Universidade do Minho
Escola de Ciências

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Extraction, isolation and synthesis of phytochemical derivatives with potential application as (bio)pesticides

december 2021

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Extraction, isolation and synthesis of phytochemical derivatives with potential application as (bio)pesticides

Master's Thesis
Master in Medicinal Chemistry

Master's Thesis prepared under the supervision of
Professor Dr. António Gil Fortes
and
Professor Dr. Maria Sameiro Gonçalves

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Statement of integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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

Resumo

Extração, isolamento e síntese de derivados fitoquímicos com potencial aplicação como (bio)pesticidas

Nos últimos anos, óleos essenciais (OEs) e outros componentes vegetais tornaram-se importantes fontes naturais de compostos biológicos ativos e/ou matérias-primas para a obtenção de novos derivados por modificações sintéticas com atividades aprimoradas adequadas para diversas aplicações, inclusive como pesticidas.

No presente trabalho, uma série de OEs, contendo anetol, estragol e eugenol, foram obtidos a partir de anis estrelado, erva-doce e cravo-da-índia, respetivamente, por hidrodestilação. Um componente vegetal menos volátil, o ácido ginkgólico C17:1, foi obtido de *Ginkgo biloba* por extração com Soxhlet. O eugenol isolado foi utilizado como material de partida na preparação de 2-metoxi-4-(oxiran-2-ilmetil)fenol e a abertura do anel epóxido foi realizada usando anilina tendo sido obtido o respetivo β-aminoálcool. Tomando como inspiração o ácido ginkgólico isolado, partindo do ácido 2-aminobenzóico e usando 1-bromododecano, obteve-se o ácido 2-(dodecilamino)benzoico. Além disso, outro composto natural, a 4-hidroxiquinolina, foi usada para obter um derivado *O*-alquilado, a 4-(3-cloropropoxi)quinolina. Considerando a pesquisa de novas alternativas sintéticas aos pesticidas convencionais, partiu-se dos ácidos 4-clorobenzoico ou 2-clorobenzoico e 3-bromoanilina, por reação com cloreto de tionilo e trietilamina, obtiveram-se as benzamidas correspondentes. Além disso, o ácido 2-clorobenzoico reagiu com 9-etil-9*H*-carbazol-3-amina, pelo mesmo procedimento e foi obtida a 2-cloro-*N*-(9-etil-9*H*-carbazol-3-il)benzamida.

Todos os compostos foram avaliados quanto à sua potencial atividade biológica contra as linhas de insetos *Sf*9, *Spodoptera frugiperda*. Os resultados mostraram que os compostos mais relevantes são o ácido ginkgólico, ácido 2-(dodecilamino)benzoico e a *N*-(3-bromofenil)-4-clorobenzamida, bioinseticidas/inseticidas semissintéticos muito promissores. Com o ácido 2-(dodecilamino)benzoico foram realizados estudos de nanoencapsulamento em lipossomas pela técnica de injeção etanólica, sendo que a eficiência de encapsulação obtida foi de 99%.

Este trabalho insere-se no projeto financiado pela FCT, PTDC/ASP-AGR/30154/2017.

Palavras-chave: Bioinseticidas, inseticidas semissintéticos, óleos essenciais, ácidos ginkgólicos.

Abstract

Extraction, isolation and synthesis of phytochemical derivatives with potential application as (bio)pesticides

In recent years, essential oils (EOs) and other plant components have become important natural sources of active biological compounds and/or raw materials for obtaining new derivatives by synthetic modifications with enhanced activities suitable for various applications, including as pesticides.

In the present work, a series of EOs, containing anethole, estragole and eugenol, were obtained from star anise, fennel and clove, respectively, by hydrodistillation. A less volatile plant component, ginkgolic acid C17:1, was obtained from *Ginkgo biloba* by Soxhlet extraction. The isolated eugenol was used as starting material in the preparation of 2-methoxy-4-(oxiran-2-ylmethyl)phenol and the opening of the epoxide ring was carried out using aniline and the respective β-amino alcohol was obtained. Taking the isolated ginkgolic acid as inspiration, starting from 2-aminobenzoic acid and using 1-bromododecane, 2-(dodecylamino)benzoic acid was obtained. Furthermore, another natural compound, 4-hydroxyquinoline, was used to obtain an *O*-alkylated derivative, namely 4-(3-chloropropoxy)quinoline. Also considering the search for new synthetic alternatives to conventional pesticides, 4-chlorobenzoic or 2-chlorobenzoic acids and 3-bromoaniline were used, and by reaction with thionyl chloride and triethylamine, the corresponding benzamides were obtained. Furthermore, 2-chlorobenzoic acid was also reacted with 9-ethyl-9*H*-carbazol-3-amine, following the same procedure, and 2-chloro-*N*-(9-ethyl-9*H*-carbazol-3-yl) was obtained.

All compounds were evaluated for their potential biological activity against the *Sf*9 insect cell lines, *Spodoptera frugiperda*. The results showed that the most relevant compounds are ginkgolic acid, 2-(dodecylamino)benzoic acid and *N*-(3-bromophenyl)-4-chlorobenzamide, being very promising bioinsecticides/semisynthetic insecticides. With 2-(dodecylamino)benzoic acid, nanoencapsulation studies in liposomes were carried out using the ethanol injection technique, and encapsulation efficiency obtained was 99%.

This work is part of the FCT-funded project, PTDC/ASP-AGR/30154/2017.

Keywords: Bioinsecticides, semisynthetic insecticides, essential oils, ginkgolic acids.

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Abbreviation list

δ	chemical shift
η	yield
ν	wavenumber (expressed in cm^{-1})
ACN	acetonitrile
d	doublet
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	dichloromethane
dd	doublet of doublets
EOs	essential oils
equiv	equivalent
et al.	*et alia* (from Latin, reference to other people)
EtOAc	ethyl acetate
EtOH	ethanol
HObt	hydroxybenzotriazole
LPs	liposomes
m	multiplet
m-CPBA	*meta*-chloroperoxybenzoic acid
MeOH	methanol
NMR	nuclear magnetic resonance
s	singlet
ls	large singlet
SNL	solid nanoparticle lipid
t	triplet
TLC	thin layer chromatography
UV	ultraviolet
vis	visible

Chapter 1: Introduction

1.1. Phytochemical pesticides

1.1.1. State of the art

In last decades, farmers and many companies in the food sector have used synthetic chemicals to produce, process and preserve food, as well as to improve agricultural production.[1] In recent years, essential oils and other plant components became important natural sources of biological active compounds and/or starting materials for obtaining new derivatives by synthetic modifications with improved activities suitable for various applications, including as pesticides.[2] In fact, insecticidal, repellent, fumigant and antifeedant activities against a wide variety of insects[3,4] are relevant for their possible use in the control of insect pests.

Synthetic pesticides are associated with environmental and human health problems such as lung, prostate, skin and throat diseases, cancer and hypertension.[1,5] More restrictive legislation on the use of synthetic pesticides and increasing resistance in populations of pests have resulted in a decline in their use.[6] Therefore, it is imperative to replace these types of harmful products by more sustainable compounds whose action continues to be the protection of crops. That substitution passes through the synthesis of new compounds from natural and semisynthetic products.

Nowadays, there is a growing interest in the evaluation of botanical insecticides as an alternative in pest control, because these compounds do not have significant adverse effects on non-target organisms, ecosystems and human health.[4] The premise of first tries was that new tools – now including RNA interference (RNAi) technology – would play an increasingly major role in future pest management, along with synthetic chemicals, cultural methods and biological control.[7] The use of biopesticides and related alternative management products is increasing.[7,8] New tools, including semisynthetic compounds and plant-incorporated protectants (PIPs), as well as botanical and microbially derived chemicals, are playing an increasing role in pest management, along with plant and animal genetics, biological control, cultural methods, and newer synthetics.[8]

Thus, in this dissertation, the synthesis of new compounds from natural or semisynthetic products was sought with insecticidal potential.

1.1.2. Plant extracts

Albrecht Kossel was the first scientist to define the concept of secondary metabolite and won the physiology or medicine Nobel prize.[9] These compounds are produced in the plant induced by biotic, (example given, pathogens) or abiotic (e.g. climatic conditions) factors.[10] Secondary metabolites are generally low molecular weight molecules and can be fat-soluble, water-soluble and/or volatile.[11]

Plants contain several types of nutrients such as carbohydrates, proteins, lipids, minerals and vitamins, as well as non-nutritive substances, such as phenolic compounds, glycosides, alkaloids and terpenoids. These substances are called phytochemicals.[12]

According to Becarre Natural, a specialized company in plant extracts, a plant extract is a substance or an active principle with desirable properties. It is isolated from the tissue of a plant, usually by treating it with a solvent, to be used for a particular goal.[13] There are many kinds of plant extracts with their major functions contained in skin medicines and cosmetics.[14] It includes anti-irritant, anti-inflammatory, wound healing, anti-infective, sterilization, wetting, protecting the skin, among others major functions.[15] With such biological properties, phytochemicals have great utility in human health and therefore more than 4000 phytochemicals have been catalogued.[16] In the Figure 1 there is a small example of phytochemicals molecules.

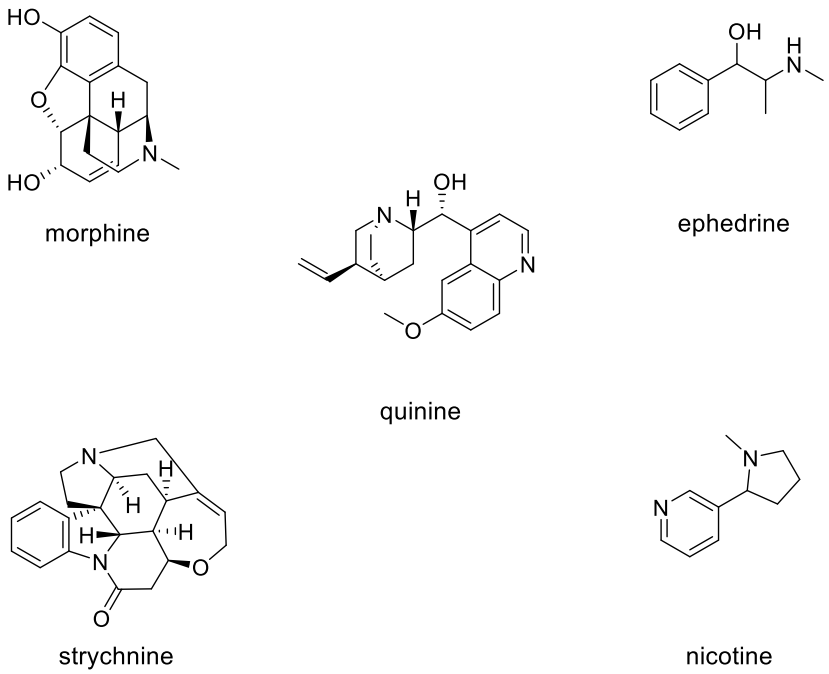


Figure 1. Illustrative image of plant extract molecules.

