**Universidade do Minho**
Escola de Ciências

Ana Sofia Pereira de Freitas

# Towards valorization of beehive natural products: unraveling Portuguese propolis bioactivities and potential

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Ana Sofia Pereira de Freitas

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# Towards valorization of beehive natural products: unraveling Portuguese propolis bioactivities and potential

Doctoral Thesis
PhD Thesis in Biology

Work supervised by
**Professor Doctor Cristina Alexandra de Almeida Aguiar**
**Professor Doctor Rui Pedro Soares de Oliveira**

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## Valorização dos produtos naturais da colmeia: desvendando as bioatividades e potencialidades do própolis português


### Resumo

A consciencialização dos consumidores para os problemas ambientais e de saúde aumentou a procura por alternativas naturais e ecológicas de matérias-primas pelas mais variadas indústrias. Os produtos naturais têm sido muito procurados, como é o caso do própolis e do mel, os produtos da colmeia mais populares, produzidos por abelhas e detentores de propriedades excecionais. Própolis, produzido a partir de exsudados de diferentes partes de plantas e misturado com substâncias do metabolismo das abelhas, é um produto natural de composição química variável e baixa produtividade, o que dificulta a sua padronização e o seu uso e comercialização. Assim, os objetivos deste trabalho são melhorar a produção do própolis, valorizá-lo através da caracterização química e biológica e promover a sua aplicação na conceção de produtos funcionais. Com este trabalho mostramos que o uso de grades e/ ou a manipulação do tamanho dos seus poros pode ajudar a aumentar a produção de própolis. Depois de se caracterizar química e biologicamente vários extratos etanólicos de amostras de própolis do Gerês colhida em diferentes anos, a amostra de 2018 (G18) foi extraída com diferentes solventes, na senda de alternativas mais ecológicas e sustentáveis, apresentando elevada capacidade antioxidante e propriedades antimicrobianas com grande potencial para combater infeções fúngicas, como as causadas por *Candida albicans*, e bactérias resistentes como *Staphylococcus aureus* resistente à meticilina (MRSA) e *Escherichia coli*, revelando-se interessante para futuras investigações e utilização em diferentes indústrias, isoladamente ou em combinação com antibióticos ou mesmo com outros produtos da colmeia. O própolis do Gerês parece também ser um agente promissor no tratamento do carcinoma de células renais, quer como alternativa, quer em combinação com os tratamentos anticancerígenos convencionais. Para explorar potenciais aplicações práticas, funcionalizou-se algodão com uma mistura de própolis e mel, tendo o têxtil resultante apresentado grande potencial antioxidante e atividade contra *Propionibacterium acnes*, com boa solidez ao processo de lavagem até pelo menos 3 ciclos de lavagem. Face aos resultados obtidos sobre o potencial do própolis do Gerês para diversas aplicações, trabalhos mais aprofundados para avaliar a possibilidade da sua exploração são essenciais para alcançar a sua padronização e consequente aceitação e comercialização.



**Palavras-chave:** Produtos apícolas, própolis, bioatividades de própolis, funcionalização de tecidos com produtos da colmeia.

## Towards valorization of beehive natural products: unraveling Portuguese propolis bioactivities and potential


### Abstract

Health-conscious awareness of consumers, along with the environmental and health problems, have increased the demand for safer and more ecologic alternatives for raw materials in the most varied industries. Natural products have been in great demand, which is the case of propolis and honey, the most popular hive products, produced by bees and owners of outstanding properties. Propolis, produced from exudates from different plant parts and mixed with substances resulting from bees' metabolism, is a natural product of high variable chemical composition and low productivity, which hampers its standardization and consequently its use and commercialization. Thus, the aims of this work are to improve propolis production, value propolis through chemical and biological characterization and to promote its application in the design of functional products. This work shows that the use of grids and/ or the manipulation of the grids pore size could help to increase propolis production. After chemical and biological characterization of several ethanol extracts of propolis from Gerês harvested from different years, the propolis sample from 2018 (G18) was extracted with different solvents, on the path towards more ecological and sustainable alternatives, and used in combination with other antimicrobial agents showing high antioxidant capacity and antimicrobial properties, presenting great potential to fight fungal infections, such the ones caused by *Candida albicans*, and resistant bacteria such as the Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*, revealing to be interesting for further investigations and use in different industries, alone or combined with antibiotics or even other beehive products. Propolis from Gerês also seems to be a promising agent to treat renal cell carcinoma, either to be used as alternative or in combination with conventional anticancer treatments. To explore possible practical applications, cotton textile was functionalized with a mixture of propolis and honey and the resulting textile exhibited great antioxidant potential and promising activity against *Propionibacterium acnes*, with good fastness to the washing process up to at least 3 washing cycles. Given the obtained results showing propolis potential for several applications, further work with propolis to assess the possibility of its use/ exploitation is essential to reach its standardization and consequent acceptance and commercialization.



