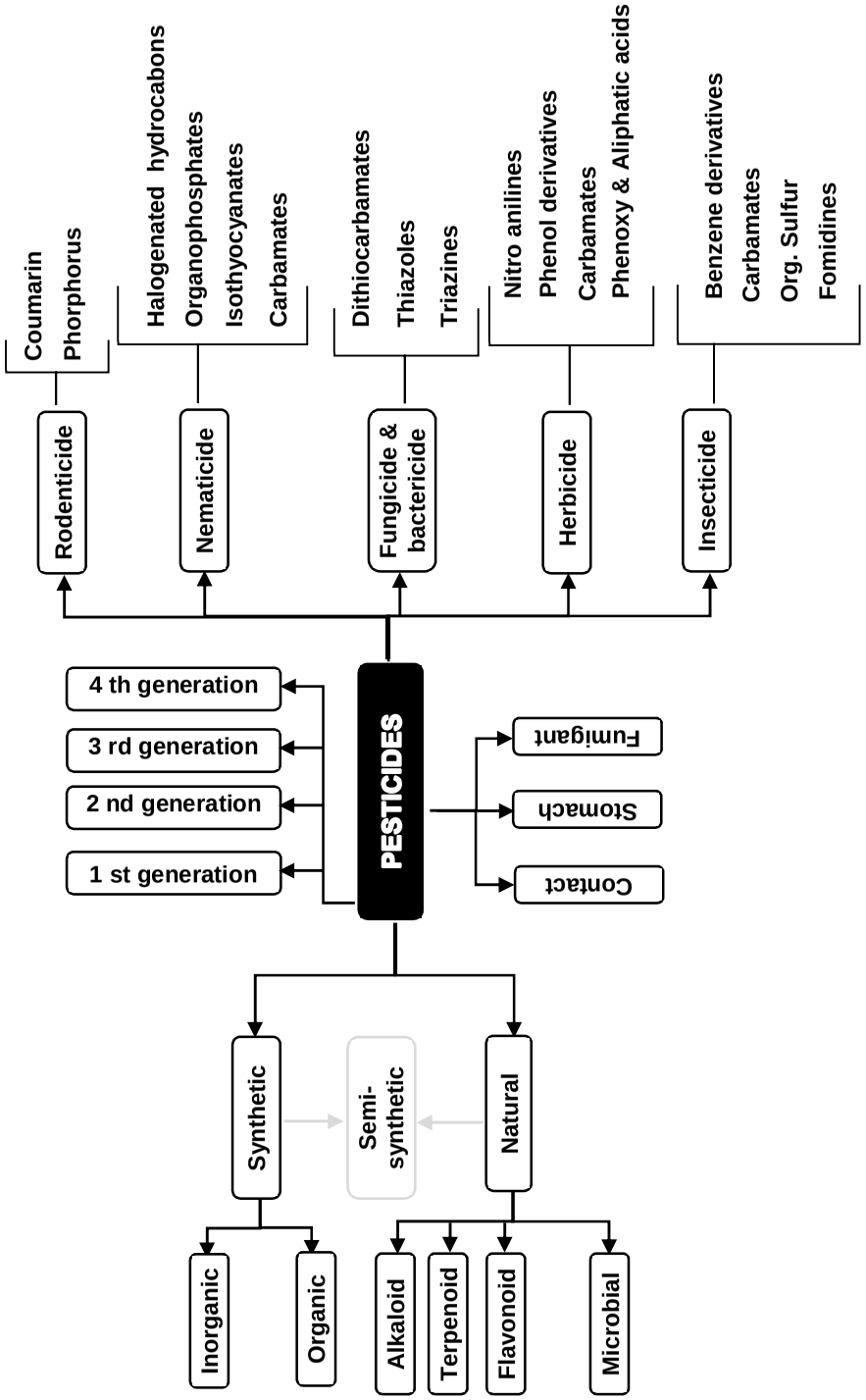


Figure A.1. Classifications of pesticides [Source: Srivastava et al. (2009)]