The process of extraction of these compounds from plants must generally be simple, fast, economic, in compliance with the regional regulation, effective and selective when needed. However, certain steps may require a long time of process.[17]

Pressing is one of the more common ways of extraction (the orange juice squeeze), pressing may be used complementary or prior to other process, for instance to prepare the materials, defat it, etc. It causes mechanical perturbation and gives a liquid product.[18] Solid/Liquid extraction consists in the extraction or separation of one or more actives compounds from solid materials working on the solubility in a liquid to obtain the soluble part and the insoluble part, or fractionation of a homogenous solution (Figure 2).[19]

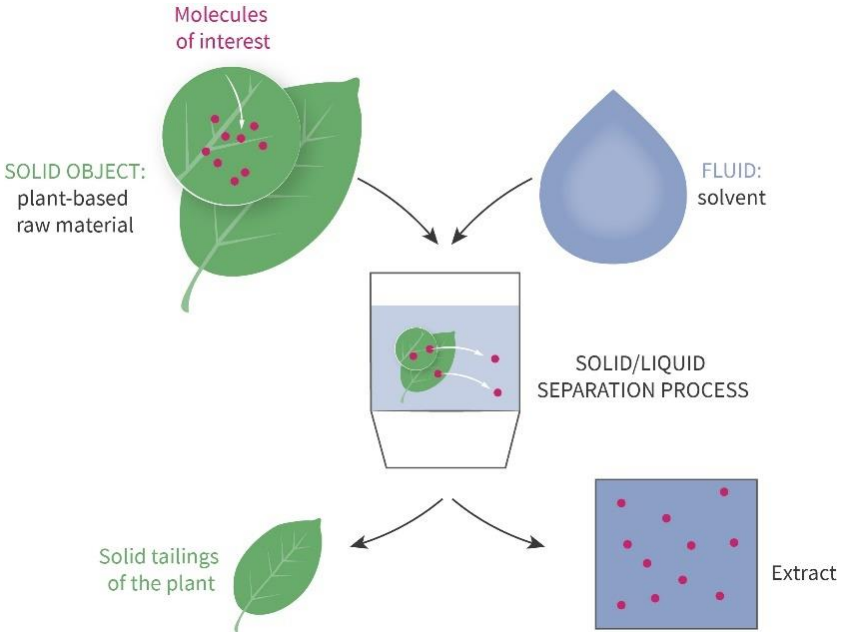


Figure 2. Extraction of molecules of interest from vegetable matter with solid/liquid method.

The typical solid/liquid extractions are maceration (solid in a solvent at room temperature); digestion (solid in a solvent over room temperature, below ebullition); reflux (solid in a solvent, temperature of ebullition); infusion (teas) (solid in a liquid at temperature of ebullition, then cooling of the suspension); elution, leaching (lixiviation) (solvent goes through the solid); lixiviation (always with cold, fresh and new solvent); and percolation (solvent goes on and through the solid).[20]

Distillation and hydrodistillation consists in the use of direct heating or steam, which is mainly used for oils and volatile components (Figure 3). It may cause degradation (oxidation, hydrolysis, enzymatic reaction).[17] In the present work, this method was used to extracting essential oils, namely estragole and eugenol in a few hours.

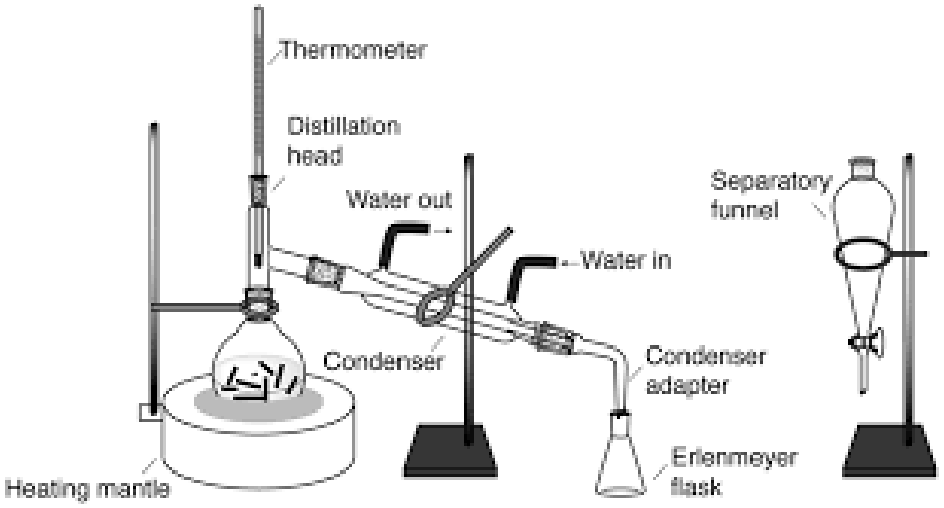


Figure 3. Experimental setup used in hydrodistillation.[21]

The Soxhlet extraction method uses a specific laboratory device, the sample is not placed directly in contact with the heat source, but in a Soxhlet extractor consisting of three main sections: a percolator (distillation flask and reflux condenser), where the extraction solvent is refluxed, a cellulose cartridge which its function is to retain solid particles (plant matter) and a siphon which periodically empties the chamber where the cartridge is placed (Figure 4).

As a result, this extraction method allows the sample to be permanently in contact with a given quantity of fresh solvent and at the end of the process, it is not necessary to carry out a filtration, leading to higher yields.[22]

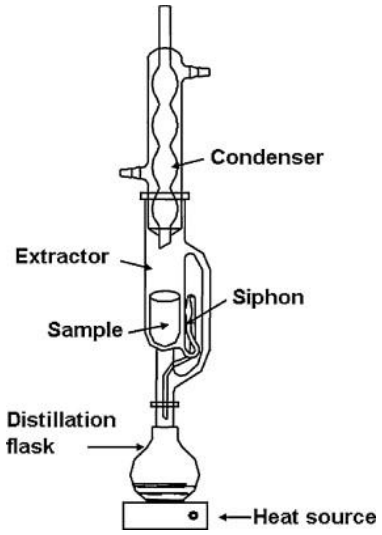


Figure 4. Experimental setup used of Soxhlet extraction.[23]

1.1.3. Essential oils

Essential oils (EOs) are aromatic and volatile liquids extracted from plant material by distillation, trivially named according to the plants from which they are derived.[24] In the present work, anethole, estragole and eugenol were extracted from the corresponding natural sources (Figure 5). Numerous literature publications on the composition of the various EOs, and their main components of economic interest are summarized by Bauer *et al.* in 2016.[25]

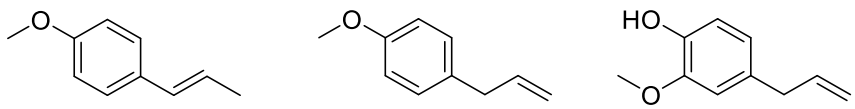


Figure 5. Similar essential oils' molecules with different uses in various industries, anethol, estragole and eugenol.

EOs can be combined with other compounds to be part of a diverse range of products including perfumes, cosmetics, soaps, cleaners, aromatic candles, food, over the counter and prescription drugs, and insecticides.[26] In addition, they can be used in aromatherapy to promote mood swings and improve health in the form of fragrances.[15] Essential oils obtained from botanically identical plants can have a significantly different set of chemical

components and therapeutic actions. This can be due to different growing conditions, location, climate, etc. When this happens, the different oils are classed as chemotypes (ct).[27] Thyme (*Thymus vulgaris*) is a great example, with Red Thyme (thyme ct thymol) containing more phenols (thymol) and Sweet Thyme (Thymus ct linalool) containing more alcohols (linalool).[28] Rosemary, Basil and Marjoram are also examples of chemotypes.[28] Oils that contain a high percentage of a single compound, are used to isolated these compounds,[29] for example, thymol of thyme (Table 1).

Table 1. Principal components of some essential oils of vulgar plants.

Plants	Principal component of essential oils
Rosemary	1,8-Cineole, α-pynene, camphor, β-pynene
Pepper mint	Menthol, menthone
Canella	Cinnamaldehyde
Thyme	Thymol, *p*-cymene, *γ*-terpinene
Lemon grass	Citral, neral
Eucalyptus	1,8-Cineole
Anise	Anethole
Fennel	Estragole
Clove	Eugenol

The presence of compounds in essence, in greater or lesser quantities, directly affects their quality, dictating the possibilities of industrial use and, consequently, the commercial value of oil.[30]

Illicium verum is a tree that is the source of star anise. It is obtained from the star-shaped pericarps of the fruit of *I. verum* which are harvested just before ripening.[31] Star anise oil is a highly fragrant oil used in cooking, perfumery, soaps, toothpastes, mouthwashes, and skin creams.[29] Roche pharmaceuticals used star anise to produce shikimic acid, a chemical intermediate used in the synthesis of oseltamivir (Tamiflu).[32]

The main component of the essential oil is anethole (80-90%), with minor components including 4-anisaldehyde, estragole and pseudoisoeugenyl-2-methylbutyrates, among others.[33] In the present dissertation, anethole was extracted from anise.

Foeniculum Vulgare, known as fennel, has a significant antioxidant activity that can be attributed to its high content of phenolic compounds (phenylpropanoid and flavonoid glycosides);[34] it also contains terpenes that give it its characteristic odour. Anethole, estragole, fenchone, limonene and α-pinene are the main compounds referenced as present in fennel oil. Fennel is widely used in cooking, food industry, beverages and cosmetics.[35] Estragole has an insecticide effect on *Ceratitis capitata*, *Bactrocera cucurbitae* (melon fruit) and act faster on *Bactrocera dorsalis*.[36] In the present work, estragole was extracted of commercial seeds of fennel.

Syzygium aromaticum, scientific name of clove specie is an aromatic flower of a tree. Eugenol represents 70-90% of the extracted essential oil and it is the responsible compound for the aroma of clove.[37] Clove is used in traditional medicine as the essential oil, used as analgesic primarily for dental emergencies and other disorders,[38] but the use of clove for any medical purpose has not been approved by the US Food and Drug Administration (FDA), and its use can cause adverse effects if taken orally by people with liver disease, blood clotting[39] and immune system disorders or food allergies.[37] Eugenol is an insecticide and was tested on *Sitophilus zeamais*[40] and its derivatives are being studied to report their insecticidal potential.[41] In the present work, eugenol was extracted of commercial clove.

Noteworthy are the applications in medicine resulting from bactericidal and fungicidal activities of essential oils and may act as antimicrobial drugs, due to the presence of phenolic compounds.[18] In any form, the use of essential oils as green pesticides rather than synthetic pesticides has ecological benefits such as decreased residual actions.[42] In addition, the increased use of essential oils as pest control could have not only ecological, but also economic benefits as the essential oil market diversifies and popularity increases among organic farmers and environmentally conscious consumers.[43,44]

1.1.4 Conventional botanics

According to Food and Drug Administration (FDA), botanics are plants, herbs and spices valued for their medicinal or therapeutic properties.[45] People are finding ways to

utilize these plant parts to improve their health, treat symptoms, and prevent disease. According to Environmental Protection Association (EPA) conventional pesticides, by contrast, are synthetic materials that usually kill or inactivate the pest.[46] The use of conventional pesticides over the past five decades has led to a range of problems in agriculture, the environment and human health.[47]

Nicotine, rotenone, pyrethrins and sabadilla alkaloids are the prominent example of first-generation botanical pesticides. For example, a pyridine alkaloid extracted from *Nicotiana tabacum* called nicotine (Figure 6), is effective against a wide range of herbivorous insect pests like leafhoppers, aphids, thrips, whiteflies, and mites.[2] After the introduction of tobacco in 1559 in Spain and Portugal from the Americas, the rest of Europe started using this product for smoking, but since the seventeenth century it has been used as an insecticide and repellent, so in the United States it was catalogued as an insecticide in 1814.[48]

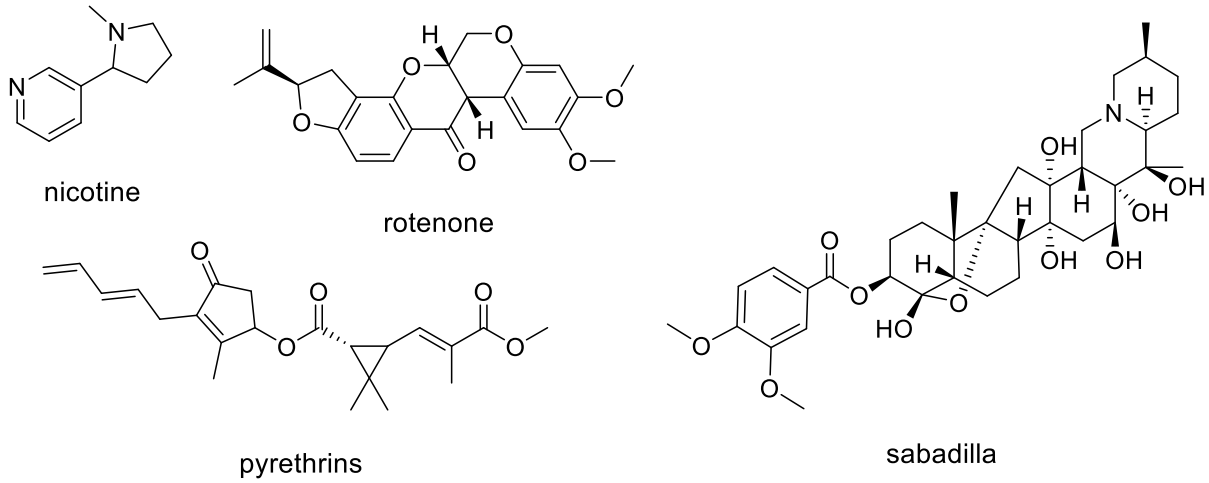


Figure 6. Examples of conventional botanics such as nicotine, rotenone, pyrethrins and sabadilla.