**Keywords:** Bee products, propolis, propolis bioactivities, textile functionalization with bee products.

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## List of abbreviations and acronyms

3WC – three washing cycles

ABV – alcohol by volume

ATCC – American Type Culture Collection

b – Bugalho

Bc – *Bacillus cereus*

Bm – *Bacillus megaterium*

Bs – *Bacillus subtilis*

BS – biofunctional solution

BS_PA – biofunctional solution with potassium alum

BTCA – 1,2,3,4-butanetetracarboxylic acid

BuOH – butanol

Ca – *Candida albicans*

CAGR – compound annual growth rate

CAIE – caffeic acid isoprenyl ester

CAPE – caffeic acid phenethyl ester

CO – cotton

CO+BS – cotton knit treated with the biofunctional solution

CO+BS_PA – cotton knit cotreated with the biofunctional solution and potassium alum

$CO+BS_PA_{3wc}$ – cotton knit cotreated with the biofunctional solution and potassium alum after three washing cycles

$CO+BS_{3wc}$ – cotton knit treated with the biofunctional solution after three washing cycles

DMCA – 3,4-dimethyl-caffeic acid

DMSO – dimethyl sulfoxide

DMSO-*d*6 – deuterated dimethyl sulfoxide

DPPH – 2,2diphenyl-1-picryl-hydrazyl

E – ethanol

Ec – *Escherichia coli*

$EC_{50}$ – concentration that reduces the free radical in 50 %

EE – ethanol extract

$EE_{70}$ – ethanol/ water (7:3) extract

EMA – European Medicines Agency

ESI-MS – electrospray ionization mass spectrometry

EtOAc – ethyl acetate

f – Felgueiras

F – fractions

FDA – Food and Drug Administration

FNAP – Federação Nacional dos Apicultores de Portugal

G – Gerês

G11 – propolis from Gerês collected in 2011

G11.EE – ethanol extract of propolis from Gerês collected in 2011

G12 – propolis from Gerês collected in 2012

G12.EE – ethanol extract of propolis from Gerês collected in 2012

G13 – propolis from Gerês collected in 2013

G13.EE – ethanol extract of propolis from Gerês collected in 2013

G14 – propolis from Gerês collected in 2014

G14.EE – ethanol extract of propolis from Gerês collected in 2014

G15 – propolis from Gerês collected in 2015

G15.EE – ethanol extract of propolis from Gerês collected in 2015

G16 – propolis from Gerês collected in 2016

G16.EE – ethanol extract of propolis from Gerês collected in 2016

G17 – propolis from Gerês collected in 2017

G18 – propolis from Gerês collected in 2018

G18.EE – ethanol extract of propolis from Gerês collected in 2018

G18.$EE_{70}$ – ethanol/ water (7:3) extract of propolis from Gerês collected in 2018

G18.HBE – honey brandy extract of propolis from Gerês collected in 2018

G18.ME – mead extract of propolis from Gerês collected in 2018

G18.PGE – propylene glycol extract of propolis from Gerês collected in 2018

G18.WE – water extract of propolis from Gerês collected in 2018

G19 – propolis from Gerês collected in 2019

G19.EE – ethanol extract of propolis from Gerês collected in 2019

GAE – gallic acid equivalents

G-r17.EE – propolis from Gerês collected in 2017 composed by the source place Roca

G-r18.EE – propolis from Gerês collected in 2018 composed by the source place Roca

G-r19.EE – propolis from Gerês collected in 2019 composed by the source place Roca

G-rt17.EE – propolis from Gerês collected in 2017 composed by a combination of source places: Roca and Toutelo

G-rt18.EE – propolis from Gerês collected in 2018 composed by a combination of source places: Roca and Toutelo

G-rt19.EE – propolis from Gerês collected in 2019 composed by a combination of source places: Roca and Toutelo

GTF – glycosyltransferase

G-tf17.EE – propolis from Gerês collected in 2017 composed by a combination of source places: Toutelo and Felgueiras

G-tf18.EE – propolis from Gerês collected in 2018 composed by a combination of source places: Toutelo and Felgueiras

G-tf19.EE – propolis from Gerês collected in 2019 composed by a combination of source places: Toutelo and Felgueiras

G-tfb18.EE – propolis from Gerês collected in 2018 composed by a combination of source places: Toutelo, Felgueiras and Bugalho

H – honey

H18 – honey collected from 2018

H18.WE – honey collected in 2018 diluted to a stock solution of 50 % (v/v) in sterile water

HB – honey brandy

HBE – honey brandy extract

HMBC – Heteronuclear Multiple Bond Correlation

HSQC – Heteronuclear Single Quantum Coherence

$IC_{50}$ – concentration that inhibits cell viability in 50 %

LB – Luria-Bertani

LBA – Luria-Bertani agar

LC-MS – liquid chromatography-mass spectrometry

M – mead

ME – mead extract

MIC – minimum inhibitory concentration

MRSA – methicillin resistant *Staphylococcus aureus*

MS/MS – tandem mass spectrometry

MSSA – methicillin sensitive *Staphylococcus aureus*

NMR – nuclear magnetic resonance

OD – optical density

PA – potassium alum

Pa – *Propionibacterium acnes*

PCA – principal component analysis

PG – propylene glycol

PGE – propylene glycol extract

PLA – polylactic acid

ppm – parts per million

PSE – Phytochemical Society of Europe

PVA – polyvinyl alcohol

QE – quercetin equivalents

r – Roca

RCC – renal cell carcinoma

rpm – revolutions per minute

RT – room temperature

Sa – *Staphylococcus aureus*

Sc – *Saccharomyces cerevisiae*

SD – standard deviation

SRB – sulforhodamine B

t – Toutelo

TCA – trichloroacetic acid

TFC – total flavonoids content

TLC – thin-layer chromatography

TOC – total *ortho*-diphenols content

TPC – total phenolics content

UHPLC-DAD-ESI/$MS^n$ – high performance liquid chromatography equipped with photodiode array detection-mass