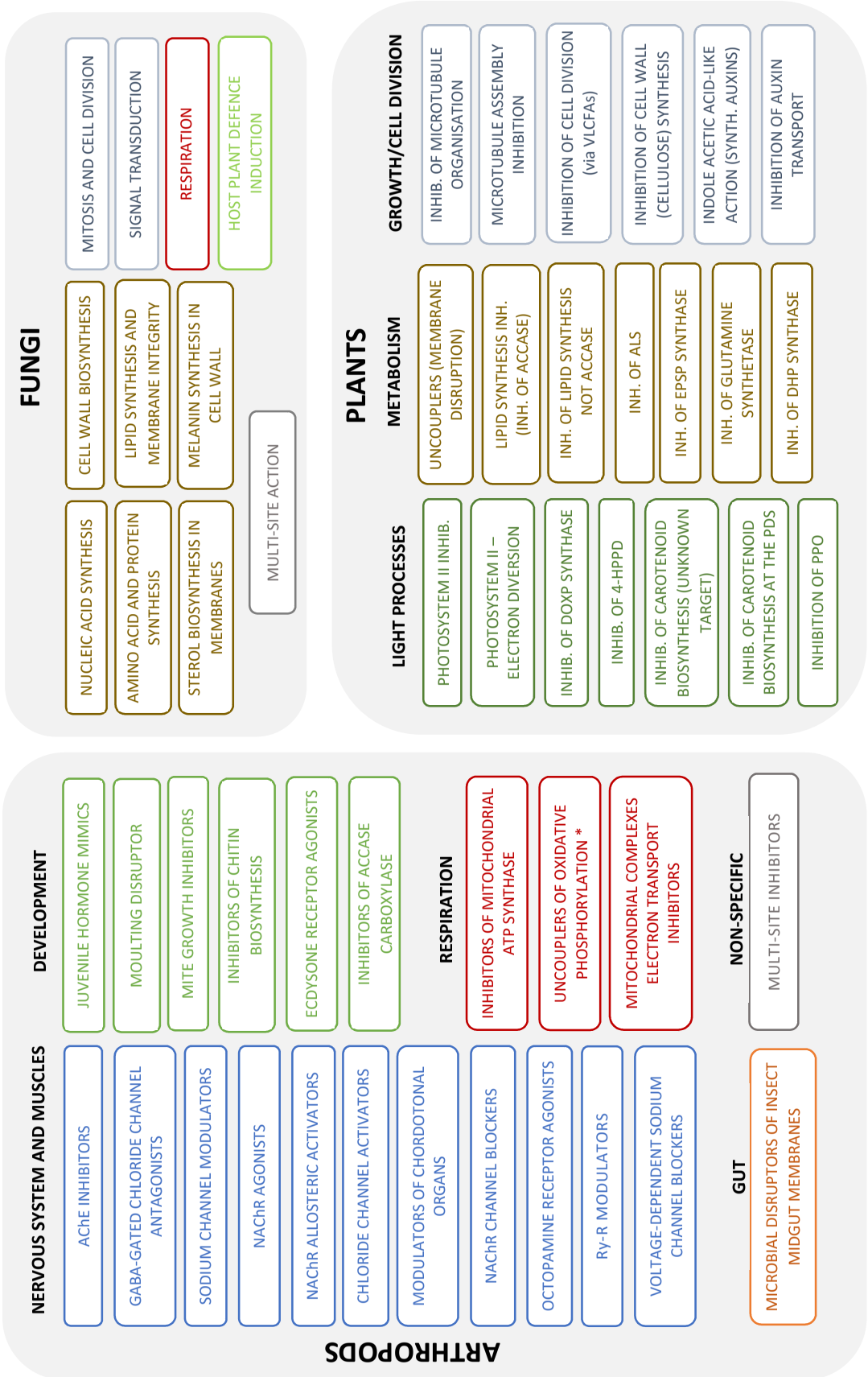

ARTHROPODS
NERVOUS SYSTEM AND MUSCLES
ACHE INHIBITORS
GABA-GATED CHLORIDE CHANNEL ANTAGONISTS
SODIUM CHANNEL MODULATORS
NACHR AGONISTS
NACHR ALLOSTERIC ACTIVATORS
CHLORIDE CHANNEL ACTIVATORS
MODULATORS OF CHORDOTONAL ORGANS
NACHR CHANNEL BLOCKERS
OCTOPAMINE RECEPTOR AGONISTS
RY-R MODULATORS
VOLTAGE-DEPENDENT SODIUM CHANNEL BLOCKERS
DEVELOPMENT
JUVENILE HORMONE MIMICS
MOULTING DISRUPTOR
MITE GROWTH INHIBITORS
INHIBITORS OF CHITIN BIOSYNTHESIS
ECDYSONE RECEPTOR AGONISTS
INHIBITORS OF ACCASE CARBOXYLASE
RESPIRATION
INHIBITORS OF MITOCHONDRIAL ATP SYNTHASE
UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION *
MITOCHONDRIAL COMPLEXES ELECTRON TRANSPORT INHIBITORS
NON-SPECIFIC
MULTI-SITE INHIBITORS
GUT
MICROBIAL DISRUPTORS OF INSECT MIDGUT MEMBRANES
FUNGI
CELL WALL BIOSYNTHESIS
LIPID SYNTHESIS AND MEMBRANE INTEGRITY
MELANIN SYNTHESIS IN CELL WALL
NUCLEIC ACID SYNTHESIS
AMINO ACID AND PROTEIN SYNTHESIS
STEROL BIOSYNTHESIS IN MEMBRANES
MULTI-SITE ACTION
MITOSIS AND CELL DIVISION
SIGNAL TRANSDUCTION
RESPIRATION
HOST PLANT DEFENCE INDUCTION
PLANTS
METABOLISM
UNCOUPLERS (MEMBRANE DISRUPTION)
LIPID SYNTHESIS INH. (INH. OF ACCASE)
INH. OF LIPID SYNTHESIS NOT ACCASE
INH. OF ALS
INH. OF EPSP SYNTHASE
INH. OF GLUTAMINE SYNTHETASE
INH. OF DHP SYNTHASE
LIGHT PROCESSES
PHOTOSYSTEM II INHIB.
PHOTOSYSTEM II – ELECTRON DIVERSION
INHIB. OF DOXP SYNTHASE
INHIB. OF 4-HPPD
INHIB. OF CAROTENOID BIOSYNTHESIS (UNKNOWN TARGET)
INHIB. OF CAROTENOID BIOSYNTHESIS AT THE PDS
INHIBITION OF PPO
GROWTH/CELL DIVISION
INHIB. OF MICROTUBULE ORGANISATION
MICROTUBULE ASSEMBLY INHIBITION
INHIBITION OF CELL DIVISION (via VLCFAs)
INHIBITION OF CELL WALL (CELLULOSE) SYNTHESIS
INDOLE ACETIC ACID-LIKE ACTION (SYNTH. AUXINS)
INHIBITION OF AUXIN TRANSPORT

Figure A.2 (of the previous page). Known mode of action (MOA) of insecticides, miticides, fungicides and herbicides [Sources: Metcalf (1948); Paranjape et al. (2014); IRAC, FRAC and HRAC-WSSA committees[1]]

*Via disruption of the proton gradient; ACCase: Acetyl Coenzyme A carboxylase; AChE: acetylcholinesterase; ALS: acetolactate synthase; DHP: dihydropteroate; DOXP: 1-deoxy-D-xylulose 5-phosphate; EPSP: 5-enolpyruvylshikimate-3-phosphate; GABA: γ-aminobutyric acid; 4-HPPD: 4-hydroxyphenyl-pyruvate-dioxygenase; PDS: phytoene desaturase; PPO: protoporphyrinogen oxidase; NAChR: nicotinic acetylcholine receptor; RyR: ryanodine receptor. VLCFAs: Very-long-chain fatty acids.

Box A.1. Classes of synthetic insecticides

Organochlorines (OC) were commonly used in the past as insecticides (e.g., DDT, aldrin, and dieldrin). They operate by disrupting the sodium/potassium balance of the nerve fiber. Many have been removed from the market because of unacceptable effects on wildlife, persistence in environment and strong evidence of their possible carcinogenic activity (Casida, 1980). Some organochlorine compounds such as pentachlorophenol (PCP) have been used as herbicide, insecticide, fungicide, algaecide, and so forth.

Organophosphates (OP) affect the nervous system by disrupting AChE the enzyme that regulates acetylcholine, a neurotransmitter. Most organophosphates are insecticides. Some are very poisonous (they were used in World War II as nerve agents). However, they usually are not persistent in the environment.

Carbamates affect the nervous system by disupting AChE an enzyme that regulates acetylcholine, but the effects are usually reversible. There are several subgroups within the carbamates, namely thiocarbamates and dithiocarbamates. These compounds are generally used as insecticide, acaricide and nematicide but some find application in the control of weeds.

Pyrethroids were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in plants of the genus *Chrysanthemum*. They have been modified to increase their stability in the environment and some of those are considerably divergent from the original pyrethrins. Some synthetic pyrethroids are toxic to insects, fish and aquatic animals but they are photolabile and not persistent (rarely present after a few days). Pyrethrins and synthetic pyrethroids acts over the nervous system at the neuronal membrane-level by keeping sodium channels opened which cause the loss of nervous impulse (knockdown) and eventually death (Sánchez-Bayo, 2012).

Neonicotinoids were introduced in the early 1990s and are now the most widely used insecticides in the world. They are neurotoxins, binding to nicotinic acetylcholine receptors (nAChRs) in the central nervous system and causing nervous stimulation at low concentrations but receptor blockage, paralysis and death at higher concentrations. Compounds can persist for years in soils. Being water soluble, neonicotinoids also contaminate freshwater and marine environment and groundwater (van der Sluijs et al., 2015; van Lexmond et al., 2015).

[1] Classification system for insecticides, fungicides and herbicides proposed by the respective following authorities: IRAC (http://www.irac-online.org/modes-of-action/), FRAC (www.frac.info/publications) and HRAC-WSSA committees (http://www.hracglobal.com/ and http://wssa.net/)

Table A.1. General principles of IPM concerning the sustainable use of pesticides. [a]

'Sustainable biological, physical and other non-chemical methods must be preferred to chemical methods if they provide satisfactory pest control.'
The decision of application plant protection measures should rely on an adequate monitoring of harmful species and *on the advice from professionally qualified advisors*.
'The pesticides applied should be as specific as possible for the target and shall have the least side effects on human health, non-target organisms and the environment.'
'The professional user should keep the use of pesticides to levels that are necessary … considering that they do not increase the risk of development of resistance in populations of harmful organisms.'
'*…. Anti-resistance strategies should be applied to maintain the effectiveness of the products*, including the use of multiples pesticides with different mode of action.'
'The professional user should check the success of the applied plant protection measures.'

[a] [Source: European Union (2009)] Retrieved from the annex III of the directive **2009/128/EC** of the European parliament and of the council, L309, p85.

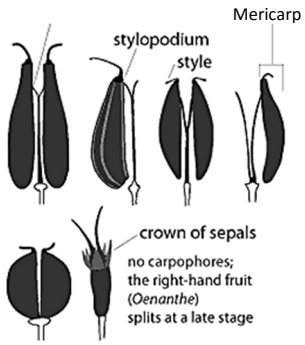
Table A.2. Substances and organisms authorized for use in organic farming for biological pest and diseases controls. [a]

Category	Type	Examples
I. Plant and animal substances	Natural plants preparations	
	Preparations on basis of pyrethrins	Extracts from *Chrysanthemum cinerariaefolium*
	Preparations on basis of rotenone	Extracts from *Derris elliptica*, *Lonchocarpus* spp.
	Preparations on basis of ryanoides	Extracts from *Ryania speciosa*
	Preparations on basis of quassinoids	Preparations from *Quassia amara*
	Preparations on basis of azadirachtin	Extracts from *Azadirachta indica* (neem tree)
	Tea from tobacco (excluding nicotine)	
	Plant and animal oils	Citronella oil, thymus oil, rosemary oil
	Sea products	Seaweed, seaweed meal, seaweed extracts, sea salts
	Propolis	
	Extract from Chlorella	
	Extract from mushroom	Shiitake fungus
	Fermented product	from *Aspergillus*
	Natural acids	Vinegar
	Proteins	Gelatin, lecithin, casein
II. Microorganisms and their substances	Bacteria, fungi and viruses	*Bacillus thuringiensis*, *Beauveria bassiana*, *Trichoderma* spp., Granulosis virus, etc.
	Avermectins	from *Streptomyces avermitilis*
	Spinosyns	from *Saccharopolyspora spinosa*
III. Mineral	Inorganic compounds	Bordeaux mixture, copper hydroxide, copper oxychloride
	Copper salts	
	Burgundy mixture	
	Sulphur	
	Mineral powders	Stone meal, silicates, clay
	Diatomaceous earth	
IV. Traps	Pheromone preparations	
	Preparations to repel higher animal species [b]	
V. Others	Homeopathic and Ayurvedic preparations	
	Herbal and biodynamic preparations	
	Sterilized insect males	
	Carbon dioxide and nitrogen gas	
	Potassium soap (soft soap)	
	Ethyl alcohol	

[a] [Source: Codex Alimentarius (2001)]; [b] Eventually containing metaldehyde, as far as applied only in traps.