Conventional botanics have a lot of benefits.[47] The most important benefits include increased crop yields, improved food safety, human health, and quality of life, and reduced labour, energy use, and environmental degradation.[49] In addition, the evolution of pesticide resistance among pest populations is another important factor driving a need to reduce our reliance on conventional pesticides.[50]

1.1.5. Quinolines and carbazoles

Quinoline or benzopyridine is an aromatic heterocyclic nitrogen-containing compound (Figure 7). It participates in both nucleophilic and electrophilic substitution reactions.[51]

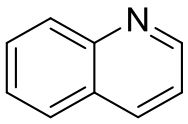


Figure 7. General representation of a quinoline.

Quinolines possess great fluorescent and electron transporting ability, high thermal and chemical stability and the possibility of chemical modifications play a significant role in applications in optoelectronics.[52,53] Quinoline possessing electron withdrawing ability is a great candidate for electron-transporter in constructed organic materials. The donor-acceptor (D-A) system of organic compounds – where donor plays a hole-transporting role and acceptor is an electron-transporter–[53] are intensively explored for applications in organic electronics.[52] The study of luminescence behaviour of aryl substituted 8-hydroxyquinoline derivatives showed the electronic effect of substituents on photophysical characteristics that electron-donating groups caused blue shift and electron withdrawing a considerable red shift in the emission.[54] Quinoline derivatives are possible to obtain by many different synthetic routes and there is a constant progress in that matter. Nowadays they are often synthesized by using a wide range of catalysts[53,55] and with the application of cross-coupling reactions.[56,57] 8-Hydroxyquinoline is a quinoline compound and antifungal with chelating properties.[54] It is a natural product found in the root exudate of the invasive plant *Centaurea diffusa*.[58] 4-Hydroxyquinoline – another quinoline derivative – is used in a large spectrum of antibiotics[59] and in this presentation of an *O*-alkylated derivative, which insecticidal activity was evaluated.

Meanwhile, carbazoles are an important class of hetero tricycles.[60] These are compounds containing a three-ring system with a pyrrole ring fused on either side to a benzene ring (Figure 8).

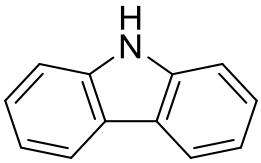


Figure 8. General representation of a carbazole.

According to Drug Bank, at least five drugs contain carbazole groups with different targets on cancers and one of them is used to treat chronic heart failure.[61]

Electrochemical oxidation of carbazole proceeds in a similar way to chemical oxidation but seems to be more selective.[62] The process of combining the carbazole moieties at the N–N′ bond, which requires the loss of the proton attached to the nitrogen atom at some point, is prevented in acidic media.[62,63] This work of Ambrose *et al.* reported that the oxidation potential depends slightly on the characteristics of the substituent. When the nitrogen atom is blocked by a substituent (e.g., a methyl group), the oxidation leads mainly to dimers. Electrochemical oxidation occurs in the same way as for carbazole but leads only to 3,3′-bicarbazyl. This results from the fact that the 9-position of carbazole is already occupied.[63] The electrochemistry of 3-substituted carbazoles is generally similar to that of pure carbazole, with some exceptions. Generally, oxidation leads to 6,6′- and *N,N*′-bicarbazyls that are formed in rather poor yield.[53] In this dissertation, 3-amino-9-ethylcarbazole was used and corresponding to two groups mentioned above – 3-substitued with an amine and 9-occupied position with an ethyl group (Figure 9). The presence of the amino group at position 3 exhibits a unique reactivity with both C-2 and C-4.[60] This compound present antifungal and antibacterial properties, which also gives it a great importance in the class of carbazoles.

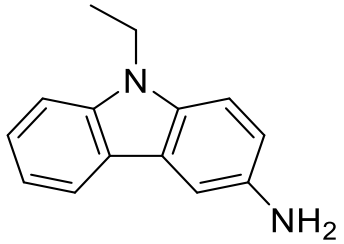


Figure 9. Example of a structure of 3-substitued and 9-occupied carbazole (3-amino-9*H*-ethylcarbazole.

1.1.6. Benzamides

Amide is the generic concept for compounds derived from oxoacids by change an acidic hydroxy group with an amino group or substituted amino group.[64] Amides are classified based on the types of oxoacids from which they are derived. The synthesis of amide is of huge importance in organic, coordination, and medicinal chemistry.[65]

For example, sulfonic acids become sulphonamides and carboxamides refer to carboxylic acids.[66] In small molecules, the presence of the amide group "-C(=O) N–" is generally easily established. It can be distinguished from nitro and cyano groups in Infrared (IR) spectra. They exhibit a moderately intense ν_{CO} band near 1650 cm^{-1}.

Carboxamides comprise a central carbon atom possessing a double bond to oxygen and a single bond to nitrogen. [67] The nitrogen lone-pair delocalisation plays a crucial role in determining the structure and reactivity of amides.[67]

Amides with an aromatic ring are designated as benzamides (Figure 10). They are the simplest amide derivative of benzoic acid. According to Drug Bank, there are at least twenty-five drugs with benzamide group on their structure.[61] Because of its effectiveness and its high tolerability in humans, benzamides have been in clinical use for decades and are till now frequently used in Europe.[68] In some studies, this group present a insecticidal potential.[69]

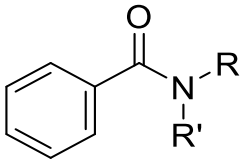


Figure 10. General representation of a benzamide where R and R' are groups linked to amide function.

1.2. Limitations of plant biopesticides

Based on evidence, world population is increasing by an estimated 97 million per year.[70] This explosive increase in world population is mostly in developing countries and this is where the need for food is greatest and starvation threatens human life.[71] So, it is necessary to establish a minimum for consumption of synthetic pesticides because their

use may have a negative impact on nontarget organisms (bird, fish, honeybee and humans) in ecosystems,[72] in rates much higher than, for example, fungicides or herbicides.[73] However, in some cases, it is difficult to determine whether a product meets the criteria for classification as a biopesticide, and the decision by local agencies might vary depending on the regulations in each country.[74] There might be specific requirements pertinent to the different categories of biopesticides.

On the other hand, the intention of biopesticides is to achieve rapid pest control, control is not reliant on the reproduction of the control agent to any significant extent within the environment.[75] So, it is important to have greater level of knowledge required by the grower to use them effectively.[71] Therefore, persistence is short, and the agent must be reapplied frequently to maintain its effectiveness in the crop environment.[76]

A slower rate of control and often a lower efficacy and shorter persistence compared to conventional pesticides combined with the contact of biopesticides to air, moisture, high temperatures, and the sunlight adequately degrades their constituents.[77]

1.3. Production and consumption of biopesticides

1.3.1 Introduction

According to EPA, safer pesticide is less persistence in the environment, causes less soil and groundwater contamination, and it is less toxic to non-target organisms and allow lesser exposure to humans, domestic animals and the environment. As compared to synthetic pesticides, phytochemical biopesticides are moderately toxic, less persistent, and biodegradable.[2]

Biopesticides are classified into three major categories:

1. Plant-pesticides – are pesticidal components that plants produce from genetic material.[78]

2. Microbial pesticides – contain a microorganism (protozoan virus, fungus, bacterium or algae) as the active ingredient. According to IUPAC website, they can control many kinds of pests, although each separate active ingredient is relatively specific for its target pest.[79] Certain other microbial pesticides act by out-competing pest organisms and it is harmful.[79] This group needs to be continuously monitored to ensure they do not become capable of harming non-target organisms, including Humanity.[78,79]

3. Biochemical pesticides – naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are synthetic materials that usually kill or inactivate the pest.[33] Biochemical pesticides include semiochemical substances that interfere with growth or mating, such as plant growth regulators, or substances that repel or attract pests.[80] Semiochemicals are secretions of plants or animals that change the function of receptor organisms of similar or different kinds. Semiochemicals include pheromones, which operates, between individuals within a species and allelochemicals act between individuals of different species. That group influences insect life situations, including feeding, mating, and egg-laying.[77] Biological control with pheromones or kairomones can identify and monitor insect populations.[81] Another strategy for controlling pests is the use of semiochemicals as feeding deterrents.[82]

Because it is sometimes difficult to determine whether a natural pesticide controls the pest by a non-toxic mode of action, EPA has established a committee to determine whether a pesticide meets the criteria for a biochemical pesticide.[7] The growth of total world production of biopesticides is rising and therefore demand and use is also increasing.[1]

1.3.2 Production of biopesticides

In general, biopesticides are prepared as dry formulations for direct applications or liquid formulations.[6] To growing concerns about food contamination with synthetic pesticide residues coupled with tighter import/export controls shifting focus to new, the scientists are searching safer alternatives.[83]

The costs associated with developing biopesticides are significantly lower compared to synthetic chemical pesticides and can be applied with spray equipment commonly used by farmers.[84]

This production occurs because these biopesticides have some benefits. One of them is no or low toxicity for vertebrates' animals is related with biological efficacy of substances contained in bioinsecticides and it is based on substances of defensive nature that are synthesised by the plants through antibiosis or antixenosis.[73] These substances are synthesised by plants as specialised molecules that have a negative impact only on pathogens and insects feeding on their tissues.[85] These substances (in commonly applied

doses) are harmless and often rather beneficial for homeothermic animals, considering their medicinal effects, which have been scientifically documented.[73]

Environmental safety is associated with products based on plant extracts and they are generally considered as safe. This assumption is because most of these products contain secondary plant metabolites,[86] which are subject to relatively fast degradation in nature and thus do not burden the environment with their residues.[87] Active substances of bioinsecticides are natural and easily degradable in natural ecosystems through common and simple degradation processes.[73]

Prevention of resistance development is the combination of physiological and behavioural actions of botanical pesticides inactivates the development of resistance. This is common phenomenon in natural ecosystem where herbivorous insects are controlled by plant allelochemicals.[73]

1.3.3 Consumption of biopesticides

Nowadays, consumers are more knowledgeable and aware about their health. To meet the growing demand for producing high-quality vegetables and fruits year-round,[88] farmers are mindful that consumers' view produce grown with less or no synthetic chemical inputs as safer to eat, healthier, and friendlier to the environment.[73]

The intensive use of synthetic pesticides in pest control activities can cause resistance and therefore resurgence of target pests.[89] Undesirable effects on the environment, including reduction in natural predation relationship (predators and parasites) and beneficial insects, are also possible.[73] A major concern is the effects of synthetic pesticides on human health. In last decades, biopesticides have emerged as a potential alternative to full synthetic insecticides.[90] Currently, biopesticides share only a small portion of global pesticide market, but growth is faster in this area than in synthetic insecticides.[91] This growth is mainly driven by a rising interest in the demand for organic agricultural products that is most pronounced in western countries.[88]

1.4. Formulations of plant based biopesticides

1.4.1 Introduction

The designation botanical pesticides or plant based biopesticides refers to pesticides developed from plant resources, being referred to as one of the most attractive green alternatives for replacing synthetic pesticides.[92] Overuse and misuse of synthetic pesticides can result in harmful effects on human beings and the environment and toxicity to non-target organisms, thus impacting negatively on biodiversity.[93]

Scientific research in this area has allowed the identification and isolation of specific active substances, some of which have been used in the production of botanical pesticides.[83,94]

1.4.2 Botanical formulations

In the end of XIX century, Felix Hoffmann and Arthur Eichengrün created the first synthetic drug, the aspirin.[95] This compound (acetylsalicylic acid) was synthesized from salicylic acid, an active ingredient of analgesic herbal medicines.[96] In this way, the production of semisynthetic substances began.