UV – ultraviolet

VLC – vacuum liquid chromatography

W – water

WE – water extract

YPD – Yeast Extract–Peptone–Dextrose

YPDA – Yeast Extract–Peptone–Dextrose agar

## List of publications

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**Published:**

**Freitas, A. S.**, Cunha, A., Cardoso, S. M., Oliveira, R., Almeida-Aguiar, C. 2019. Constancy of the bioactivities of propolis samples collected on the same apiary over four years. *Food Research International*, 119, 622-633.

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*Equal senior authorship

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# Chapter 1

General introduction

## 1.1. Chemical industry: chemicals consumption and health-conscious awareness of consumers

In 2020, the global market of chemicals amounted to 1.86 and 1.78 trillion euros in importations and exportations, respectively. Europe was, in the first place, responsible for the highest value of both chemical imports and exports in 2020, followed by Asia-Pacific. Worldwide chemical consumption registered more than 3.53 trillion euros in 2020 and is expected to grow during the next years (Statista, 2021). With Asia becoming the center of the chemical industry, to face the pressure of the industry competitiveness, Europe will continue to play its role as pioneer in the development of innovative products associated to the global mega trends that have potential to generate new industries. Within these mega trends are the natural resources, environment, technology, innovation - environmental technology for clean air and water or waste treatment -, and intelligent materials - nano materials and functional textiles (Kearney, 2012).

Humans are daily exposed to synthetic chemicals in their most varied forms. They can be found under the form of food, clothes, tools, cosmetics and medicines. However, despite their usefulness, many of these substances can be harmful to our health and to the environment (Prüss-Ustün *et al.*, 2011). Exposure to toxic chemicals can lead to the development of different health problems such as the case of cancers (e.g., skin and kidney; Clapp *et al.*, 2008), and several skin diseases (e.g., dermatitis, chloracne and hyperkeratosis; Spiewak, 2001). Agencies such as the European Medicines Agency (EMA) and the United States of America (USA) Food and Drug Administration (FDA) work together on the constant monitoring of the adverse effects of chemical compounds and often determine the prohibition of the use of certain substances, acting to promote health. However, the health-conscious awareness of consumers regarding the potential toxicity and unforeseen effects of the continuous use of chemicals in their daily lives has been increasing the demand for natural products as replacements for the commonly used synthetic compounds (Montenegro and Mejías, 2013). Known to own properties with numerous benefits that can be applied in the most varied industries, natural products are also of clinical interest for the development of new drugs, both to retard the outbreak of bacterial resistance and to decrease its impacts. Consisting in mixtures of several compounds, natural products, alone or combined with other agents with antimicrobial properties, are less likely to trigger microbial resistance. Also, their use in combination with antibiotics can contribute to decrease their overuse by reducing the required amount of the drug. Natural products such the ones produced by bees, as is the case of propolis and honey, are chemically complex and the need to somehow standardize their chemical and biological profiles is acknowledge by several authors (Kapare *et al.*, 2017; Sadhana *et al.*, 2017), in order to enable its use and acceptance by industries. Nevertheless, the

valuable bioactivities of these natural products still attract worldwide scientific community and industry for research and innovation.

## 1.2. Beehive products: propolis and honey

Bees have existed for over a thousand million years and have sparked interest around the world mostly due to the remarkable properties of their products (Bankova *et al.*, 2014). Bees´ products include honey, propolis, bee pollen, bee wax, bee venom and royal jelly, and have been used since ancient times due to their nutritional value and healing properties (Koç *et al.*, 2011; Pranskuniene *et al.*, 2016; Pasupuleti *et al.*, 2017). They can be used as food or active products for medicinal and cosmetic purposes (Nesheim *et al.*, 2006), playing an important role in human health and well-being (Yadav *et al.*, 2015). Honey and propolis, the most popular bee products, both commercialized in different parts of the world, are known as important sources of bioactive compounds with properties for several applications (Moreira *et al.*, 2008; Pasupuleti *et al.*, 2017; Sforcin *et al.*, 2017). By 2028, the Global Honey and Propolis Markets are expected to reach a value of USD 17.34 billion - with a compound annual growth rate (CAGR) of 8.2 % - and USD 60.9 million - with a CAGR of 4 % -, respectively (Market, 2019; Grand View Research, 2021).