Table A.3. Morphological description, reproductive characteristic and important genera of the family Apiaceae

Plants	Herbaceous or rarely shrubby
Stems	Stem often hollow or pith-filled stems
Leaves	Considerable variation in leaf morphology, even within the Apioideae subfamily. Mostly compound, alternate, lamina usually deeply dissected, pinnately divided. Leaf-sheath usually present, more or less developed. Many genera of the Apioideae exhibit interspecific transitions from pinnately lobed or divided leaves, with broad leaflets at one extreme to decompound leaves with linear or filiform leaflets at the other extreme (e.g., *Ammi, Apium, Ligusticum, Lomatium, Seseli*, and *Tauschia*, among many others).[b]
Inflorescences	Umbellate inflorescence, simple or compound. Rarely heads.
Flowers	Small, actinomorphic, pentamerous perianths and androecia. Frequently unequal in size with the largest ones on the centrifugal side of the outer umbellules. Calyx: absent or small sepals (5); Corolla: distinct petals (5), white, red-tinged, yellow or greenish yellow; Carpels and styles: bicarpellate gynoecia, (1-)2 styles that are fused at their base forming a nectar secreting disc (stylopodium); Ovary: inferior; Stamens: 5.
Fruit	Usually dry schizocarps, rarely drupes. Specialized fruits with or without wings. Presence or absence of surface hairs, ridges, bristles or spines. Mericarps are more or less compressed laterally or dorsally with longitudinal veins (ridges) on the abaxial side separated by valleculae. In some genera, immersed oil ducts (vittae) may be found in ripe fruits between the primary ridges on both side of the mericarp. Vittae and companion canals are schizogenous oil ducts (companion canals are associated with vascular bundles; vittae are located between vascular bundles).[b]
Reproduction	Some species have only bisexual flowers, many are andromonoecious (having both bisexual and male flowers on the same individual). Usually, primary and secondary umbels have more bisexual flowers, while male flowers are fewer (or even absent); male flowers prevail in the later developed tertiary umbels. Most species are insect-pollinated generalists visited by, e.g., coleopterans, dipterans and hymenopterans; in many species, umbellules have enlarged outer petals serving as means of attraction (thus mimicking single flowers); a few species are autogamous. Many fruits have traits which help them to be spread with wind (well-developed ridges or wings), water (large, air-filled spaces in the ridges), or animals (hooks, hooked spines or hairs) are frequent. A few species are adapted to self-dispersal (e.g., in *Scandix*).
Important genera [c]	*Eryngium* (250 species), *Ferula* (190 species), *Pimpinella* (150 species), *Angelica* (110 species), *Bupleurum* (100 species)
Deadly poisonous species	Fool´s parlsey (*Aethusa cynapium*), hemlock water-dropwort (*Oenanthe crocata*), poison hemlock (*Conium maculatum*) and water hemlock (*Cicuta virosa*).



[a] Apiaceae for Flora Nordica, URL: http://www.floranordica.org/Review/-Review_public/accounts/Apiaceae-x/Apiaceae-ram.html, accessed 2015, April 7; [b] Plunkett & Downie (1999); [c] Bennet (online).

Table A.4. Physical and organoleptic properties of pure compounds identified in Apiaceae essential oils.

Compounds	**Mw** ($g\ mol^{-1}$)	**Boiling point** (ºC) [a]	**Vapor pressure** ($mm\ Hg^{-1}$) [b]	**log P** (o/w) [b,c]	**Solubility in water** ($mg\ L^{-1}$) [b]	**Odor type and description**
trans-Anethole	148.21	234-237	0.069	3.390	98.7	Sweet, anise, liquorice
Apiole	222.24	294-295	0.003	3.481	27.7	Herbal, parsley faint
1-Allyl-2,3,4,5-tetramethoxybenzene	238.28	307-309	0.001	2.427	78.8	–
Carotol	222.37	295-296	0.00015	4.465	8.5	Pleasant mild
(+)-(*S*)-Carvone	150.22	231	0.066	3.070	367.1	Spicy, warm, caraway-like
O-Cymene	134.22	177-179	1.500	4.380	23.3	–
p-Cymen-8-ol	150.22	–	0.020 (20.0 ºC)	–	–	Fruity
p-Cymen-7-ol	150.22	246-248	0.012	2.370	–	strong cumin-like, spicy, herbal
Cuminaldehyde	148.21	235-236	0.065	3.170	282.0 (20 ºC)	Spicy, cumin-like, green
Dillapiole	222.24	285-292	0.006	2.343	27.7	Woody-spice
Dill ether	152.24	207-208	0.331	2.576	184.4	Ethereal, floral, herbaceous, minty
α-*p*-Dimethylstyrene	132.21	186-189	0.834	3.782	35.29	Phenolic, spicy
Elemicin	208.26	–	0.007	2.298	132.2	Spicy, floral
Estragole	148.21	215-216	0.210	3.151	84.6	Anise, spice, green
Falcarinol	244.38	368-370	–	6.257	0.748	–
(+)-(1*S*)-Fenchone	152.24	192	0.463	2.089	73.1	Herbal, warm, sweet, woody
(+)-(*R*)-Limonene	136.24	176-177	1.541	4.581	4.38	Citrus-like
(-)-(*S*)-Limonene	136.24	176-177	1.541	4.380	4.58	Terpenic, pine, herbal, peppery
p-Mentha-1,3-diene-7al	150.22	234-235	0.510	2.772	124.6	Fatty, spicy
1,3,8-*p*-Menthatriene	134.22	196-197	0.555	4.031	2.29	Turpenic, camphor, herbal, woody
Myrcene	136.24	176-177	2.29	4.170	6.92	Terpenic, spicy, green, woody
Myristicin	192.21	–	0.008	2.586	46.43	Spicy, warm, woody, nutmeg-like
(+)-(*S*)-α-Phellandrene	136.24	175-176	1.856	4.408	2.862	Weak herbaceous
(-)-(*R*)-α-Phellandrene	136.24	171-173	1.856	4.408	2.862	Terpenic, medicinal
β-Phellandrene	136.24	171-174	1.573	4.386	2.45	Minty, terpenic
(+)-α-Pinene	136.24	155-156	3.489	4.440	4.071	Harsh, terpenic, minty
(+)-β-Pinene	136.24	164-165	2.399	4.366	7.061	Sweet, fresh, pine, woody
(-)-β-Pinene	136.24	–	–	–	–	Dry, terpenic, woody, resinous
α-Terpinene	136.24	173-175	1.638	4.250	5.915	Woody, terpenic, citrusy
γ-Terpinene	136.24	181-183	1.075	4.500	–	Terpenic, sweet, citrusy
α-Terpineol	154.25	136-137	0.028	2.670	–	Pine, terpenic, woody, floral

[a] At 1 atm of pressure; [b] Physical properties measured at 25 ºC; [c] Partition coefficient in oil and water.
The presented data were obtained from the website **http://www.thegoodscentscompany.com** [Accessed May, 2015].

ANETHUM GRAVEOLENS L.

Synonyms: *Anethum sowa* Roxb. ex, Fleming; *Peucedanum graveolens* (L.) C.B. Clarke; *Peucedanum sowa* (Roxb. ex Fleming) Kurz

ITS AGROINDUSTRIAL VALUE

Dill plant has long been known as a popular spice crop and seasoning herb. It is cultivated worldwide, namely in India, Pakistan, Egypt, Hungary, Germany, Mexico and USA (Gupta et al., 2012). Most dill seed exported internationally is of the subsp. *sowa* (Small, 2006).

Young chopped leaves are used as seasoning herb in salads, soups, sauces, fish and seafood dishes (Huopalahti & Linko, 1983). Dry seeds are a common ingredient of pickles, liqueurs and pastries (Góra et al., 2002; Gupta et al., 2012).

Dillweed and dillseed EOs are valuable flavoring agents used for pickling and beverages preparation (Callan et al., 2007). The fruits EO and oleoresin find also some application in the cosmetic and pharmaceutical industries (Kapoor et al., 2002; Gupta et al., 2012).

EOs PRODUCTION

EO occurs in all vegetative organs, mainly in the blooming umbels and fruits (2.5 - 4 %) (Góra et al., 2002).

A low density planting or a late harvest is suitable to obtain dill seed with a high-carvone content (Callan et al., 2007).

It was estimated that 114 and 23 tons of EOs from dill leaves and seeds, respectively, are annually produced worldwide (Douglas et al., 2005).

ETHNOBOTANICAL USES

Dill is traditionally used in the ailments of children and women (Gupta et al., 2012) and to relief digestive disorders, namely dyspepsia, flatulence, stomach and colic pain (da Cunha et al., 2007; Gupta et al., 2012). The Ayurveda medicine, used dill as stomachic, galactogogue, diuretic and carminative (Jana & Shekhawat, 2010).

A SOURCE OF SECONDARY METABOLITES

Umbels with unripe seeds contain (R)-limonene, (S)-carvone, α-phellandrene, and dillapiole as major odorant compounds (Fig. A.4) (Góra et al., 2002; Kapoor et al., 2002). Beside the volatile terpenoids found in EOs, fruits and leaves contain flavonoids, xantones, coumarins, triterpenes and polyacetylenes (da Cunha et al., 2007).

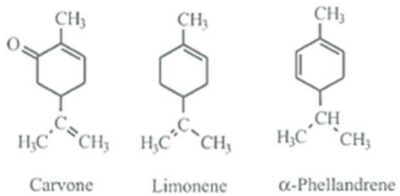


Figure A.4. Predominant monoterpenes of dill fruit EO.



Figure A.5. Dill fruit.