The development of botanical biopesticides is mainly based on the use of secondary plant metabolites, such as flavonoids, alkaloids, among other compounds.[97] But these compounds have some issues like is mention in soon after point.

Generally, preparations containing mixtures of fatty acids, essential oils and extracts present problems related to the combined effect of the biological properties of their constituents, which are only revealed after their separation.[98]

Molecular modification, naturally occurring on biological active substances, is one of the main strategies to improve biological effects as well as to reduce any harmful effects.[99]

Like was mentioned above, scientists are researching in botanical formulations area. One example is neem oil, widely used in India for the control of agriculture as insecticide. Oil from seeds and other parts of the neem tree contain several bioactive metabolites,

including azadirachtin, (Figure 11), which is used in various botanical products for pest control.[100]

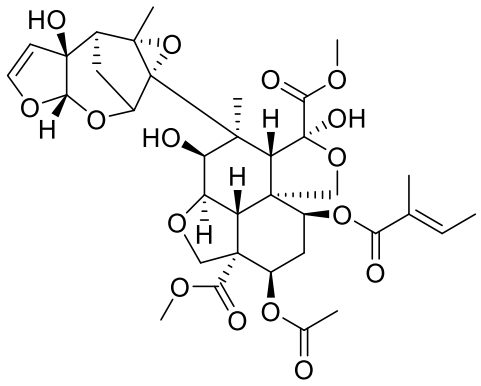


Figure 11. Chemical structure of azadirachtin, a bioactive metabolite of neem oil.

In some cases, the obtained extracts were purified and a main component was isolated. This was the case of eugenol the main component of clove extract. In the present work, eugenol is used as a precursor for the synthesis of derivatives, through epoxidation reaction followed by epoxide ring opening attempts with aniline as nucleophile.

The partial or total chemical synthesis of a derivative of natural origin can be complex. Obtaining the basic structure of the compound, the introduction of functional groups at certain positions and the stereochemistry of the molecules are the main research challenges in this area under study.[98]

The potential toxic effects of unknown compounds with similar chemical structures are predicted using this approach, and it is important to understand how changes in chemical structure affect the magnitude and type of biological effect.[101]

In the future, it is expected that the sustainable control of agricultural pests will be carried out using isolated botanical products and their semisynthetic derivatives.

The use of nanoencapsulated formulations, although allowing an increase in efficiency, can substantially reduce the amount of active compound present and the toxic effect by limiting its exposure.[83]

1.4.3 Problems associated with botanical formulations

If the product tested is not characterized and authenticated plant product studies cannot be considered scientifically valid to prevent reproducibility in the manufacturing

of the product in question.[102] Many studies refer the use of standardized material, but they are referring to chemical standardization. While chemical standardization is important, its utility is limited when the starting material is not well characterized botanically.[103] Although the resulting studies are sound with respect to the actual product tested, adequate authentication of the product cannot be compared to other products on the market.[67,68] Could be as straightforward as botanical/morphological identification or as elaborate as genetic or chemical profiling. Authenticated raw material is the basic starting point for the development of a botanical product.[106]

However, harvesting, storing, processing and formulating methods may dramatically affect the quality and consistency of the final product by altering the desired marker components or by increasing the possibility of unwanted contaminants.[102] Thus, validated methods to ensure quality control in manufacturing and storage are required tools for optimal efficacy and safety of the products. These controls are also critical for the evaluation of pharmacological, toxicological and clinical studies of the botanical supplements.[107]

1.5. Nanotechnology and plant biopesticides

Encapsulation is the technology of encasing substances in gaseous, liquid or solid states in matrices, which can release their payload under certain conditions at a controlled rate.[108] Use of nanoencapsulated formulations (Figure 12), although allowing an increase in efficiency, can substantially reduce the amount of active compound present and the toxic effect by limiting its exposure.[83]

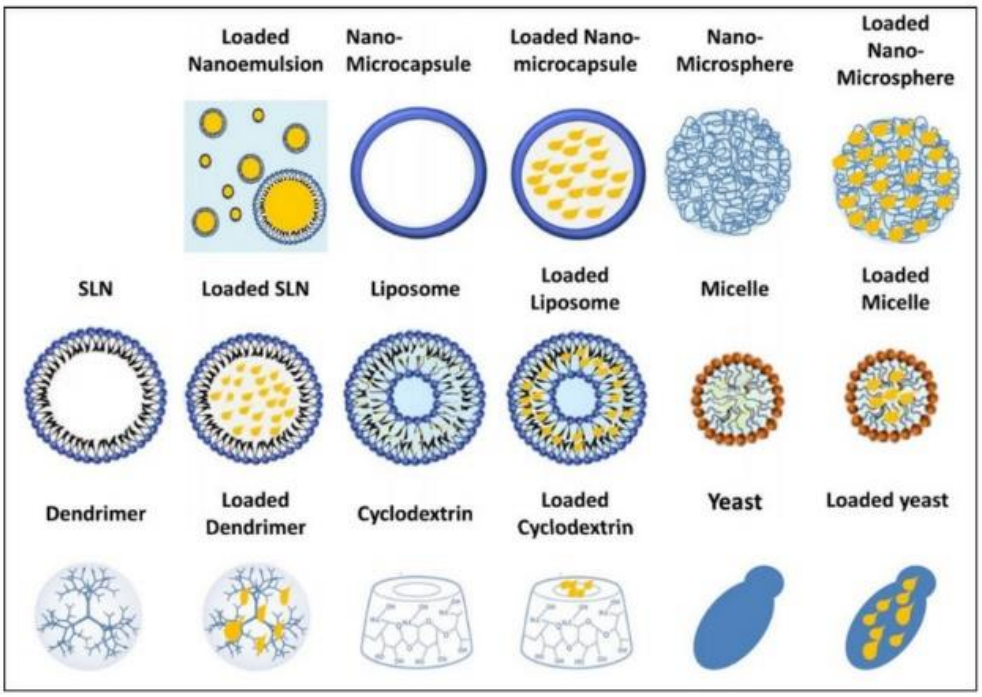


Figure 12. Different encapsulation structures for bioactive oils, comparing loaded and unloaded nanosystems.[109]

The encapsulation system is selected according to the intended use in the final formulation, which may vary depending on size, shape or nature. Microparticles, nanoparticles, lipid nanoparticles, liposomes, micelles, nanoemulsions, ethanol injection and polymer-based nanoemulsions stand out. For nanoencapsulation studies in this dissertation, the compound 2-(dodecylamino)benzoic acid **8** was used. Liposomes were prepared using a commercial lipid mixture used in food industry, EggPC (egg phosphatidylcholine): cholesterol (7:3)[110] and the tecnhique used was the ethanolic injection method,[43] where simultaneous injection of the extract and lipid were carried out, under vigorous vortexing, in an aqueous buffer solution.

Nanotechnology is exploited for many applications in the biological, agricultural, chemical, medical and cosmetics industries and many more. However, the use of (nanoparticles) nanotechnology in agriculture is still in its premature stage.[73] The use of nanoencapsulated formulations, although allowing an increase in efficiency, can substantially reduce the amount of active compound present and the toxic effect by limiting its exposure.[83]

Nanosilica, which prepared from silica, is used as nano-pesticide. The exoskeleton of insects is composed of lipid which is used as a water barrier to protect the insects and

thereby prevent death from desiccation.[73] However, nanosilica of 3–5 nm gets absorbed into the exoskeleton (cuticle) lipids by physisorption and thereby causes death of insects.[111]

Chapter 2 – Results and discussion

2.1. Introduction

In recent years, essential oils and other plant components became important natural sources of biological active compounds and/or starting materials for obtaining new derivatives by synthetic modifications with improved activities suitable for various applications, including as pesticides. In fact, insecticidal, repellent, fumigant and antifeedant activities against a wide variety of insects[3,4] are relevant for their possible use in the control of insect pests.

In the present dissertation extraction of essential oils, such as anethole, estragole and eugenol was carried out, as well as a less volatile plant component, a ginkgolic acid.

The isolated eugenol was used as starting material for the preparation of two derivatives, while the ginkgolic acid served as an inspiration for the preparation of 2-(dodecylamino)benzoic acid. In addition, another natural compound, 4-hydroxyquinoline, was used for obtaining an *O*-alkylated derivative.

Considering the demand for also new alternative synthetic pesticides with less harmful properties than the conventional, a series of benzamides were prepared using chlorobenzoic acids and bromoaniline or aminoethylcarbazole.

All compounds were evaluated for their potential biological activity against *Sf9* insect cell lines. Studies on the encapsulation of one of the most promising compounds were also carried out.

This work is part of a funded FCT project (PTDC/ASP-AGR/30154/2017), which main application interests are related to environment-friendly preservatives and (bio)pesticides preparation.

2.2. Extraction of plant components

For obtention of the essential oils and other plant components, the hydrodistillation and Soxhlet extraction are among the most used techniques and were chosen to use in this work. As before mentioned, the hydrodistillation comprises the immersion of the vegetable raw material in water, boiling heating and subsequent liquid-liquid extraction of the distillate obtained with an organic solvent in which the oil is soluble followed by

evaporation on the rotary evaporator. The Soxhlet extraction method uses a specific laboratory device, named Soxhlet extractor comprising three main units: a percolator (distillation flask and reflux condenser), where the extraction solvent is refluxed, a cellulose cartridge with the function of retaining solid particles (plant matter) and a siphon that periodically empties the chamber where the cartridge is located.[112]

2.2.1. Extraction of essential oils

Star anise (*Illicium verum*) was subjected to hydrodistillation to give an off-white oily extract. ^{1}H NMR spectrum analysis revealed a pure compound, 1-methoxy-(4-prop-1-en-1-yl-benzene), anethole **1** (Figure 13). The main characteristics of the spectrum are the presence of a singlet at δ 3.83 ppm corresponding to the OCH_3 group, double bond protons as a multiplet at δ 6.09-6.18 ppm ($CH=CH_2CH_3$) and a double doublet at δ 6.40 ppm ($CH=CH_2CH_3$), as well as the CH_3 group as quartet at δ 1.91 ppm.

In case of fennel (*Foeniculum vulgare*), seeds were hydrodistilled to give a yellow oily extract. ^{1}H NMR spectrum analysis revealed a presence of a pure compound, 1-allyl-4-methoxybenzene, estragole **2** (Figure 13). The main characteristics of the spectrum are the presence of a singlet at δ 3.82 ppm corresponding to the OCH_3 group, the double bond protons as multiplets at δ 5.02-5.51 ppm ($CH=CH_2$), and δ 5.95-6.02 ppm ($CH=CH_2$).