### 1.2.1. Propolis

Propolis has been used in traditional medicine since primordial times and acquired popularity in many civilizations due to its valuable properties such as anti-putrefactive, anti-pyretic, antiseptic and due to its capability to heal wounds and burns, sore throat and stomach ulcers (Wollenweber *et al.*, 1990; Burdock, 1998; Moreira *et al.*, 2008). In the Second World War propolis was used by Soviets to treat tuberculosis due to the observed decrease of lung problems upon propolis administration to patients (Wollenweber *et al.*, 1990). Being registered as an official drug in London's pharmacopeia in the 17$^{th}$ century, propolis has become very popular in Europe during the following years particularly owing to its notable bioactivities (Fokt *et al.*, 2010).

Propolis, also known as bee glue, is a complex mixture composed by resinous and balsamic material, produced by honeybees from branches, flowers, pollen, buds and exudates of trees and mixed with salivary enzymes, waxes and other compounds resulting from bees' metabolism (Fokt *et al.*, 2010). The word propolis derived from the Greek *pro* (for 'in front of') and *polis* (for 'community'), meaning that this natural product acts protecting the hive from pests resulting from putrefaction (Sforcin, 2007), being used to immobilize the invaders carcasses. In the hive, propolis is also used to

fill openings, functioning like a mechanical and thermal isolator (Moreira *et al.*, 2008; Simone-Finstrom and Spivak, 2010; Silva-Carvalho *et al.*, 2015).

The chemical and biological profiles of propolis are very variable according to the source plant species existent around the hive (Katalinić *et al.*, 2004; Falcão *et al.*, 2010), the bee species (Pereira *et al.*, 2002), environmental factors such as the climate characteristics (Falcão *et al.*, 2010), the season and technique of harvest (Sales *et al.*, 2006; Tomazzoli *et al.*, 2015) as well as the extraction method and solvent used (Sheng *et al.*, 2006). Different types of propolis are currently recognized according to the botanical origin, being characterized by a set of chemical constituents that are responsible for valuable properties (Bankova, 2005; Silva-Carvalho *et al.*, 2015). However, even samples collected from the same apiary at different times can display different chemical and bioactive profiles (Marques, 2015; Miguel *et al.*, 2010; Veloz *et al.*, 2015). Nevertheless, this complex mixture is mainly composed of around 50 % resins and vegetable balsams, 30 % wax, 10 % essential oils, 5 % pollen and 5 % of other substances, including organic compounds (Pasupuleti *et al.*, 2017).

More than 850 compounds have been identified in different propolis samples (Bankova *et al.*, 2000; de Castro *et al.*, 2001), with phenolics being the most important. The most common phenolic compounds in propolis from temperate zones are flavonoids like pinocembrin, galangin and chrysin and phenolic acids such as caffeic acid, ferulic acid and cinnamic acid (Bankova *et al.*, 2000; Huang *et al.*, 2014). Flavonoids (flavones, flavonols and flavonones), phenolic acids and their esters are the main bioactive compounds found in European propolis (Huang *et al.*, 2014).

Propolis has been subject of intensive studies concerning its biological and pharmacological properties (Boisard *et al.*, 2015; Szweda *et al.*, 2015; Oliveira *et al.*, 2017). Due to the plant diversity, there are different types of propolis containing numerous chemical constituents responsible for its biological properties, which include antibacterial activity (Falcão *et al.*, 2010; Castro *et al.*, 2011, 2012), antifungal (Moreira *et al.*, 2008; Falcão *et al.*, 2010; Castro *et al.*, 2011, 2012), anti-protozoan (Castro *et al.*, 2011, 2012), anti-viral (Borrelli *et al.*, 2002; Sheng *et al.*, 2006; Moreira *et al.*, 2008) anti-HIV (Falcão *et al.*, 2010; Castro *et al.*, 2011, 2012), antioxidant (Sheng *et al.*, 2006; Falcão *et al.*, 2010; Cruz *et al.*, 2016), anti-inflammatory (Sforcin, 2007, Moreira *et al.*, 2008; Falcão *et al.*, 2010; Araújo *et al.*, 2022), anti-tumor (Sforcin, 2007; Moreira *et al.*, 2008; Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Freitas *et al.*, 2022; Oliveira *et al.*, 2022), hepato-protective (Sheng *et al.*, 2006), anti-neurodegenerative (Chen *et al.*, 2008), local-anaesthetic (Moreira *et al.*, 2008), anti-tuberculosis (Falcão *et al.*, 2010), free-radical-scavenging (Sheng *et al.*, 2006; Castro *et al.*, 2012), immunostimulating (Borrelli *et al.*, 2002; Lotfy *et al.*, 2006) and cytotoxic (Matsuno *et al.*, 1997).