Drawing by Osterkamp M., from http://www.floranordica.org/Review/-ill-html-filer/ill_Anethum-Foeniculum.html

PLANT DESCRIPTION



Figure A.3. *A. graveolens* in the fruit growing stage

Probable origin and distribution: SW Asia or SE Europe. It grows wild in Spain, Portugal and upon the coast of Italy (de Carvalho & da Fonseca, 2006).

Climate and soil conditions: Grows well under warm/cool weather (from 7 to 30 ºC). Tolerates dry conditions. Requires well-drained soils with a good fertility level.

Life cycle: Annual herbaceous plant (Fig. A.3) with a culture cycle of 130-150 days (Gupta et al., 2012). Seeds may be harvested 45-70 days after anthesis, when mature (Boonsong & Sutevee, 2008).

Vegetative features: Herbaceous plant (grows up to 90-120 cm). *Stems*: erect, hollow, finely-ridged. *Leaves*: blue-green, alternate, 3 to 4-fold pinnate and feathery-like; sheaths well-developed.

Inflorescences and flowers: Compound umbels (2-9 cm diam.) with 15 to 30 umbellets. *Flowers*: Small, corolla yellow. Bracts absent. Polygamous species (Gupta et al., 2012).

Fruits: schizocarpic, light brown, ovoid (5-6 cm length). Mericarps are winged and flattened with ridged and oil ducts (Fig. A.5).

CUMINUM CYMINUM L.

Synonyms: *Cuminum odorum* Salisb.; *Cuminum sativum* J. Sm.; *Cuminia cyminum* J. F. Gmel.; *Selinum cyminum* E.H. Crantz

ITS AGROINDUSTRIAL VALUE

Cumin is one of the oldest and most important spices (Kafi et al., 2006). There are three types of cumin seeds in the market (Indian, Iranian and Middle Eastern) which differ in the color, flavor and EO content (Amin, 2012). Cumin is cultivated in several countries worldwide. India and Iran are the major producers and exporters (Lim, 2013).

Dry fruits are traditionally consumed as condiment in India and Middle Easter countries, being a key ingredient in curry powders and a variety of dishes (soups, rice, sausages, stews, pickles, cheese, grilled meat, bread and biscuits) (Behera et al., 2004; Lim, 2013). It is also employed as a flavoring ingredient and food preservative (Amin, 2012), and the EO is used in trace amounts in perfumes (Beis et al., 2000).

EOS PRODUCTION

Cumin fruit is highly aromatic and its EO content varies from 2 to 5 % (Behera et al., 2004) while roots, stems and leaves, and flower accumulate much lower content (0.03, 0.1 and 1.7%) (Bettaieb et al., 2010).

Around 15 tons of cumin fruit EO, estimated at 0.9 Million US$, are produced annually (Douglas et al., 2005).

Figure. A.7. Cuminaldehyde, the chief oxygen-containing monoterpene produced by cumin plant.

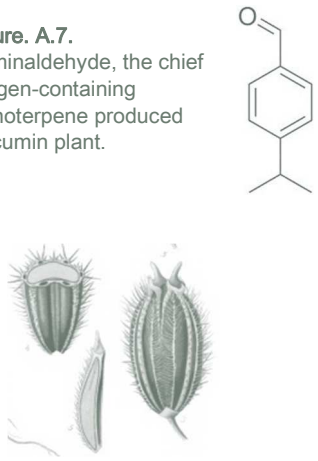


Figure A.8. Fruits of cumin. *Illustration from Koehler's medicinal-plants (1887).*

A SOURCE OF SECONDARY METABOLITES

Cuminaldehyde is the main component of herb (40.5 %) and seed (53.6 %) EO (El-Sawi & Mohamed, 2002) (Fig. A.7). Fruits EO may contain substantial amounts of β-pinene, p-cymene, terpinolene, terpinen-7-al, safranal, acoradiene, *etc*. (Baser et al., 1992; El-Sawi & Mohamed, 2002; Hashemi et al., 2008).

The several parts of the plant accumulate flavonoids, phenolic acids and tannins in different amounts (Bettaieb et al., 2010).

ETHNOBOTANICAL USES

The plant has a long history of therapeutic uses in Asia, namely for the treatment minor digestive disorders (stomach, colic, dyspepsia and diarrhea, *etc*.) and as a stimulant of appetite (Behera et al., 2004; Lim, 2013). Cumin fruit is reported to be antispasmodic, carminative, anti-inflammatory, galactogogue, antimicrobial and aphrodisiac (Amin, 2012; Lim, 2013).

PLANT DESCRIPTION



Figure A.6. *C. cyminum* plant with infrutescences. *Illustration from Bentley R. & Trimen H., Medicinal Plants (1880).*

Origin and Distribution: Probably native to the eastern Mediterranean and SW Asia. Widely cultivated.

Climate and soil conditions: Requires warm temperatures (25-30 ºC). Grows better on well-drained sandy loam to loamy soils. Tolerate semi-arid areas.

Life cycle: Annual, about 120 days.

Vegetative features: Herbaceous plant growing to a height of 15-50 cm. *Stem*: Glabrous, divided in uniform secondary branches. *Leaves*: alternate, narrow (4-6 cm long), 2-3-pinnatisect, thread-like and deep green colored.

Inflorescences and flowers: Small compound umbels (5-7 umbellets), with bracts and bracteoles. *Flowers:* Slender covered with tiny or longer hairs; petals either white or pink (Fig. A.6) (Amin, 2012).

Fruits (Fig. A.8)**:** Schizocarpic, lateral fusiform to ellipsoid (3-6 mm long), yellowish-brown, with 9 longitudinal pale ridges. Paired or separate carpel (Amin, 2012; Lim, 2013).

Note: The species is susceptible to frosting, pests and diseases.

FOENICULUM VULGARE Mill.

Synonyms: *Foeniculum dulce* Mill.; *Foeniculum azoricum* Mill.; *Anethum foeniculum* L.; *Anethum dulce* DC.; *Meum foeniculum* (L.) Spreng.

ITS AGROINDUSTRIAL VALUE

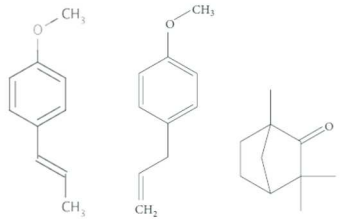
This aromatic and medicinal plant is a traditional and popular herb used since ancient times. It is widely cultivated worldwide, namely in India, Egypt, Turkey, Greece, Spain and France (Hunault et al., 1989).

Wild and cultivated fennel has different size, odor, taste, quality and yield potential (Ehsanipour et al., 2012). The var. *azoricum* (Florentine fennel) is cultivated for its bulb and used as a vegetable. The bulb, shoots, foliage, and fruits are edible and added to many culinary preparations (fish and sea food dishes, salads, *etc*.) as seasoning herb, spice or garnish (Lim, 2013). Inflorescences and fruits of sweet fennel (var. dulce) give a pleasant anise-flavor to alcoholic beverages, herbal tea, sweets, *etc*. (Lim, 2013). Dry seeds, EO and its constituent *trans*-anethole are important ingredients of the flavor and fragrance industry (da Cunha et al, 2007).

EOs PRODUCTION

EO occurs in all plant parts, more abundantly in fruits, which contain about 0.7-6.0 % of volatile oil depending on the genotypes. The EO content from commercial fruits is variable (5-51 mL kg^{-1}) (Raal et al., 2011).

It was estimated that 255 and 28 tons of EO, valued at US$ 8.4 Million, are annually produced worldwide from sweet fennel and bitter fennel fruits, respectively (Douglas et al., 2005).



trans-Anethole *Estragole* *Fenchone*

Figure A.10. Chief volatile constituents of fennel.

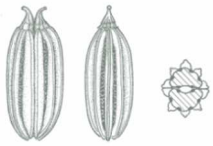


Figure A.11. Fennel fruit.

Drawing by Osterkamp M., from http://www.floranordica.org/Review/-ill-html-filer/ill_Anethum-Foeniculum.html

A SOURCE OF SECONDARY METABOLITES

trans-Anethole, estragole, fenchone and limonene are the major EO constituents of the *dulce* and *vulgare* variety (da Cunha et al., 2007) (Fig. A.10). Fennel bulb and fruits are rich in vitamins and dietary fiber (Lim, 2013; Badgujar et al., 2014). Fruits contain coumarins, phenolic acids, flavonoids, phytosterols and triterpenoids (Tanira et al., 1996; Badgujar et al., 2014 and ref. therein).

ETHNOBOTANICAL USES

Fennel has been used for more than forty types of disorders (Badgujar et al., 2014). The plant has several digestive properties (Ehsanipour et al., 2012) and the fruit is widely utilized in folk medicine systems as carminative, analgesic, antipyretic, expectorant, anti-inflammatory, antispasmodic, antimicrobial, emmenagogue diuretic and galactogogue (Tanira et al., 1996; da Cunha et al., 2007).