Clove (*Syzygium aromaticum*) was also hydrodistilled and an off-white oily extract was obtained. ^{1}H NMR spectrum analysis revealed a pure compound identified as 4-allyl-2-methoxyphenol, eugenol **3** (Figure 13). The main characteristics of the spectrum are the presence of a singlet at δ 3.89 ppm corresponding to the OCH_3 group, double bond protons, as multiplets at δ 5.13-5.18 ppm ($CH=CH_2$), and δ 6.0-6.07 ppm ($CH=CH_2$).

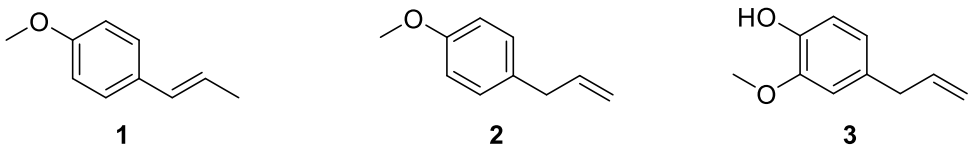
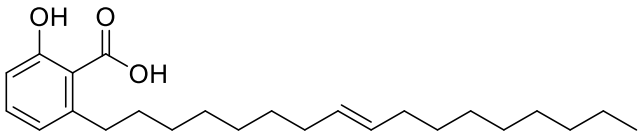


Figure 13. Structure of anethole **1**, estragole **2**, and eugenol **3**.

2.2.2. Extraction of Ginkgo Biloba components

Ginkgo biloba fresh leaves were subjected to Soxhlet extraction with dichloromethane for 4 hours. The off-white extract obtained was characterized by ^{1}H NMR, which has shown the presence of the main component, ginkgolic acid **4** (C 17:1) (Figure 14). Main features of the ^{1}H NMR spectrum are the presence of signals at the aromatic region, namely H-5 and H-3 as double doublets at δ 6.77 and 6.87 ppm, respectively, and H-4 as a triplet at δ 7.36 ppm, the alkenic protons showed up at δ 5.35 ppm as a triplet and methyl protons as a multiplet at δ between 0.87 and 0.92 ppm.



4

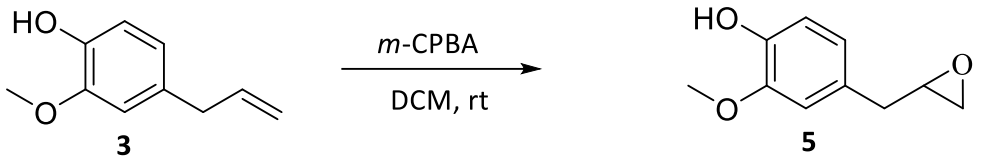
Figure 14. Structure of 2-(heptadec-8-en-1-yl)-6-hydroxybenzoic acid, ginkgolic acid C17:1 **4**.

2.3. Synthesis of eugenol derivatives 5 and 6

Estragole, anethole and eugenol are phenylpropanoids, which have a double bond with potential for further functionalization, namely through epoxidation reaction. The epoxide itself can also be further reacted with nucleophiles (O-, N- and S-) in order to obtain the corresponding derivatives. The use of epoxides, three-membered heterocyclic rings, as intermediates is very useful in pharmaceutical and agrochemical industries. The reaction of epoxides with amines resulted in β-amino alcohols, which are present in several biologically active compounds; they are important pharmacophores[113,114] and also interesting building blocks in the preparation of added-value chemicals.[115,116]

In addition, eugenol derivatives are potentially relevant as pesticides. Thus, the obtained eugenol **3** was reacted with *m*-chloroperoxybenzoic acid (*m*-CPBA), in dichloromethane, at room temperature for 24 hours to give the corresponding epoxide, 2-methoxy-4-(oxiran-2-ylmethyl)phenol **5** in 16 % yield (Scheme 1). The ^{1}H NMR spectrum shows the presence of compound **5** through the signals of the CH of oxirane as

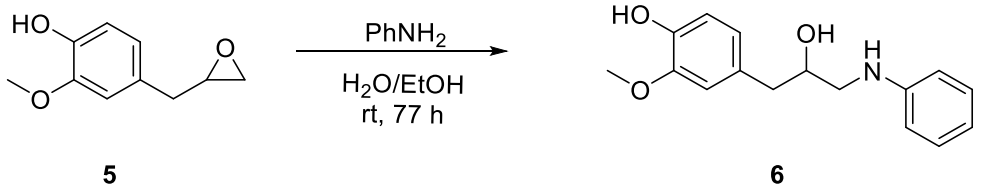
a multiplet δ 3.12-3.16 ppm, and new signals of the CH_2 of oxirane, a quartet at δ 2.55 and a multiplet at δ 2.79-2.82 ppm.



Scheme 1. Synthesis of 2-methoxy-4-(oxiran-2-ylmethyl)phenol **5**.

Epoxide **5** ring opening was performed with aniline in water/ethanol (2:1), with heating at 50ºC for 5.5 hours. 4-(2-Hydroxy-3-(phenylamino)propyl)-2-methoxyphenol **6** was obtained in 36%, and its structure was confirmed by NMR (Scheme 2).

The ^1H NMR showed the signals for protons of the CHOH, CH_2N, and OCH_3 groups. The CH_2N protons appeared as two distinct doublet of doublets (δ 3.10 and 3.31 ppm); the CHOH proton displays as a multiplet in all compounds (δ 4.02-4.09 ppm); and the OCH_3 group show up as a singlet (δ 3.87 ppm). The ^{13}C main features are the CH_2N (δ 41.2 ppm), CHOH carbon (δ 71.1 ppm), OCH_3 carbon (δ 55.9 ppm).



Scheme 2. Synthesis of 4-(2-hydroxy-3-(phenylamino)propyl)-2-methoxyphenol) **6**.

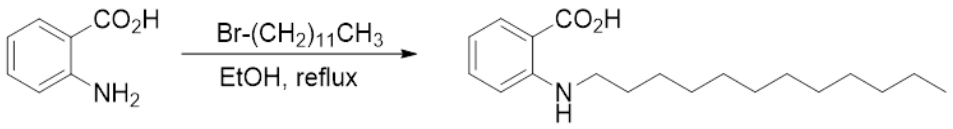
2.4. Synthesis of 2-(dodecylamino)benzoic acid 8

2-Amino-benzoic acid **7**, trivially named anthranilic acid, is a metabolite produced in L-tryptophan-kynurenine pathway in the central nervous system, being an important intermediate in the biosynthesis of acridone and quinolinone alkaloids, which occur in greatest abundance in some plant families.[117]

Also inspired in the structure of ginkgolic acids, one of which was isolated in the present work, possessing long aliphatic chains in their structures, it was decided to synthesize a *N*-alkylated derivative of 2-aminobenzoic acid, bearing a quite long alkyl

chain. Thus, by reaction of 2-aminobenzoic acid **7** with 1-bromododecane, in ethanol, by reflux, for 64h (Scheme 3). After purification by column chromatography, 2-(dodecylamino)benzoic acid **8** was obtained in 50% yield.

The ^{1}H NMR spectra showed the signals of aliphatic protons, such as from methylenic groups of the amino substituent, directly linked to the nitrogen atom NH*CH*$_2$ as a triplet (δ 3.13 ppm), as well as the group close to the same atom NHCH$_2$*CH*$_2$ as a multiplet (δ 1.53-1.60 ppm), and the terminal methyl group as a triplet (δ 0.84 ppm). In addition, spectra showed the aromatic protons as four signals at δ 6.52-7.76 ppm. The ^{13}C NMR spectra showed the signals of methylenic groups, namely directly linked to the nitrogen atom NH*CH*$_2$ (δ 41.95 ppm), as well as the others in the range δ 22.08-31.28 ppm; the terminal methyl group show occurred at δ 13.93 ppm. The carbon of carboxylic acid group appeared at δ 170.06 ppm.



7 8

Scheme 3. Synthesis of 2-(dodecylamino)benzoic acid **8**.

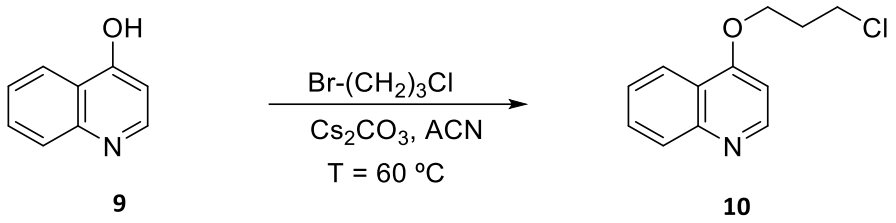
2.5. Synthesis of 4-(3-chloropropoxy)quinoline 10

The quinoline core is present in natural products that display bioactivity, and together with its derivatives have attracted great attention in the field of drug development, and in pesticides uses. 4-Hydroxyquinoline is a hydroxylated quinoline derivative with antimicrobial activity.[118]

Considering the importance of quinoline-based compounds, in the present work, 4-hydroxy quinoline was *O*-alkylated by reaction with 1-bromo-3-chloropropane in acetonitrile, under heating at 60 ºC, in the presence of caesium carbonate as base (Scheme 4).

The 4-(3-chloropropoxy)quinoline **10** obtained in 26% yield, was characterized by ^{1}H and ^{13}C NMR. In the ^{1}H NMR spectrum, signals related to the methylene protons of the new substituent are showed as a quintet (δ 2.40 ppm) and triplets (δ 3.83-4.35 ppm), in addition to the aromatic signals related to the quinoline core (δ 6.76-8.74 ppm). The ^{13}C

NMR spectrum showed the carbons of the methylene groups (δ31.78-60.35 ppm), as well as the aromatic carbons (δ100.64-161.43 ppm).

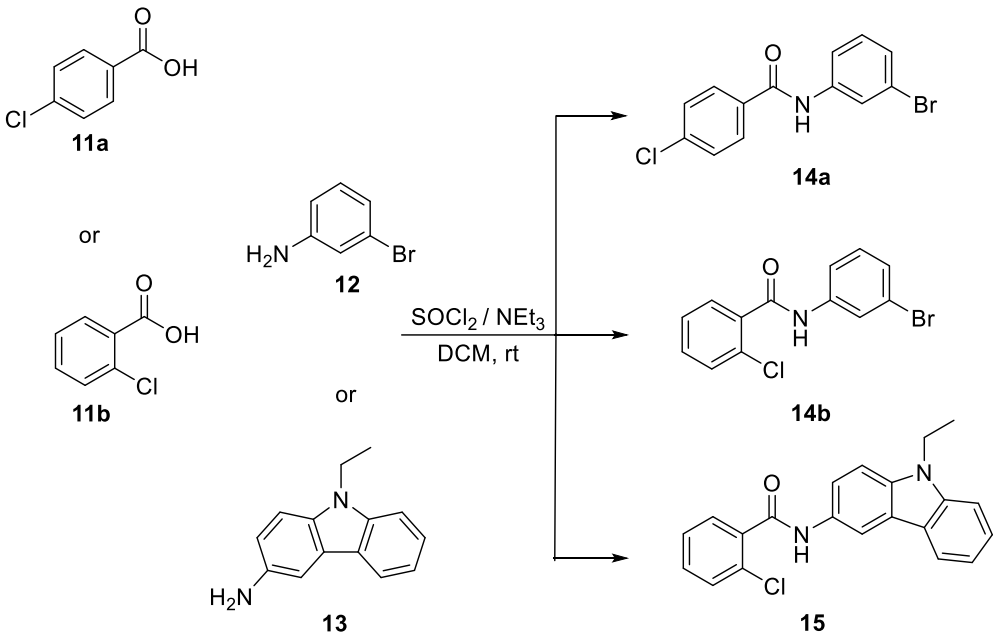


9 **10**

Scheme 4. Synthesis of 4-(3-chloropropoxy)quinoline **10**.