### 1.2.2. Honey

For thousands of years honey has been used in traditional medicine. In the Old Egypt by Egyptians, and by Chinese, Indians, Ancient Greeks, Romans, and Arabs, honey has been used in ointments to treat any kind of wounds, such as several types of ulcers, and many diseases (Boukraâ, 2013), also stimulating the growth of wound tissues and accelerating the process of healing by initiating an anti-inflammatory process (Oryan *et al.*, 2006; Bittmann *et al.*, 2010). Honey is used not only for nutrition purposes but also as an alternative treatment for clinical conditions (Khan *et al.*, 2007). Honey is a natural mixture produced by honeybees from the nectar collected from flowers and/ or other parts of plants and mixed with substances resulting from bees´ metabolism (Montenegro and Mejías, 2013; Jantakee and Tragoolpua, 2015). Honey is one of the most consumed natural products, being characterized by its properties capable to promote health and prevent diseases, in addition to its nutritional value (Muhammad *et al.*, 2012, 2016).

Honey composition, color, aroma and flavor depend on several factors such as the type of nectar bees fed (floral source) (Al-Mamary *et al.*, 2002; Molan, 2002; Yao *et al.*, 2003; Kaškonienė and Venskutonis, 2010; Escriche *et al.*, 2014; Al-Nahari *et al.*, 2015), climatic conditions (Escriche *et al.*, 2014; Al-Nahari *et al.*, 2015), geographic origin (Estevinho *et al.*, 2008; Kaškonienė and Venskutonis, 2010; Escriche *et al.*, 2014), season of collection (Molan, 2002), storage time, preservation conditions (Al-Waili and Boni, 2003; Soares *et al.*, 2017), production methods (Molan, 2002) and extraction methods (Kadri *et al.*, 2017). Regarding the botanical origin, honey can be monofloral if the nectar collected by bees has one main floral source, owing distinct organoleptic characteristics such as color, fragrance and percentage in phenolic compounds. On the other hand, a multifloral honey is obtained from the nectar collected from different botanical origins (Machado *et al.*, 2020). Still, honey is mainly composed by carbohydrates (up to 80 %), particularly fructose and glucose, water (17-20 %) and other bioactive substances such as organic acids, proteins, amino acids, enzymes, lipids, flavonoids, phenols and vitamins (Montenegro and Mejías, 2013; Oryan *et al.*, 2016; Pasupuleti *et al.*, 2017). The concentration of fructose and glucose, as well as the ratio between these monosaccharides, are common indicators for the classification of monofloral honeys. Usually, fructose is found in greater quantity than glucose (Silva *et al.*, 2016). Organic acids, either naturally present in nectar or resultant from sugars through the action of enzymes secreted by bees, provide a slight acidity to honey (Cherchi *et al.*, 1994). Like sugars, organic acids are also used to distinguish honey origin and influence its color, flavor and chemical properties such as pH and electrical conduction capacity (Mato *et al.*, 2006). Proteins and amino acids are found in lower amounts in honey and result essentially from the bees'

salivary secretions during its production. The amino acids lysine, proline, γ-aminobutyric and arginine can trigger the *Maillard* reaction, that can occur during the storage time, or in the presence of heat, where sugars start to undergo a non-enzymatic glycation that occurs between reducing sugars and compounds with free amine groups, causing changes in the color, flavor and fragrance of honey (Silva *et al.*, 2016; Gupta *et al.*, 2018). The glucose oxidase enzyme, also present in honey, plays an important role in the production of glucuronic acid and hydrogen peroxide, with bactericidal activity (Kwakman and Zaat, 2012; Silva *et al.*, 2016; Cornara *et al.*, 2017). Phenolic compounds, in particular phenolic acids, are mainly responsible for the bioactivities of honey, similarly to what is seen in propolis and other natural products (Silva *et al.*, 2016).

There are several studies regarding the determination of honey polyphenols (Kaškonienė and Venskutonis, 2010; Chan *et al.*, 2013; Campone *et al.*, 2014; Sergiel *et al.*, 2014), the most common phytochemicals found in different honey samples being flavonoids (apigenin, chrysin, galangin, hesperetin, kaempferol, luteolin, myricetin, pinobanksin, pinocembrin, quercetin, genistein, naringenin and tricetin), phenolic acids (caffeic, chlorogenic, coumaric, benzoic, ellagic, ferulic, gallic, homogentisic, phenyllactic, protocatecuic, syringic, ellagic and vanillic acids) and their derivatives (Gómez-Caravaca *et al.*, 2006; Trautvetter *et al.*, 2009; Kaškonienė and Venskutonis, 2010; Kassim *et al.*, 2010; Alvarez-Suarez *et al.*, 2012; Candiracci *et al.*, 2012; Chan *et al.*, 2013; Sergiel *et al.*, 2014). Such constituents, particularly flavonoids and phenolic acids are normally related to honey biological activities (Kaškonienė and Venskutonis, 2010).