PLANT DESCRIPTION



Figure A.9. *F. vulgare* var. *vulgare* leaves and umbellets with flowers and fruits.

Origin/distribution: Native to the southern Mediterranean region. Worldwide naturalized.

Climate and soil conditions: Adapted to sub-temperate climates. Grows well in moderately fertile soil with good light exposure.

Life cycle: Hemicryptophyte, branching perennial or biennial herb.

Vegetative features: upright plant, quite variable in size (up to 2 m).

Stem: Erect, branched, glaucous, glabrous solid or secondarily hollow. *Leaves:* triangular-ovate shape; up to 15 cm; threadlike segments; 2-4 times pinnatisecta. Petiole (7-14 cm) with broad sheath at base (Fig. A.9) (Lim, 2013).

Inflorescences and flowers: Compound, slightly convex umbels, 4-10 cm across; with 4 to 30 umbellets (Fig. A.9). Bracts and bractlets absent. *Flowers:* hermaphrodite, protrandrous with yellow corolla.

Umbels of the subsp. *vulgare* have more than 10 rays, whereas subsp. *piperitum* have less.

Fruits (Fig. A.11): Schizocarpic, ovoid-oblong, brown or green. Paired mericarps (4-10 mm long) with 5 prominent ridges and 6 vittae (da Cunha et al., 2007).

Note: *Occurrence of polymorphism within the species and/or variety.*

PETROSELINUM CRISPUM (Mill.) Nym ex Hill.

Synonyms: *Petroselinum sativum* Hoffm.; *Petroselinum hortense* Hoffm.; *Apium petroselinum* L.

ITS AGROINDUSTRIAL VALUE

Parsley is cultivated as an annual culinary herb in Europe and Western Asia. The varieties crispum and neapolitanum are traditionally used for their foliage, whereas the turnip rooted or Hamburg type parsley (var. *tuberosum*) is produced for its edible root (Charles, 2012). Chopped leaves are consumed either fresh or dried, as a garnish or condiment (Petropoulos et al., 2008), and are part of the gastronomic tradition of many countries.

EO is obtained from both seeds and leaves and is used as a food flavoring agent or as a fragrance in perfumery and cosmetics (Kurowska & Gałązka, 2006; Azzeez & Parthasarathy, 2008).

EOS PRODUCTION

The EO is present in all parts of the plant. Usually commercial EO is derived from mature seeds (fruits) and has a distinct flavor from the oil extracted from the foliage (Kurowska & Gałązka, 2006; Charles, 2012). Herb oil of the finest quality has a flavor that resembles the fresh herb but EO yield from this tissue (≤ 0.4 %) (Charles, 2012) is low compared to fruits (2-6 %) (da Cunha et al., 2007).

The EO composition is influenced by plant type, tissue, growth stage, climate, season and hydric stress (Petropoulos et al., 2004 and 2008).

The annual production of parsley EO from aerial parts and fruits (total of 12 tons) was estimated at US$ 1.7 Million (Douglas et al., 2005).

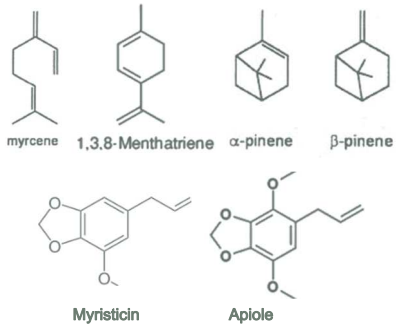


Figure A.13. Chief compounds of parsley EOs.

ETHNOBOTANICAL USES

Preparation from parley fruits, leaves and roots are used in modern herbal medicine to treat gallstones, kidney stones, urinary infections, menstrual disorders, water retention, coughs, gout, rheumatism and arthritis. In Ayurvedic medicine, it is used as a diuretic and expectorant, and as a remedy for gastric disorders (Charles, 2012).

A SOURCE OF SECONDARY METABOLITES

Parsley produces several monoterpenes and volatile phenylpropene (Lamarti et al., 1993) (Fig. A.13), vitamins (Azzeez & Parthasarathy, 2008), furanocoumarins (Beier et al., 1994), flavonoids, including flavones glycosides (i.e, apiin, 6''-Acetylapiin) (Yoshikawa et al., 2000).

The plant is a source of several unusual compounds, namely the aristolane sersquiterpenoids, crispane and crispanone (Appendino et al., 1998), the monoterpene petranol and its glycoside (petroside) (Yoshikawa et al., 2000) and the halogenated furocoumarin, saxalin (Beier et al., 1994).

PLANT DESCRIPTION



Figure A.12. Italian parsley (*P. crispum* var. *neapolitanum*). Mature fruits and young plant with green foliage.

Origin and Distribution: Origin uncertain (SE Europe or SW Asia). Cultivated and escaped in almost the whole temperate region.
Climate and soil conditions: It grows in drained, rich and moist soils.
Life cycle: Hemicryptophyte
Vegetative features: Heterophyllous, much branched plant, upright, growing to 1 m tall. *Stems*: Erect, glabrous, angular (Charles, 2012), slightly glaucous, solid or often hollow. *Leaves*: Dark green, stalked, 3-pinnate, with triangular outline and narrow sheathing bases. Large basal leaves and narrower upper leaves. Petiole 4–9.5(–15) cm. [2]
Inflorescences and flowers: Compound slightly convex umbels (3.5–6.5 cm wide) with (5–)10–19 umbellets; rays and pedicels straight and glabrous. [15] Bracts and bractlets persistent. *Flowers*: Greenish yellow petals, (10–)15–28 per umbellet.
Fruits: Schizocarpic, ovoid, laterally compressed (Charles, 2012); carpophore divided (Fig. A.12). Greenish, brown mericarps (1.9–2.7 mm long) with 5 fine ridges and dark-brown vittae. [2]

[2] http://www.floranordica.org/Review/-Review_public/accounts/Apiaceae-x/Apiaceae-ram.html

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Appendix B

Table B.1. Information concerning the experimental field location.

Region	Northern of Portugal
Geographic coordinates	41°27'04.5"N; 8°36'14.5"W
Shorter distance from the Atlantic coast	15 km
Altitude	~ 170 m

Table B.2. Average temperatures and total rainfall registered in the geographic zone of the experimental field during the period of cultivation. [a]

Months	Temperatures (º C)				Total precipitation (mm)	
	2010		2011		2010 [f]	2011 [g]
	Min. [b]	Max. [c]	Min. [d]	Max. [e]		
January	4.5-6.0 *	13.0-15.0	6.0-8.0	12.0-14.0	150-200	200-300 §
February	5.0-7.0	≥ 14	4.0-6.0 *	14.0-16.0	150-200	150-200
March	6.0-8.0	15.0-17.0	6.0-8.0	16.0-18.0	150-175	50-100
April	9.0-11.0	20.0-22.0	10.0-12.0	22.0-24.0	50-75	25-50
May	9.0-11.0	21.0-23.0	12.0-14.0	24.0-26.0	45-60	50-100
June	12.0-14.0	23.0-25.0	12.0-14.0	24.0-26.0	40-60	1-5 *
July	14.0-16.0	27.0-29.0	12.0-14.0	24.0-26.0	2-4 *	10-25
August	14.0-16.0	28.0-30.0 §	14.0-16.0	26.0-28.0 §	2-5 *	25-50
September	12.5-14.0	25.0-26.5	12.0-14.0	24.0-26.0	10-20	25-50
October	10.5-12.0	20.0-21.5	-	-	250-350 §	-
November	5.5-7.5	14.0-16.0	-	-	≥ 150	-
December	4.0-6.0 *	13.0-15.0	-	-	175-200	-

***** Lowest values; § Highest values; [a] The presented data were obtained from graphs available in the Portuguese Institute of Sea and Atmosphere (IPMA) website.