2.6. Synthesis of benzamides 14a,b and 15

Considering the importance of new alternatives to synthetic conventional pesticides, in the present work, it was made an attempt to find (semi)synthetic alternatives with high and selective activity to insects, but nontoxic for human cells and environmentally safe. Thus, by reaction of 4-chlorobenzoic acid **11a** or 2-chlorobenzoic acid **11b** and 3-bromoaniline **12**, with thionyl chloride and triethylamine, at room temperature, *N*-(3-bromophenyl)-4-chlorobenzamide **14a** and *N*-(3-bromophenyl)-2-chlorobenzamide **14b** were obtained. Furthermore, 2-chlorobenzoic acid **11b** was also reacted with 9-ethyl-*9H*-carbazol-3-amine **13**, through the same procedure, and 2-chloro-*N*-(9-ethyl-*9H*-carbazol-3-yl)benzamide**15** was obtained (Scheme 5). After flash chromatography purification, all compounds were isolated in moderate yields, and ^{1}H and ^{13}C NMR confirmed their structures.



Scheme 5. Synthesis of benzamides *N*-(3-bromophenyl)-4-chlorobenzamide **14a**, *N*-(3-bromophenyl)-2-chlorobenzamide **14b** and *N*-(3-bromophenyl)-2-chlorobenzamide **15**.

The ^{1}H NMR of compounds **14a,b** and **15** showed the aromatic protons of the carboxylic acid units, in addition to the amines protons (δ 7.85-8.06 ppm), highlighting the H-3 and H-5 protons of 4-Cl-Ph as double triplets (δ 7.44-7.48 ppm, **14a**) and of 2-Cl-Ph as multiplets (δ 7.22-7.47 ppm, **14b**, **15**), and also the H-2 and H-5 protons of 3-Br-Ph as triplets (δ 7.14-7.90 ppm, **14a**,**b**), and of the carbazole nucleus as doublets, double doublets or double triplets (7.22-8.47 ppm, **15**). In the ^{13}C NMR stands out the carbon signal of the amide linkage (δ at about 164.5 ppm).

2.7. Insecticidal studies

2.7.1. Assays in *Sf9*

The assays of the biological activity of compounds **1**, **3**-**8**, **10**, **14a** and **15** performed in *Spodoptera frugiperda* (*Sf9*) cells were carried out at REQUIMTE of the Faculdade de Farmácia da Universidade do Porto, within the scope of the project "BioP&FoodP - Biopesticides and Food Preservatives of Nanoencapsulated Plant Extracts: Biological Assessment, Modeling and Molecular Synthesis", in which this dissertation is integrated. All studies were performed with the same concentration of compound (100 μg/mL), so

that a direct comparison of all results was possible. These results are generated based on the loss of cell viability, in percentage (%).

Cell viability assays allow analysing cell proliferation, determining how metabolically active cells are in a cell culture, and are often performed to identify molecules that have effects on cell proliferation or toxic effects that can eventually induce cell death.[112]

The study of the insecticidal activity of compounds **1**, **3**-**8**, **10**, **14a** and **15** was carried out in two-dimensional (2D) cultures of *Sf9* cells, which are derived from ovary cells of *Spodoptera frugiperda*, a common plague. In these assays, it is expected that the compound presents a low percentage of cell viability, that is, the lower the percentage of viability associated with the greater the loss of viability, the compound being more toxic, having greater insecticidal activity.

Figure 15 summarizes the biological activity results obtained in *Sf*9 as a function of percent cell viability of all compounds tested, **1**, **3**-**8**, **10**, **14a** and **15**. Based on these results, some considerations can be drawn regarding the potential of the compounds obtained to act as (bio)insecticides/semisynthetic insecticides.

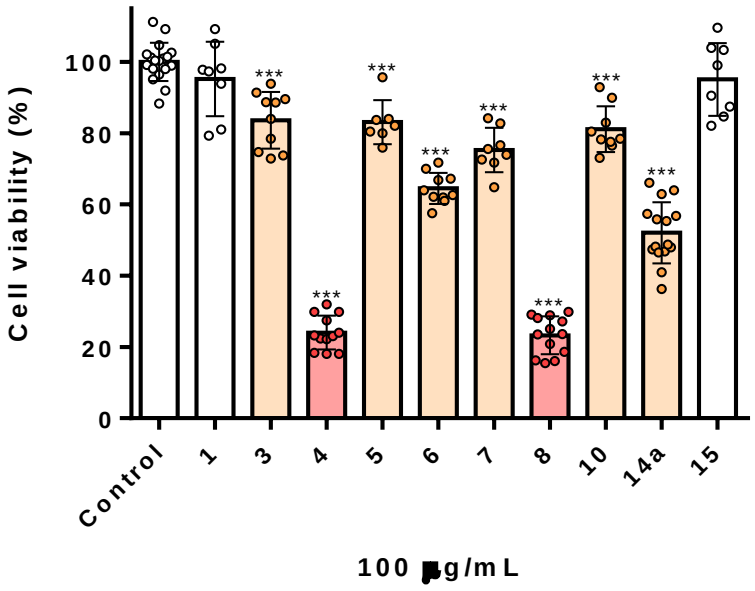


Figure 15. Percent cell viability of control, anethole **1**, eugenol **3**, ginkgolic acid C17:1 **4**, 2-methoxy-4-(oxiran-2-ylmethyl)phenol **5**, 4-(2-hydroxy-3-(phenylamino)propyl)-2-methoxyphenol) **6**, 2-amino-benzoic acid **7**, 2-(dodecylamino)benzoic acid **8**, 4-(3-chloropropoxy)quinoline **10**, *N*-(3-bromophenyl)-4-chlorobenzamide **14a**, *N*-(3-bromophenyl)-2-chlorobenzamide **15**. Results correspond to the mean ± SD of at least three independent assays.

Regarding the EOs anethole **1** and eugenol **3** it is possible to see that both show a high percentage of cell viability in *Sf*9, at about 95% and 85%, respectively, meaning that only eugenol **3** has some activity, but very low. In the case of the less volatile natural component extracted from *Ginkgo Biloba*, the ginkgolic acid C17:1, it is found to have high activity, with a percentage of cell viability of approximately 20%.

In order to evaluate the behaviour of eugenol **3** derivatives, its epoxide, 2-methoxy-4-(oxiran-2-ylmethyl)phenol **5** and also the β-amino alcohol resulting from the epoxide ring opening with aniline, namely 4-(2-hydroxy-3-(phenylamino)propyl)-2-methoxyphenol) **6** were tested. In comparison with the eugenol activity **3**, the results show that its conversion to the corresponding epoxide practically does not affect cell viability, remaining around 80%. However, its transformation into the corresponding β-amino alcohol induce a very relevant increase in activity, changing the cell viability to approximately 60%. Although the transformation of eugenol **3** into epoxide **5** is not relevant to the activity in *Sf*9, its synthesis was very important, as it worked as an intermediate for the synthesis of amino alcohol **6** from eugenol **3**.

Regarding 2-(dodecylamino)benzoic acid **8** in comparison with its precursor, 2-aminobenzoic acid **7**, which display a cell viability in *Sf*9 inferior to 80%, its activity is much higher being the cell viability at about 20%. Compound **8** has a long aliphatic chain and a carboxylic acid function, and as mentioned before its synthesis was inspired in ginkgolic acid **4**. By comparison of the activity in *Sf*9 cells of compounds **8** and **4**, it is possible to conclude that their insecticidal activity is similar.

The 4-(3-chloropropoxy)quinoline **10** obtained by *O*-alkylation of quinoline shows a cell viability of approximately 80%. This result encourages to the synthesis of other derivatives in order to evaluate the effect of these alterations in the biological activity.

Finally, were tested two benzamides: *N*-(3-bromophenyl)-4-chlorobenzamide **14a** and *N*-(3-bromophenyl)-2-chlorobenzamide **15**, and the results show that compound **15** has almost no activity, but compound **14a** display a cell viability of 50%, which is a very relevant value.

Thus, from the evaluated compounds, those with best activity in *Sf*9, and the most promising as insecticides are compounds **4**, **8** and **14a**.

Overall, biological activity of compounds **1**, **3**-**8**, **10**, **14a** and **15** performed in *Spodoptera frugiperda* cells allowed to identify compounds that have high activity and

generate knowledge to be applied in the design of new derivatives through structural changes in natural or synthetic precursors, in order to obtain molecules with insecticidal potential that can function as interesting alternatives such as (bio)insecticides or semisynthetic insecticides to the currently existing conventional synthetic insecticides.

2.8. Nanoencapsulation studies

2.8.1. Encapsulation efficiency

For nanoencapsulation studies, the compound 2-(dodecylamino)benzoic acid **8** was used. Liposomes were prepared using a commercial lipid mixture used in food industry, EggPC (egg phosphatidylcholine): cholesterol (7:3),[110] and ethanolic injection method for the preparation of extract-loaded nanocarriers was used. The ethanolic injection method has been shown to be adequate for enhanced encapsulation of poorly water-soluble compounds.[43] Table 2 shows the determined encapsulation efficiencies only for ethanolic injection preparation method.

Table 2. Encapsulation efficiency, EE (%) ± SD (%), of 2-(dodecylamino)benzoic **8** acid in liposomes prepared by the ethanolic injection method (SD: Standard Deviation).

Sample	Ethanolic injection	EE (%) ± SD (%)
1	99.4	
2	99.2	99.0 ± 0.4
3	98.4	

The encapsulation efficiencies are very good for the ethanolic injection method. Nevertheless, the high EE% values obtained (Table 2) point to promising future applications of the extract-loaded soybean liposomes as green insecticides, with the possibility of controlled release of the encapsulated compounds.

Chapter 3: Conclusions and future perspectives

With the accomplishment of the experimental work related to this dissertation, it was possible to extract three essential oils, namely anethole **1**, estragole **2** and eugenol **3** by hydrodistillation, and a less volatile component ginkgolic acid C17:1 **4** by Soxhlet extraction, from the corresponding natural materials.

Two eugenol derivatives, the epoxide **5** and a β-amino alcohol **6** were synthesised. 2-(Dodecylamino)benzoic acid **8** and 4-(3-chloropropoxy)quinoline **10** were also synthesised.

In addition, using 4-chlorobenzoic or 2-chlorobenzoic acids and 3-bromoaniline or 9-ethyl-9*H*-carbazol-3-amine, three benzamides **14a**, **14b** and **15** were obtained.

All compounds were adequately purified, usually by column chromatography, and characterised by the usual analytical techniques.

As part of a collaboration, biological activity results were obtained in *Spodoptera frugiperda*, *Sf*9, cells of compounds **1**, **3**-**8**, **10**, **14a** and **15**.

Compounds **1** and **3** were submitted to tests of the biological activity in *Sf9* to evaluate its application as potential (bio)insecticides. Cell viability remains high, with eugenol **3** showing approximately 85% of cell viability, which means a reduced insecticide potential. Ginkgolic acid **4**, extracted from *Ginkgo Biloba* was submitted to biological activity tests in *Sf9* to evaluate its potential as bioinsecticide. This compound has a cell viability of approximately 20% so it is a potential good insecticide.

In order to evaluate the behaviour of eugenol **3** derivatives, its epoxide, 2-methoxy-4-(oxiran-2-ylmethyl)phenol **5**, and also the β-amino alcohol resulting from the epoxide ring opening with aniline, namely 4-(2-hydroxy-3-(phenylamino)propyl)-2-methoxyphenol) **6** were tested. In comparison with the eugenol activity **3**, the results show that its conversion to the corresponding epoxide practically does not affect cell viability, remaining around 80%. However, its transformation into the corresponding β-amino alcohol induce a very relevant increase in activity, changing the cell viability to approximately 60%.

The synthesised 2-(dodecylamino)benzoic acid **8** display a biological activity similar to ginkgolic acid **4**, with 20% of cell activity in *Sf*9, which is a relevant result and a potential candidate as semisynthetic insecticide.