Biological activities of honey are known since ancient times, and they have been studied by many researchers (Molan, 1999; Al-Nahari *et al.*, 2015; Jantakee and Tragoolpua, 2015; Grego *et al.*, 2016; Miguel *et al.*, 2017). Honey biological activities include antibacterial (Visavadia *et al.*, 2006; Baltrušaitytė *et al.*, 2008; Estevinho *et al.*, 2008; Tan *et al.*, 2009; Al-Hindi *et al.*, 2011; Al-Nahari *et al.*, 2015; Jantakee and Tragoolpua, 2015; Grego *et al.*, 2016), antifungal (Koç *et al.*, 2009, 2011), antioxidant (Estevinho *et al.*, 2008; Omotayo *et al.*, 2010; Al-Hindi *et al.*, 2011; Jantakee and Tragoolpua, 2015; Attanzio *et al.*, 2016), mitogenic and immunomodulatory (Muhammad *et al.*, 2014); anti-inflammatory (Kassim *et al.*, 2010), gastroprotective (Omotayo *et al.*, 2010), antiulcer (Almasaudi *et al.*, 2017), nematicidal (Azim and Sajid, 2009), and anti-tyrosinase (Jantakee and Tragoolpua, 2015), among others (Miguel *et al.*, 2017).

## 1.3. Research objectives

Investigation on Portuguese propolis has been carried out by our research group at the Department of Biology of the University of Minho (Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Cruz *et al.*, 2016; Freitas *et al.*, 2019; Peixoto *et al.*, 2021, 2022; Araújo *et al.*, 2022; Oliveira *et al.*, 2022; Pereira *et al.*, 2022). Among the various propolis samples studied, a propolis sample from Gerês (coded G) is produced in a standardized and reliable way by the beekeeper, who practices an organic beekeeping, and is characterized by an unusual constancy in their chemical and biological profiles (Freitas *et al.*, 2019), having shown to possess several bioactivities, such as antimicrobial, antioxidant, free-radical-scavenger, anti-inflammatory and antitumor. Further work with this Portuguese propolis sample from Gerês is essential and guided this work to outlook its use/ exploitation as a whole and/ or of its isolated compounds in several applications. Firstly, it was important to recognize and understand propolis hindrances that are hampering its acceptance and introduction in some industries, such as the cosmetic and pharmaceutical ones. This research was essentially focused to overcome two of those major barriers - propolis low production and its complex and variable chemical composition - together with the investigation of propolis properties to value this beehive product that is quite neglected, and even unknown, in Portugal. Thus, it is important to call the attention of the research and non-academic communities, and particularly of the beekeepers, to the importance and high value of propolis, which can be an added source of income to the beekeeping sector.

According to Federação Nacional dos Apicultores de Portugal (FNAP, 2010), propolis annual production per hive is 100 – 200 g when the traditional method of scrapping is used, being possible to reach 500 g with the use of grids. However, based on data from the beekeeper who provided the propolis samples used in this work, the annual production of propolis from Gerês is around 27 g/ hive (Amadeu Fortunas, personal communication). This way, we decided to investigate the possibility of improving propolis production by placing different pore sized grids (0.63 mm, 1.8 mm, 2.8 mm) and the one normally used by the beekeepers (3 mm) in beehives in an apiary located in Gerês over 3 consecutive years (2017 - 2019). Isogenic queen bees were obtained by controlled fertilization and used in the beehives to decrease genetic variability. Propolis production was recorded every year and the influence of propolis production on honey yield was also verified to evaluate if the production of the biggest incoming source of the beekeeping sector, honey, could be affected by alterations in propolis production (**Chapter 2**).

Propolis from Gerês, usually composed by a mixture of three source places from Gerês (Bugalho, Toutelo and Felgueiras), is characterized by an unusual constancy regarding its chemical and biological

profiles (Freitas *et al.*, 2019), which is an important attribute considering the struggle for propolis standardization and consequent use and commercialization (Bankova *et al.*, 2000; Falcão *et al.*, 2010). However, the occurrence of wildfires in the year of 2017 and the destruction of part of the hives from which Gerês propolis was usually harvested (Bugalho), made us question to what extent this constancy would be maintained and rethink the way we study propolis from Gerês. To replace those hives, new ones were added in a different location (Roca) and, despite the installation of new beehives at the Bugalho place, in 2018, the swarms did not resist, and the beehives had to be removed. Thus, we have performed a chemical characterization and evaluation of the antioxidant and antimicrobial activities of several ethanol extracts of propolis sampled from the different apiary locations of Gerês (**Chapter 3**). This study was conducted over three consecutive years (2017-2019) as the beehives suffer a new reconfiguration, to search for the usual constancy observed, questioned by the stated variations, and to help choosing the propolis sample to proceed for further investigation (**Chapters 4-7**). In parallel to this study, and to overcome the low propolis productivity, I collaborated in the research of a Master student from the University of Minho, Marta Peixoto, in the investigation of the potential of blending propolis samples from the same apiary, but from different harvesting years (Peixoto *et al.*, 2021) and the potential of blending propolis samples from different apiaries and harvesting years (Peixoto *et al.*, 2022).