[b] https://www.ipma.pt/pt/oclima/monitorizacao/index.jsp?selAno=2010&selAna=me&selVar=tn&selTipo=m&print=true

[c] https://www.ipma.pt/pt/oclima/monitorizacao/index.jsp?selAno=2010&selAna=me&selVar=tx&selTipo=m&print=true

[d] https://www.ipma.pt/pt/oclima/monitorizacao/index.jsp?selAno=2011&selAna=me&selVar=tn&selTipo=m&print=true

[e] https://www.ipma.pt/pt/oclima/monitorizacao/index.jsp?selAno=2011&selAna=me&selVar=tx&selTipo=m&print=true

[f] https://www.ipma.pt/pt/oclima/monitorizacao/index.jsp?selAno=2010&selAna=to&selVar=rr&selTipo=m&print=true

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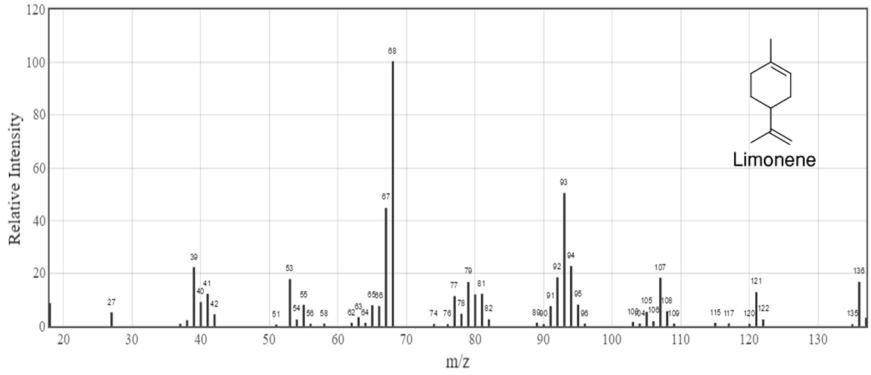
Table B.3. Composition of *Foeniculum vulgare* subsp. *vulgare* var *vulgare* essential oils isolated from leaves more stems and green infrutescences collected in 2010 from a wild population located in *S. Paio de Merelim*, Braga (Portugal)

	KI			Relative content (%) [d]	
Compounds [a]	Rtx-5	TR-5	Ref.	**Lv &St** 06-2010	**Green infrut.** 10-2010
Tricyclene [b,c]	—	916	926	0.1	—
α-Tujene [b,c]	925	925	931	0.1	tr
α-Pinene [b,c]	932	942	939	5.8	3.1
Camphene [b,c]	—	950	953	tr	tr
Sabinene [b,c]	966	979	975	1.0	0.5
β-Pinene [b,c]	971	977	980		0.6
Myrcene [b,c]	977	996	990	2.1	0.8
α-Phellandrene [b,c]	994	1014	1002	13.7	4.4
α-Terpinene [b,c]	1009	1020	1016	1.6	0.3
ortho-Cymene [b,c]	—	1014	1026	0.5	tr
β- Phellandrene [b,c]	1019	1035	1030	7.1	2.6
Z-β-Ocimene [b,c]	1023	1040	1037	0.1	0.2
E-β-Ocimene [b,c]	—	1048	1050	tr	0.2
γ-Terpinene [b,c]	1050	1059	1062	0.1	0.1
Fenchone [b,c]	1093	1091	1087	8.8	11.8
Isopentyl isovalerate [b,c]	1104	1103	1105	tr	tr
exo-Fenchol [b,c]	1114	1113	1117	—	0.1
***allo*-Ocimene** [b,c]	1128	1127	1129	1.6	0.1
Camphor [b,c]	1141	1136	1146	0.1	—
Terpinen-4-ol [b,c]	1169	1167	1177	tr	—
Estragol [b,c]	1200	1200	1198	28.2	59.2
endo-Fenchyl acetate [b,c]	1226	1226	1221	tr	—
cis-Anethole [b,c]	1255	—	1258	0.2	tr
***trans*-Anethole** [b,c]	1298	1288	1287	27.7	16.2
Geranyl acetate [b,c]	1381	1380	1382	0.1	—
Methyl eugenol [b,c]	1400	1400	1402	—	tr
E-Caryophyllene [b,c]	1423	1421	1417	0.1	—
Germacrene D [b,c]	1464	1472	1480	tr	—
Myristicin [b,c]	1522	1517	1523	0.1	tr
Elemicin [b,c]	1559	1560	1556	tr	tr
Identified content % (± SD)				**99.2** (±0.02)	**99.9** (±0.03)

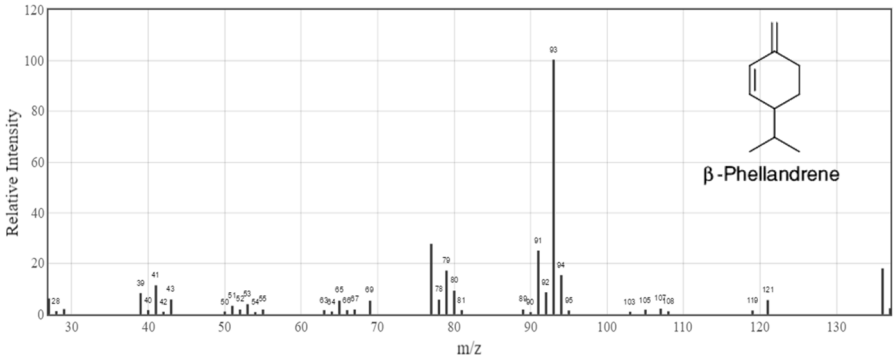
[a] Compounds listed by order of elution on both nonpolar capillary columns. Rtx-5 and TR-5 (fused silica capillary columns coated with 5 % phenyl methyl polysiloxane); [b] Identification based on peaks mass spectra (MS) comparison with MS available in the NIST library and data bases (Wiley, Mainlib, tr, etc.); [c] Identification confirmed by Kovats indices calculation and comparison with those reported in the literature and in the online databases (http://webbook.nist.gov/ and http://www.pherobase.com/kovats/) for equivalent stationary phases; [d] Mean values of relative peak areas based on three or more injections.

KI- Kovats retention indices determined for a Rtx-5 capillary column and reference KI found in the literature and databases; *St and Lv*- stems with leaves; *Infut.*- green infrutescences; tr- Trace amount with relative content below 0.05 %; — - Not detected.

Table B.4. Comparison of limonene and β-phellandrene mass spectra (MS)[a] and their Kovats retention indices.



KI	Column	Authors
1028	DB-5MS	Angioni, et al., 2006
1031	DB-5	Adams, 1995
1031	RTX-5	dos Santos et al., 2015



KI	Column	Authors
1031	DB-5	Adams, 1995
1033	DB-5MS	Angioni et al., 2006
1035	RTX-5	Masanetz & Grosch, 1998

[a] Mass Spectra retrieved from the NIST Chemistry WebBook at: http://webbook.nist.gov/chemistry

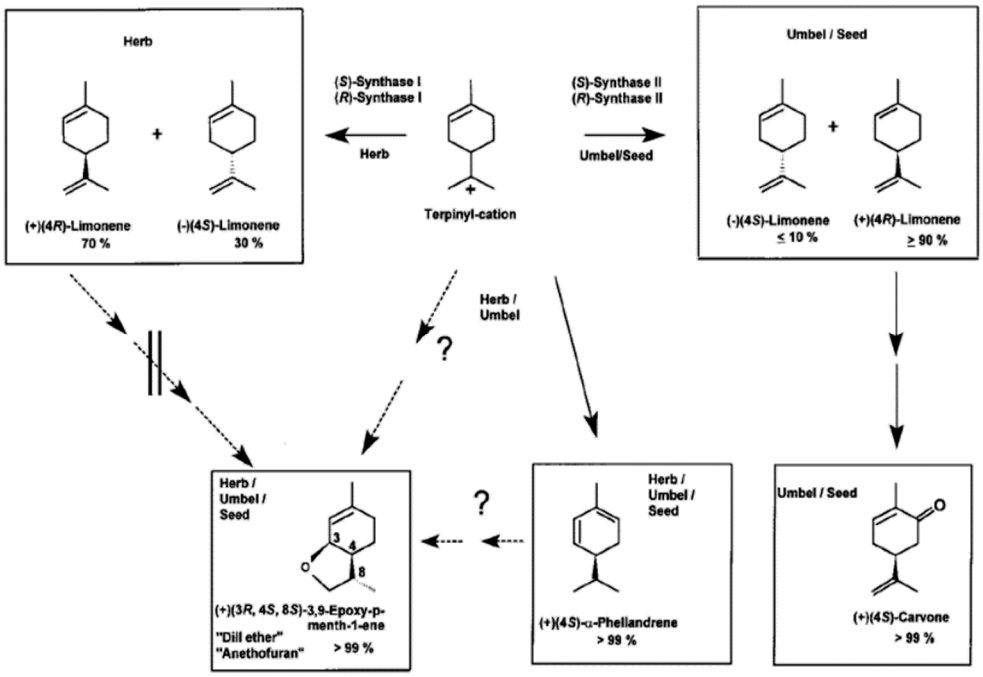


Figure B.1. Biosynthesis of monoterpenes and their enantiomeric forms in *Anethum graveolens* organs. [Source: Faber et al. (1997)]

The presented biochemical pathway was postulated by Faber and collaborators (1997) with basis on their investigation and a previous biochemical study concerning monoterpenes formation in dill (Bouwmeester et al., 1995).

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Appendix C

(1) **Calculation of the Kovats retention indices:**

For temperature programmed chromatography (Shibamoto, 1987)

$$KI = 100\,n + 100\frac{t'R(x) - t'R(n)}{t'R(n+1) - t'R(n)}$$

where $t'R(x)$ is the substance x retention time, $t'R(n)$ and $t'R(n+1)$ are the retention times of the *n*-alkanes with n *and* $n+1$ carbon atoms and eluting immediately before and after x, respectively.

(2) **Calculation of the column void volume (V_0)**

For gravity column chromatography based on the column dimensions

$$Vo = {}^{1}/_{4}\ (\pi d^2 \varepsilon h)$$

where V_0 is the void volume of the column (mL), d is the diameter of the column (2.3 or 2.5 cm), ε is the porosity of the silica (0.71-0.78 mL g^{-1}), and h is the length of the column (18-22 cm).