The 4-(3-chloropropoxy)quinoline **10** obtained by *O*-alkylation of quinoline shows a cell viability of approximately 80%. This result encourages us to the synthesis of other derivatives in order to evaluate the effect of these alterations in the biological activity.

From the three benzamides obtained, two of them, *N*-(3-bromophenyl)-4-chlorobenzamide **14a** and *N*-(3-bromophenyl)-2-chlorobenzamide **15** were evaluated in *Sf*9 cells. The results show that compound **15** has almost no activity, but compound **14a** display a cell viability of 50%, which is a very relevant value.

Overall, the results show that the most relevant compounds are ginkgolic acid **4**, 2-(dodecylamino)benzoic acid **8** and *N*-(3-bromophenyl)-4-chlorobenzamide **14a**, being very promising (bio)insecticides/semisynthetic insecticides.

Nanoencapsulation studies carried out with one of the most relevant active molecules, 2-(dodecylamino)benzoic acid **8** in liposomes, by ethanolic injection method, show high encapsulation efficiencies (99%), which point to promising future applications in green insecticides formulations, with the possibility of controlled release of the encapsulated compounds.

In the near future, it is expected to proceed with the design and synthesis of new derivatives of natural compounds, in order to obtain semisynthetic insecticides formulations by combining the experimental results of activity in *Sf9* with ongoing computational studies, as well as carrying out the corresponding nanoencapsulation and controlled release assays. Thus, it is intended to contribute to the development of derivatives of compounds of botanical origin that work as promising alternatives to currently available harmful synthetic insecticides.

Chapter 4: Experimental tests

4.1. Material and Methods

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III 400 apparatus at a frequency of 400 MHz to ^{1}H and 100.6 MHz to ^{13}C, using the solvent peak as an internal reference, at 25° C. The chemical shifts are reported in ppm, using the value δ_H Me_4Si = 0 ppm as reference, and the coupling constants (*J*) are in Hz. The assignment of the ^{1}H and ^{13}C signals was executed by comparison of chemical shifts, multiplicity of peaks and *J* values, and it was also used double-decoupling techniques, as well as two-dimensional heteronuclear spectroscopic correlation, namely HMQC and HMBC. The deuterated solvent used was chloroform with deuteration degree greater than 99.8% of Eurisotop® and DMSO from same brand. TLC analyses were performed on 0.25 mm thick silica gel plates (Merck Fertigplatten Kieselgel 60F254) and in the development, visual detection and ultraviolet light (λ_{max} = 254 nm) were used in a CN-6 chamber. Column and dry flash chromatographies used Kieselgel Merck 60 (230-400 mesh). The solvents used were p.a. products supplied by Fisher Chemical.

The *Gingko Biloba* leaves were collected in Tenões-Nogueiró, Braga in November and December 2019. Leaves of green and yellow colour were used due to the changes of seasons. The other vegetal materials were commercially obtained.

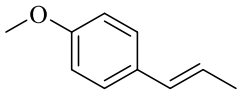
4.2. Extraction of plant components

4.2.1 General procedure to hydrodistillation for essential oils extractions

To a round-bottom flask containing distilled water (200 mL) was added the material in pre-crushed or cut pieces (20 g-40 g) and the mixture was refluxed during 2 h.

The distillate was extracted with dichloromethane (3 × 150 mL), the organic phase was dried over anhydrous magnesium sulphate, and solvent evaporation under vacuum.

4.2.1.1. Extraction of anethole **1** from *Illicium verum* (star anise)



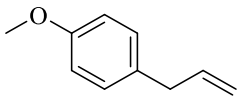
1

Using *Illicium verum* (star anise) (40.0 g), 1-methoxy-(4-prop-1-en-1-yl-benzene), anethole **1**was obtained as an off-white oily.

η = 3 % (w/w)

^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 1.91 (3H, q, *J* = 1.6 Hz, CHC*H*$_3$), 3.83 (3H, s, OC*H*$_3$), 6.09-6.18 (1H, m, CH=C*H*CH$_3$), 6.40 (1H, dd, *J* =14 Hz and 1.6 Hz, C*H*=CHCH$_3$), 6.88 (2H, d, *J* = 8.8 Hz, H-2 and H-6), 7.30 (2H, d, *J* = 8.8 Hz, H-3 and H-5) ppm.

4.2.1.2. Extraction of estragole **2** from *Foeniculum vulgare* (fennel)



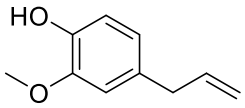
2

Using *Foeniculum vulgare* (fennel) (40.0 g), 1-allyl-4-methoxybenzene, estragole **2** was obtained as a yellow oil.

η = 1 % (w/w)

^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 3.36 (2H, d, *J* = 6.4 Hz, C*H*$_2$Ph), 3.82 (3H, s, OC*H*$_3$), 5.02-5.51 (2H, m, CH=C*H*$_2$), 5.95–6.02 (1H, m, C*H*=CH$_2$), 6.88 (2H, d, *J* = 6.4 Hz, H-3 and H-5), 7.13 (2H, d, *J* = 6.4 Hz, H-2 and H-6) ppm.

4.2.1.3. Extraction of eugenol **3** from *Syzygium Aromaticum*



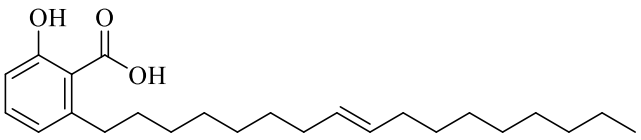
3

Using *Syzygium aromaticum* (cloves) (21.4 g), 4-allyl-2-methoxyphenol, eugenol **3** was obtained as an off-white oil.

η = 14 % (w/w)

^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 3.39 (2H, d, *J* = 6.8 Hz, C*H*$_2$Ph), 3.89 (3H, s, OC*H*$_3$), 5.13-5.18 (2H, m, CH=C*H*$_2$), 5.81 (1H, broad s, OH), 6.00–6.07 (1H, m, C*H*=CH$_2$), 6.74-6.79 (2H, m, H-3 and H-5), 6.93 (1H, d, *J* = 8.4 Hz, H-6) ppm.

4.3. Soxhlet Extraction of ginkgolic acid C17:1 4 from *Ginkgo Biloba*



4

To a flask containing DCM (750 mL) was coupled a Soxhlet having a cellulose cartridge where the leaves of *Ginkgo Biloba* (25.3 g) were placed. The extraction system was assembled and the solvent was refluxed during 4 h. The solvent was evaporated and the extract was purified by column chromatography, using petroleum ether/ethyl acetate (1:3), as the eluent. 2-(Heptadec-8-en-1-yl)-6-hydroxybenzoic acid, ginkgolic acid **4** was obtained as a beige oily solid.

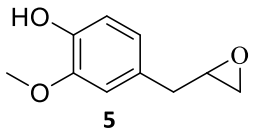
η = 4 % (w/w)

^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 0.92-0.87 (3H, m, C*H*$_3$), 1.32-1.23 (16H, m, 10×C*H*$_2$), 1.64-1.57 (2H, m, C*H*$_2$), 2.08-1.99 (4H, m, 2×C*H*$_2$), 2.98 (2H, t, *J* = 8.0 Hz, Ph-C*H*$_2$), 5.35 (2H,

t, *J* = 4.8 Hz, *H*C=C*H*), 6.77 (1H, dd, *J* = 7.6 and 0.8 Hz, H-5), 6.87 (1H, dd, *J* = 8.4 and 1.2 Hz, H-3), 7.36 (1H, t, *J* = 8.0 Hz, H-4), 11.07 (1H, brs, CO_2H) ppm.

4.4. Synthesis of eugenol derivatives 5 and 6

4.4.1. Synthesis of 2-methoxy-4-(oxiran-2-ylmethyl)phenol 5



5

A solution of eugenol **3** (0.400 g 2.44 mmol; 1 equiv) dissolved in dichloromethane (10 mL) was added dropwise to a suspension of 70% *m*-chloroperbenzoic acid (0.60 g; 3.48 mmol; 1 equiv) in dichloromethane (10 mL) at 0 ºC. After stirring for 1 h, *m*-chloroperbenzoic acid was again added (1 equiv), and the reaction mixture was stirred for another 24 h at room temperature. A 10% aqueous solution of sodium sulphate (2 × 20 mL) was added, and the resulting solution was washed with 5% aqueous solution of sodium hydrogen carbonate (2 × 20 mL). The organic phase was dried with anhydrous magnesium sulphate, and the solvent was evaporated to afford the compound **5** as a dark yellow oil.

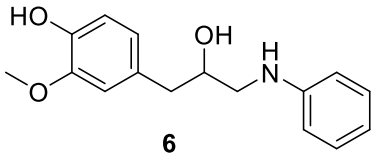
η = 67 % (0.293 g)

R_f = 0.27 (DCM)

^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 2.55 (1H, q, *J* = 2.8 Hz, C*H*$_2$ oxirane), 2.79–2.82 (3H, m, C*H*$_2$Ph and C*H*$_2$ oxirane), 3.12–3.16 (1H, m, C*H* oxirane), 3.90 (3H, s, OC*H*$_3$), 5.54 (1H, s, OH), 6.73–6.78 (2H, m, H-3 and H-5), 6.87 (1H, d, *J* = 8 Hz, H-6) ppm.

^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C 38.37 (CH_2Ph), 46.79 (CH_2 oxirane), 52.67 (CH oxirane), 55.90 (OCH_3), 111.54 (C-3), 114.32 (C-6), 121.64 (C-5), 129.03 (C-4), 144.39 (C-1), 146.46 (C-2) ppm.

4.4.2. Synthesis of 4-(2-hydroxy-3-(phenylamino)propyl)-2-methoxyphenol) 6



6

To a suspension of 2-methoxy-4-(oxiran-2-ilmethyl)phenol **5** (0.174 g, 0.97 mmol, 1 equiv) in H_2O/EtOH 2:1 (2 mL) was added aniline (0.4 mL, 3.78 mmol, 3.9 equiv), and the resulting mixture was heated at 50 ºC for 5.5 h. Then, water (2 mL) was added, and the resulting mixture was extracted with EtOAc (2 mL). The organic phase was collected, dried with anhydrous $MgSO_4$, and the solvent was evaporated to afford an oil (0.284 g), which was subjected to column chromatography using DCM/MeOH as eluent of increasing polarity to give compound **6** as a yellow oil.

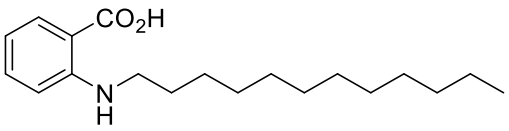
η = 36 % (0.095 g)

R_f = 0.7 (MeOH/DCM 5:95)

^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 2.75 (1H, dd, *J* = 14 and 8 Hz, C*H*$_2$CH(OH)), 2.83 (1H, dd, *J* = 14 Hz and 5.2 Hz, C*H*$_2$CH(OH)), 3.10 (1H, dd, *J* = 12.4 and 8 Hz, C*H*$_2$NH), 3.31 (1H, dd, *J* = 12.4 Hz, 7.2 Hz, C*H*$_2$NH), 3.87 (3H, s, OC*H*$_3$), 4.02-4.09 (1H, m, CH$_2$C*H*(OH)), 6.65 (2H, d, *J* = 8 Hz, Ar-H), 6.75 (3H, m, Ar-H), 6.88 (1H, d, *J* = 8 Hz, Ar-H), 7.19 (t, *J* = 7.2 Hz, 2 H, Ar-H) ppm.

^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C 41.2 (CH_2), 49.5 (CH_2), 55.9 (OCH_3), 71.1 (CH), 111.8 (Ar-C), 113.5 (Ar-C), 114.5 (Ar-C), 118.1 (Ar-C), 122.0 (Ar-C), 129.3 (Ar-C), 129.4 (Ar-C), 144.4 (Ar-C), 146.6 (Ar-C), 147.9 (Ar-C) ppm.