Consumer's health-conscious awareness led to an increase in the demand for more natural and sustainable solutions. One of the several factors that influence propolis chemical composition and, consequently, its biological profile is the solvent used to extract propolis bioactive portion. Ethanol, the most commonly solvent used for propolis extraction (Asgharpour *et al.*, 2020) has its drawbacks and limited applicability in certain industries (Kubiliene *et al.*, 2015). To search for new and ecological solvents for propolis extraction capable to provide propolis extracts with great potential for different applications, the chosen propolis sample from Gerês, collected in 2018 (G18), was extracted with different solvents: absolute ethanol, ethanol/ water (7:3), honey brandy, mead, water and propylene glycol. Honey brandy and mead, never used for propolis extraction according to our knowledge, were obtained using honey from the same apiary. The chemical characterization and antioxidant and antimicrobial properties of the six extracts were evaluated. Additionally, chemical composition of the same extracts was determined by the LC-MS technique and possible associations between both chemical and biological profiles were made through the principal component analysis (PCA; **Chapter 4**).

Along with its low productivity, propolis complex and variable chemical composition have been hampering its use for production at industrial scale and consequent commercialization in cosmetic and

pharmaceutical products (Bankova *et al.*, 2000; Falcão *et al.*, 2010; Sforcin, 2017; Cornara *et al.*, 2017). To contribute to overcome this problem that hinders propolis standardization, compound fractioning and isolation from the ethanol extract of Gerês propolis harvested in 2018 (G18.EE) was subsequently performed to find and identify the main bioactive compounds, establishing a chemical fingerprint for the studied propolis. Firstly, G18.EE was obtained and liquid-liquid partition was performed to obtain four different fractions (*n*-hexane, ethyl acetate, *n*-butanol and water fractions). Techniques such as vacuum liquid chromatography and column chromatography were used to isolate compounds from the fractions. As result of these procedures, it was possible to obtain some propolis sub-fractions and to isolate compounds from the *n*-hexane (**Appendix 1**) and ethyl acetate fractions. Following the previous procedures, the ethanol extract, the four fractions obtained (*n*-hexane, ethyl acetate, *n*-butanol and water) and three samples isolated from the ethyl acetate fraction (P1 – P3) were tested for their activity on A498, 786-O and Caki-2 renal cell carcinoma cell lines and the non-neoplastic HK2 kidney cells for toxicity assessment (**Chapter 5**). In addition, the ethanol extract and three fractions (*n*-hexane, ethyl acetate and *n*-butanol) were tested in A375 and WM9 melanoma cell lines by a Master student from the University of Minho, Rafaela Oliveira, with whom I collaborated by providing the propolis sample and respective fractions to test, as well as with the phenolic compound analysis (**Chapter 5 – Figure S5.3** and **Table S5.1 Supplementary material**).

Despite the great improvements in medical care, traditional medicine/ therapies based on natural products remain interesting alternatives due to the diversified bioactive profiles that can be used in the treatment of different diseases while allowing to overcome some limitations such as the increase of microbial resistance (Pereira and Bártolo, 2016). The overuse and misuse of antibiotics has been leading to the development of resistant bacterial strains, limiting the efficacy of the antibiotics currently available (Jantakee and Tragoolpua, 2015; Almasaudi *et al.*, 2017). In addition, bacteria are developing new mechanisms of antibiotic resistance, allowing them to spread across the world, contributing to the scalation of this threat (Mayor, 2018; WHO, 2018) and making urgent the identification of new antimicrobial agents. Both propolis and honey act on the modulation of bacterial resistance, containing several components that may act in a synergistic manner to compromise resistance (Carnwath *et al.*, 2014; Al-Nahari *et al.*, 2015). Also, the possibility of using honey and/ or propolis in combination with the commercially available antibiotics, or other antimicrobial agents, is a very promising subject for further studies. Aiming to investigate those topics to foresee the potential application of Gerês propolis in this field, antibacterial and antioxidant effects of several ethanol extracts of Portuguese propolis from

Gerês combined with gentamicin, a widely used aminoglycoside antibiotic, or with honey collected from the same apiary, were evaluated (**Chapter 6**).

Lastly, the production of one bioactive product using beehive resources, namely propolis and honey, was a major aim of this work, envisaging possible applications of a new natural solution with antimicrobial activity as well as the valorization of beehive resources. For the development of this product, a mixture of propolis and honey, both harvested in Gerês and showing great antioxidant and antimicrobial activities, was used for cotton textile dyeing. Textile dyeing with natural products has benefits when compared to the synthetic chemicals commonly used that are often associated with adverse health and environmental side effects. Additionally, natural dyes came with the ability to improve the performance of the textiles by providing functionalization. The dyed meshes were then tested for antioxidant potential and antimicrobial activity, and further evaluated after 3 washing cycles given the importance of textile fastness to washing (**Chapter 7**).

All the approaches aimed not only to circumvent the problems of standardization and scarcity of this natural product, but also go deeper characterizing propolis and evaluating its potential in different applications, simultaneously contributing to enhance the value of this national bee product.

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# Chapter 2

## Improvement of propolis production by decreasing the grid pore size

*The work presented in this chapter has been submitted and was published in a national technical-scientific journal in the Portuguese language aiming to target a wider audience, namely the national beekeeping sector.*

Freitas, A. S., Fortunas, A., Oliveira, R.*, Almeida-Aguiar, C.* 2022. Otimização da produção de própolis: utilização de grades com poros de menores dimensões. *AGROTEC* (45), 56-60.