(3) **Abbott' s formulae** (Abbott, 1925)

Correction of mortality data with relation to the negative control

$$\text{Mortality}\ (\%) = 100\left(\frac{Obs.\ Mortality\ - mortality\ control}{100 - Mortality\ control}\right)$$

with all mortality values being expressed in percentage.

(4) **The Feeding Deterrence Index** – According to Huang et al. (1997)

$$\text{FDI}\ (\%) = [(C - T)/(C)] \times 100$$

where *C* is the weight of diet consumed in control and *T* is the weight of the diet consumed in the treatment, both being expressed as dry weights.

(5) **The Growth inhibition** – According to El-Aswad et al. (2003)

$$\text{GI}\ (\%) = [(C_L - T_L)/(C_L)] \times 100$$

where C_L is the mean larval weight gain in the control and T_L is the mean larval weight gain in the treatment, both being expressed as dry weights.

Nutritional indices - According to Waldbauer (1968) and modified by Scriber & Slansky Jr. (1981)

(6) Approximated Digestibility (%): $\text{AD} = 100\ (\text{D} - \text{F})\ /\ \text{D}$

(7) Efficiency Conversion Index of Ingested food (%): $\text{ECI} = 100\ \text{L}\ /\ \text{D}$

(8) Efficiency Conversion Index of Digested food (%): $\mathrm{ECD} = 100\ \mathrm{L} / (\mathrm{D} - \mathrm{F})$

(9) Metabolic Cost (%): $\mathrm{MC} = 100 - \mathrm{ECD}$

(10) Relative Metabolic Rate (mg mg^{-1} day^{-1}): $\mathrm{RMR} = \mathrm{M} / I \times t$

(11) Relative Growth Rate (mg mg^{-1} day^{-1}): $\mathrm{RGR} = \mathrm{L} / I \times t$

(12) Relative Consumption Rate (mg mg^{-1} day^{-1}): $\mathrm{RCR} = \mathrm{D} / I \times t$

Where t is the duration of the experimental period, $\mathbf{D}$ is the mean dry weight of consumed diet during t, $\mathbf{L}$ is the mean dry weight gain of larvae during t, I is the mean dry weight of larvae, $\mathbf{F}$ is the dry weight of feces produced during t and $\mathbf{M}$ is the metabolized food during t ($\mathrm{M}=\mathrm{D}-\mathrm{F}-\mathrm{L}$). All calculations were performed on a dry weight basis.

(13) **Inhibition of the AChE activity** - According to Ellman's colorimetric assay (1961)

$$\text{Inhibition } (\%) = 100 - [100\ (\frac{Vmax\ \ treatment}{Vmax\ \ control})]$$

were $Vmax$ *is* the reaction rate determined for each concentration of the substance been tested and for the control.

$Vmax$ is estimated using the following equation:

(14) $$Vmax = \frac{\Delta Abs}{time}$$

were ΔAbs is the variation of the absorbance measured at 415 nm during the time of reaction (0, 150, 300 and 450 s).

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Larvicidal activity of EOs from *Anethum graveolens*, *Cuminum cyminum* and *Petroselinum crispum* and some constituents against *Anopheles atroparvus*

Overview

In line with the study developed with bitter fennel (*Foeniculum vulgare* var *vulgare*) EOs and their two major phenylpropanoids (*trans*-anethole and estragole) against the mosquito species *A. atroparvus* (Chapter III and the corresponding publication Sousa et al., 2015), we have equally appraised the larvicidal potential of EOs from the three other species of the Apiaceae family encompassed in this work: dill (*A. graveolens*), parsley (*P. crispum*) and cumin (*C. cyminum*). The results of this research conduct in the context of the PhD thesis were presented as a poster communication at the 43rd International Symposium of Essential Oils (ISEO2012, Faculty of Sciences, University of Lisbon, Portugal, 5-8 Sept 2012).

With basis on the comparison of the estimated lethal parameters (LCs) determined for a 24 h susceptibility test (**table D.1**), we verified that the EO contained in parsley fruit was significantly more active (LCs < 50 µg mL^{-1}) than the other tested EOs, while dill infrutescences EO, which is rich in (+)-carvone, was the least active (LCs > 100 µg mL^{-1}) toward *A. atroparvus* third-instar larvae. Overall, *trans*-anethole was recognized as the most effective treatment (LCs < 30 µg mL^{-1}). In addition, cumin EO and its major compound showed moderate but equivalent level of effectiveness. Amongst the five tested standard, the two ketone (+)- fenchone and (+)- carvone showed very low activity in the first screening test. Therefore, on the account of the WHO recommendation as regard to the minimum level of effectiveness for purified substances of natural origin with promising larvicidal activity (WHO, 2005), we have omitted the estimation of the lethal parameters for both compounds since their LCs values after 24 h of treatment are expected to be much superior to 100 µg mL^{-1}.

In our research of potential sources of bio-insecticides from Apiaceae species, more specifically with mosquitocidal activity, we confirmed the superior toxicity of *trans*-anethole against the larval form of this mosquito species. Furthermore, we have found some strong potential for parsley fruit EO. Nevertheless, we were not able to conduct this evaluation with the major compounds identified in this EO, namely the likely active phenylpropene-derivatives, for a large amount of compounds would be indispensable to obtain valid data[2].

Methodology

The adopted procedures for the estimation of LCs as well as the insect rearing conditions were identical to those previously described in the in the point 3.4 of chapter III. All bioassays with *A. atroparvus*

[2] **Requisite for valid experimental procedures**: a pre-screening test with replicates to define the range of concentration; For assays: at least 3 independent assays consisting of 5-6 concentrations with 4 replicates each. Large volume of tested solution (around 200-250 mL) are necessary for a number of 25 larvae.

third-instar larvae were carried out at the IHMT (Univ. Nova de Lisboa) and performed according to the WHO laboratory guidelines for larvicidal screening assays (2005).

Table D.1. Lethal concentrations (µg mL^{-1}) of Apiaceae essential oils and some compounds against the third-instar larvae of *Anopheles atroparvus*, assessed at 24 h of exposure.

EOs/ compounds	***n*** [a]	**CL_{50}** [b, c] (95% CL)	**CL_{90}** [b, c] (95% CL)	**Slope** ± SEM [b, c]	***H*** [b, d]
Ag-I	300	143.1 (136.8 – 150.4) e	225.3 d (206.5 – 253.4)	6.5 ± 0.55	1.09
Fv-I [e]	250	51.9 (47.5 – 56.2) cd	110.0 c (98.8 – 125.7)	3.9 ± 0.30	1.40
Fv-L [e]	275	53.0 (48.7 – 57.3) cd	109.0 c (98.4 – 124.0)	4.1 ± 0.31	2.24
Cc-F	300	47.7 c (44.3 – 51.1)	91.0 c (82.9 – 102.2)	4.6 ± 0.35	2.13
Pc-F	300	22.9 b (19.7 – 25.1)	41.3 b (37.8 – 47.4)	5.0 ± 0.72	0.013
trans- Anethole [e]	300	15.7 a (14.8 – 16.7)	26.9 a (24.2 – 31.5)	5.5 ± 0.59	1.04
(+)- Carvone	100	>100 [f]	>100 [f]	–	–
Cuminaldehyde	300	51.0 cd (47.4– 54.6)	98.2 c (89.1 – 111.2)	4.5 ± 0.35	0.98
Estragole [e]	300	56.1 (52.2 – 60.0) d	107.4 c (97.5 – 122.1)	4.5 ± 0.39	0.51
(+)- Fenchone	100	> 250 [e]	> 250 [e]	–	–

[a] Total number of larvae used per concentration and the negative control.

[b] Estimated LC_{50}, LC_{90}, 95% confidence limits (CL) and *H* were determined by probit analysis based on mean values of mortalities after 24 h of exposure. Values are expressed in µg mL^{-1} of essential oils required to cause larvae death;

[c] LC values and slopes within a column followed by the same letter are not significantly different based on nonoverlapping of 95% CL;

[d] *H*, heterogeneity factor (χ^2/degree-of-freedom);

[e] These results were presented and integrated in the discussion concerning the biopesticidal activity of *Foeniculum vulgare* var. *vulgare* (Chapter III).

[f] LCs of these compounds were not estimated due to their lower activity on the basis on the pre-screening test.

In vitro assessment of the anticholinesterasic properties of Apiaceae EOs and some constituents

Overview

As discussed in the Chapter I (Point 3.5.1), the insecticidal properties exhibited by EOs and their constituents have been frequently related to a neurotoxic mode of action achieved, in part, through an inhibitory action on the acetylcholinesterase (AChE), in the same measure as several synthetic pesticides namely carbamates and organophosphates (Box 1 of appendix A).

As our first attempt to relate the biological activities of EOs and compounds produced by the four studies Apiaceae (results reported chapters III-VI) with a possible MOA, we proposed to start investigating their effect on the AChE activity.