4.5. Synthesis of 2-(dodecylamino)benzoic acid 8



8

1-Bromododecane (0.398 g, 2.90 mmol) was added to a solution of 2-amino-aminobenzoic acid **7** (0.2 g, 1.50 mmol). The reaction mixture was refluxed in ethanol (14 mL) for a certain time (64 h) and monitored by TLC (DCM/MeOH 9:1). The solvent was evaporated and the residue obtained was subjected to purification by column chromatography, with DCM/MeOH, as eluent, to afford compound 2-(dodecylamino)benzoic acid, **8** as a grey solid.

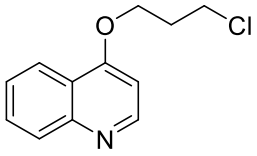
η = 50 % (0.23 g)

R_f = 0.33 (DCM/MeOH 9:1).

^{1}H RMN (DMSO, 400 MHz): δ_H 0.84 (3H, t, *J* = 6.8 Hz, C*H*$_3$), 1.35-1.25 (18H, m, 9×CH_2), 1.60−1.53 (2H, m, NHCH$_2$*CH*$_2$), 3.13 (2H, t, *J* = 7.2 Hz, NH*CH*$_2$), 6.52 (1H, t, *J* = 6.8 Hz, H-5), 6.69 (1H, d, *J* = 8.4 Hz, H-3), 7.33 (1H, dt, *J* = 6.8 and 1.6 Hz, H-4), 7.76 (1H, dd, *J* = 6.4 and 1.6 Hz, H-6) ppm.

^{13}C RMN (DMSO, 100.6 MHz): δc 13.93 (CH_3), 22.08 (CH_2), 26.52 (CH_2), 28.56 (CH_2), 28.70 (CH_2), 28.72 (CH_2), 28.95 (CH_2), 28.99 (CH_2), 29.02 (CH_2), 31.28 (CH_2), 41.95 (NH*CH*$_2$), 109.71 (C-1), 111.08 (C-3), 113.90 (C-5), 131.67 (C-6), 134.45 (C-4), 150.96 (C-2), 170.06 (CO_2H) ppm.

4.6. Synthesis of 4-(3-chloropropoxy)quinoline 10



10

Caesium carbonate (1.122 g, 3.45 mmol) and 1-bromo-3-chloropropane (0.068 mL, 0.69 mmol) were added to 4-quinolinol **9** (0.100 g, 0.69 mmol) in acetonitrile at room temperature. Then, the reaction mixture was set to 65º C in oil bath, stirred for 35 hours and monitored by TLC (silica: dichloromethane/methanol (9:1)). The recovery of the reaction product was performed by evaporating the solvent under reduced pressure and the residue obtained was purified by column chromatography,

using dichloromethane/methanol, mixtures of increasing polarity as the eluent. Compound **10** was isolated as yellow oil.

η = 26 % (0.028 g)

R_f = 0.42 (DCM/MeOH 9:1)

M.p. = 150.0-152.0º C

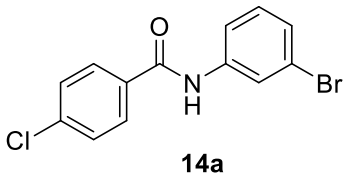
^{1}H RMN ($CDCl_3$, 400 MHz): δ_H 2.40 (2H, quint, *J* = 6.0 Hz, $CH_2CH_2CH_2Cl$), 3.83 (2H, t, *J* = 6.4 Hz, $CH_2CH_2CH_2Cl$), 4.35 (2H, t, *J* = 6.0 Hz, $CH_2CH_2CH_2Cl$), 6.76 (1H, d, *J* = 5.2 Hz, H-2), 7.51 (1H, dt, *J* = 6.8 and 1.2 Hz, H-7), 7.70 (1H, dt, *J* = 6.8 and 1.2 Hz, H-6), 8.07 (1H, d, *J* = 8.0 Hz, H-8), 8.18 (1H, dd, *J* = 8.4 and 1.2 Hz, H-5), 8.74 (1H, d, *J* = 5.2, H-3) ppm.

^{13}C RMN ($CDCl_3$, 100.6 MHz): δc 31.78 ($CH_2CH_2CH_2Cl$), 41.12 ($CH_2CH_2CH_2Cl$), 60.35 ($CH_2CH_2CH_2Cl$), 100.64 (C-2), 121.20 (C-4a), 121.58 (C-5), 125.75 (C-6), 128.49 (C-8), 129.95 (C-7), 148.65 (C-8a), 151.00 (C-3), 161.43 (C-4) ppm.

4.7. Synthesis of benzamides 14a,b and 15

Chlorobenzoic acids **11a** or **11b** (1 equiv) was added to 3-bromoaniline **12** or 3-amino-9-ethylcarbazole **13** (1 equiv) and triethylamine (3 equiv) in dichloromethane (3 mL). Then, thionyl chloride (2 equiv) was added at room temperature. The mixture was stirred for 5 days at room temperature and monitored by TLC (silica: dichloromethane). The recovery of the reaction product was performed by evaporating the solvent under reduced pressure. The resulting residue was taken up in dichloromethane and washed first with 1 M hydrogen chloride (40 mL) and then with 1 M sodium hydroxide (40 mL). The organic phase was dried with magnesium sulphate, evaporated to dryness and purified by flash chromatography (silica: dichloromethane/light petroleum, mixture of increasing polarity) to afford benzamides **14a,b** and **15** as solids.

4.7.1. Synthesis of *N*-(3-bromophenyl)-4-chlorobenzamide 14a



14a

Starting from 4-chlorobenzoic acid **11a** (0.200 g, 1.28 mmol), and using 3-bromoaniline **12** (0.150 mL, 1.28 mmol), triethylamine (0.054 mL, 2.56 mmol) and thionyl chloride (0.27 mL, 3.84 mmol). After purification, compound **14a** was obtained as a yellow solid.

η = 10 % (0.061 g)

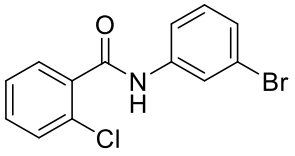
R_f = 0.72 (DCM/Light petroleum 60:40).

M.p.= 123.0-125.0 ºC.

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 7.23 (1H, t, *J* = 8.4 Hz, H-5 Ph-Br), 7.29-7.31 (1H, m, H-4 Ph-Br), 7.46 (2H, d, *J* = 8.8 Hz, H-3 and H-5 Ph-Cl), 7.53-7.56 (1H, m, H-6 Ph-Br), 7.80 (2H, d, *J* = 8.4 Hz, H-2 and H-6 Ph-Cl), 7.89 (2H, t, *J* = 2.0 Hz, H-2 Ph-Br and NH) ppm.

^{13}C NMR (100.6 MHz, $CDCl_3$) δ_C: 118.71 (C-6 Ph-Br), 122.72 (C-3 Ph-Br), 123.21 (C-2 Ph-Br), 127.76 (C-4 Ph-Br), 128.47 (C-2 and C-6 Ph-Cl), 129.12 (C-3 and C-5 Ph-Cl), 130.37 (C-5 Ph-Br), 132.83 (C-1 Ph-Cl), 138.46 (C-4 Ph-Cl), 138.91 (C-1 Ph-Br), 164.68 (C=O) ppm.

4.7.2. Synthesis of *N*-(3-bromophenyl)-2-chlorobenzamide 14b



14b

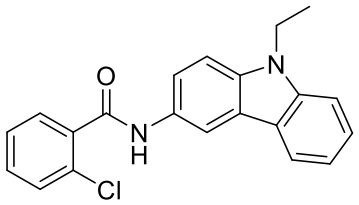
Starting from 2-chlorobenzoic acid **11a** (0.200 g, 1.28 mmol), using 3-bromoaniline **12** (0.150 mL, 1.28 mmol), triethylamine (0.054 mL, 2.56 mmol) and thionyl chloride (0.27 mL, 3.84 mmol). After purification, compound **14b** was obtained as a yellow solid.

η = 14 % (0.085 g)

R_f = 0.68 (silica: dichloromethane).

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 7.23 (1H, t, *J* = 8.0 Hz, H-5 Ph-Br), 7.22-7.35 (4H, m, 4 × H Ph-Cl), 7.47-7.53 (2H, m, H-4 and H-6 Ph-Br), 7.86 (1H, t, *J* = 1.6 Hz, H-2 Ph-Br), 7.89 (1H, s, NH) ppm.

4.7.3. Synthesis of *N*-(3-bromophenyl)-2-chlorobenzamide 15



15

Starting from 2-Chlorobenzoic acid **11b** (0.372 g, 2.74 mmol), using 3-amino-9-ethylcarbazole **13** (0.500 g, 2.74 mmol), triethylamine (0.995 mL, 7.13 mmol) and thionyl chloride (0.345 mL, 4.76 mmol). After purification, compound **15** was obtained as a green solid.

η = 36 % (0.264 g)

R_f = 0.65 (silica: dichloromethane).

M.p. = 162-164 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.44 (3H, t, *J* = 7.2 Hz, C*H*$_3$), 4.38 (2H, q, *J* = 7.2 Hz, C*H*$_2$), 7.24 (1H, dt, *J* = 8.0 and 1.2 Hz, H-Ar), 7.39-7.43 (4H, m, 4 × H Ph-Cl), 7.47-7.51 (2H, m, 2 × H-Ar) 7.64 (1H, dd, *J* = 8.8 and 2.0 Hz, H-Ar), 7.84 (1H, dd, *J* = 6.8 and 2.4 Hz, H-Ar), 8.06 (1H, s, NH), 8.12 (1H, d, *J* = 8.0 Hz, H-Ar), 8.47 (1H, d, *J* = 2.0 Hz, H-Ar) ppm.

^{13}C NMR (100.6 MHz, $CDCl_3$) δ_C 13.79 (CH_3), 37.63 (CH_2), 108.57 (2 × C-PhCl), 113.07 (C-Ar), 118.84 (C-Ar), 119.50 (C-Ar), 120.77 (C-Ar), 122.77 (C-4b), 123.13 (C-4a), 125.95

(C-Ar), 127.29 (C-Ar), 129.31 (C-PhCl), 130.36 (C-Ar), 130.44 (C-PhCl), 130.69 (C-PhCl), 131.52 (C-PhCl), 135.50 (C-Ar), 137.52 (C-9a), 140.48 (C-8a), 164.68 (C=O) ppm.

4.8. Nanoencapsulation studies

For nanoencapsulation studies, the compound 2-(dodecylamino)benzoic acid **8** was used. Liposomes were prepared using a commercial lipid mixture used in food industry, EggPC (egg phosphatidylcholine): Cholesterol (7:3)[110] and the technique used was the ethanolic injection method,[43] where simultaneous injection of the extract and lipid were carried out, under vigorous vortexing, in an aqueous buffer solution.

4.8.1. Encapsulation efficiency

To determine a calibration curve (absorbance *vs.* concentration), concentration dilutions of 1×10^{-6} - 1×10^{-5} mg/mL were performed. Loaded liposomes were subjected to centrifugation at 3000 rpm for 10 minutes using Amicon® Ultra centrifugal filter units 100 kDa. Then, the filtrate (containing the non-encapsulated compound) was pipetted out, the water was evaporated and the same amount of ethanol was added. After vigorous agitation, its absorbance was measured, allowing to determine compound concentration using a calibration curve previously obtained in the same solvent. Absorption spectra were performed in a Shimadzu UV-3600 Plus UV-vis-NIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Three independent measurements were performed for each system. The encapsulation efficiency, EE (%), was obtained through equation 1:

$$EE(\%) = \frac{Total\ amount - Amount\ of\ nonencapsulated\ extract}{Total\ amount} \times 100 \quad \text{(Equation 1)}$$

Chapter 5: Bibliography

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