The inhibitory activity of EOs/compounds determined *in vitro* are exhibited in the **table D.2**. With basis on this preliminary study we obtained evidences that EOs from parsley and cumin fruits (185 and 235 µg ML^{-1}, respectively) have higher inhibitory action over the hydrolytic activity of the AChE than the remainders EOs. Moreover, among the purified substances included in our investigation cuminaldehyde was the compounds that most effectively inhibited the enzymatic activity (IC_{50} value of 214.5 µg mL^{-1}, equivalent to 1.4 mM). Therefore, it seems that the compound might have a significant role as inhibitor of AChE.

The results obtained by now are not conclusive but they give some evidence of the possible action of the studied EOs and compounds on the cholinergic system. Nonetheless, several major compounds, namely those found in parsley EO (myristicin, apiole), remain to be evaluated.

Methodology

The inhibitory effect of EOs and compounds on acetylcholinesterase (AChE) activity was screening by in *vitro* assay using purified AChE from electric eel (*Electrophorus electricus*) and following the method of Ellman et al. (1961). Ellman' s colorimetric assay was adapted to 96-well microplates and performed with some modification in accordance with Barreto et al. (2012). The hydrolysis of the substrate (ATChI, acethylcholine iodide) was monitored by repeated spectrophotometric readings (absorbance at 415 nm) for different time of reaction: 0, 150, 300 and 450 s. The assessment of EO/Compounds' activity consisted in 3 individual experiments, each one including a range of 6-7 concentrations (from 16 to 1000 µg mL^{-1}) with 4 replicates per concentration. After determining the reaction rate *Vmax* from the formulae **(14)** of the appendix D for each concentration, mean percentages of inhibition were calculated using the formulae **(13)** and plotted against treatments' concentrations (dose-response curve). IC_{50} values (concentration which causes 50% inhibition of AChE activity) and the respective 95% confidence limits (CL) were estimated by probit analysis using IBM *SPSS statistics* package 20.0 (IBM corp., USA).

Table D.2. *In vitro* assessment of EOs and some constituents' inhibition on acetylcholinesterase (AChE) activity.

EOs/Compounds	**IC_{50}** (CL 95%) [a]	**Slope** ± SEM	**Intercept** ± SEM	χ^2 [b]
Ag-F	n.d	n.d	n.d	n.d
Ag-I	370.5 (288.2 – 523.6) bc	1.35 ± 0.16	1.54 ± 0.35	1.94
Fv-I	465.4 (331.0 – 759.3) c	1.04 ± 0.12	2.22 ± 0.26	0.85
Fv-L	377.7 (281.0 – 581.4) bc	1.12 ± 0.15	2.10 ± 0.33	0.34
Pc-F	**185.1** (143.3 - 253.5) a	1.00 ± 0.11	2.72 ± 0.23	2.61
Cc-F	**234.9** (184.9 – 316.9) ab	1.17 ± 0.12	2.23 ± 0.25	1.90
trans-Anethole	n.d	n.d	n.d	n.d
Apiole	n.d	n.d	n.d	n.d
(+) -Carvone	368.1 (245.7 – 667.7) ab	0.83 ± 0.11	2.87± 0.23	0.80
Cuminaldehyde	**214.5** (176.6 – 269.7) a	1.44 ± 0.13	1.64 ± 0.27	2.13
Estragole	605.7 (400.0 – 1221.7) c	0.97 ± 0.15	2.30 ± 0.34	1.78
(+) -Fenchone	726.6 (515.7 – 1187.4) bc	0.92 ± 0.11	2.36 ± 0.27	0.85
Miristicin	n.d	n.d	n.d	n.d
Ursolic acid [c]	190 ± 14			

[a] Estimated IC_{50} and 95% confidence limits (CL) were determined by probit analysis based on determined mean values of inhibition. Values are expressed in µg mL^{-1} of EO/compound required to inhibit 50% of the enzymatic activity; [b] Qui-square values were determined for 4 degree of freedom; [c] IC_{50} of ursolic acid was determined by Oliveira et al. (2015) following the same methodology; *n.d* Not determined or results lacking statistical validation.

Design of chitosan micro/nanogels as delivery system for the release of EOs

Overview

In order to improve the stability and biological activity of EOs and volatile compounds, we proposed to study one method of encapsulation, considering the use of chitosan, as a non-toxic biopolymer. Empty Chitosan microcapsules (or loaded with EO) were obtained through the ionotropic gelation method[3], following the procedure described by Anchisi et al. (2006) (with some modifications). 75-85 % Deacetylated chitosan dissolved in acetic aqueous solution (2 mg mL^{-1}, pH=4.7) was emulsified with parsley EO in a proportion of 1:1 w/w using a Ultra-Turrax at 16,000 rpm. The polymeric dispersion was dropped through a disposable syringe onto a solution containing citrate as crosslinking agent (1 mg/mL) and maintained under gentle magnetic stirring at room temperature for 40 min. Chitosan nanoparticles were separated from suspension by centrifugation using ultra centrifugal filters.

The mean diameter of particles was determined by dynamic light scattering (DLS) (Zetasizer Nano ZS Instrument, Malvern) at day1 and day15. The Z-average, PDI and Z-potential measurement were performed in triplicate. The incorporation of EO into chitosan microcapsules was visually confirmed through the observation of the fluorescence emitted by the aromatic compounds constituting the EO (in the range of 420-460 nm) when these were exposed to the UV light (mercury lamp) (Fig. D.1).

Although the encapsulation efficiency, and the kinetic of compounds' release have not been determined yet, we have confirmed that the chitosan/citrate micro/nanogels contained EOs and did not show substantial physical changes, or swelling, over 15 days (Table D.3). The distribution of particle size was monomodal in both loaded and unloaded formulations, and micro/nanocapslules containing *Petroselinum crispum* EO had a slightly superior mean diameter.

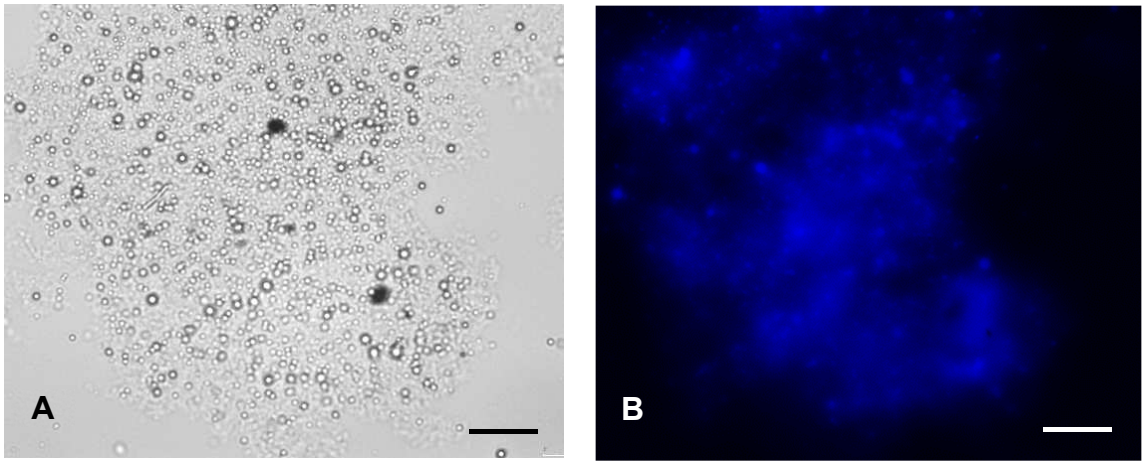


Figure D.1. Observation with the fluorescence microscope under visible light **(A)** and UV light **(B)**, of aggregated chitosan microcapsules loaded with *Petroselinum crispum* essential oil (EO) (proportions of polymer/EO weights of 1:1). Scale bars: 25 µm.

[3] In acidic solution, the $-NH_2$ of a chitosan molecule is protonated ($NH3^+$) being able to it interacts with an anion (counter ion) such as tripolyphosphate (TPP) or citrate by ionic interaction to form microgel particles.

Table D.3. Physical characterization of chitosan-based micro/nanogels containing *Petroselinum crispum* essential oil performed at day 1 and day 15 after their preparation.

Chitosan: citrate (4:1) [a]	Time (Days)	Z-Average (r, nm) [b]		Polydispersity index (PDI) [b]		Zeta- Potential (mV) [b]	
		Mean	SD	Mean	SD	Mean	SD
Unloaded	1	130.8	2.56	0.253	0.014	33.1	1.77
	15	101.0	0.882	0.219	0.013	34.0	1.90
Loaded	1	160.8	2.708	0.235	0.045	38.6	2.38
	15	143.6	5.352	0.297	0.032	36.9	2.63

[a] Proportion used on a weight basis; DLS measurements conditions: Temp=25 °C, scattering angle= 173 °, Samples diluted in acetic acid pH 4.7. [b] the average size distribution intensity and Zeta-potential were obtained from three measurements.

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