*Equal senior authorship

## Abstract

Honey and propolis, the most popular bee products, are both commercialized in different parts of the world and recognized as important sources of bioactive compounds with properties for several applications. However, propolis production is limited and is still overlooked by most of the beekeepers, being unvalued and discarded. Thus, it is important to call the attention for the value of propolis as an additional income source to the beekeeping sector and simultaneously develop procedures to optimize propolis production. In this work, propolis production was studied over three consecutive years (2017 – 2019) by placing different pore sized grids (0.63 mm, 1.8 mm and 2.8 mm) as well as the one normally used by the beekeeper (3 mm) in beehives of an apiary located in Gerês, Portugal. Propolis production was higher when the smaller pore sized grids were used, and honey yield was not affected. Our results suggest that the design of beehive grids with small pore would optimize propolis production and increase the valorization of this bee product.

**Keywords:** Beekeeping, bee products, propolis production, beehive grid pore size.

## 2.1. Introduction

Beekeeping is the activity of prolonging, sustaining, and retaining health of bee colonies by the beekeeper, who benefits from beekeeping by using bees' products (Pranskuniene *et al.*, 2016; Pasupuleti *et al.*, 2017). In the last years beekeepers have lost over a third of their hives, mostly due to the exposure to chemicals used to treat pathogens and parasites, such as the well-known parasite *Varroa destructor* and the fungus *Nosema ceranae* (Bordin *et al.*, 2022). Poor nutrition and habitat loss, or a combination of several factors also contribute to bees' population decline (Johnson *et al.*, 2010; Mullin *et al.*, 2010; Sanchez-Bayo and Goka, 2014), leaving farmers worried about their ability to meet demands for bee-pollinated crops (Misiewicz *et al.*, 2015). Some studies have shown that organic farms provide more support to the pollinators than the conventional ones (Kremen *et al.*, 2002; Andersson *et al.*, 2014; Kehinde and Samways, 2014), promoting their health and conservation by prohibiting the use of toxic pesticides and supporting higher levels of landscape diversity (Misiewicz *et al.*, 2015). Organic beekeeping favors sustainable agricultural management, high-quality products and promotes human health and animal welfare, strongly restricting the use of external products, especially synthetic chemicals.

Interest in natural products, as the ones produced by bees - which include honey, bee bread, bee venom, bee pollen, bee wax, propolis, and royal jelly - has been increasing due to their nutritional value

and healing properties (Pranskuniene *et al.*, 2016; Pasupuleti *et al.*, 2017) and, as such, play an important role in human health and have potential for the development of new drugs (Yadav *et al.*, 2015). Propolis, for instance, is a complex mixture composed by resinous and balsamic material, produced by honeybees from branches, flowers, pollen, buds and exudates of trees, which are mixed with salivary enzymes, waxes and other compounds resulting from bees´ metabolism (Sforcin, 2007). Propolis is used against invaders to immobilize their carcasses, protecting the hive against microbial infections, to seal hive walls and to fill eventual cracks or openings (Moreira *et al.*, 2008), thus contributing for hive defense preserving its integrity and health balance (Sforcin, 2007; Fokt *et al.*, 2010).

The chemical profile and biological activities of propolis are very variable according to several factors, namely the diversity of plants around the hive (Katalinić *et al.*, 2004; Falcão *et al.*, 2010), the bee species (Pereira *et al.*, 2002), the climatic characteristics (Falcão *et al.*, 2010) and harvest time and technique (Sales *et al.*, 2006; Tomazzoli *et al.*, 2015). Different types of propolis are currently recognized based on their botanical origin, being characterized by a set of chemical constituents responsible for various properties (Bankova, 2005; Silva-Carvalho *et al.*, 2015). However, even samples collected from the same apiary at different times can present different chemical and biological profiles (Marques *et al.*, 2015; Miguel *et al.*, 2010; Veloz *et al.*, 2015). Still, this complex mixture is mainly composed by 50 % plant resins and balsams, 30 % wax, 10 % essential oils, 5 % pollen, and 5 % other substances, including organic compounds (Pasupuleti *et al.*, 2017).

Propolis has been subject of intensive studies regarding its biological and pharmacological properties (Boisard *et al.*, 2015; Szweda *et al.*, 2015; Oliveira *et al.*, 2017), which include antibacterial, antifungal, and antiviral activity. as anti-HIV, antioxidant, anti-inflammatory, antitumor, hepatoprotective, anesthetic, antineurodegenerative and immunostimulant (Borrelli *et al.*, 2002; Lotfy *et al.*, 2006; Sheng *et al.*, 2006; Sforcin, 2007; Chen *et al.*, 2008; Moreira *et al.*, 2008; Falcão *et al.*, 2010; Castro *et al.*, 2011, 2012; Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Oliveira *et al.*, 2022; Freitas *et al.*, 2019, 2022; Peixoto *et al.*, 2021).

Propolis can be collected by scratching the walls of the hive - the traditional method which can result in a lower quality propolis with high percentage of impurities - or by using grids designed to mimic hive openings and thus promoting propolis production with higher quality and increased yield (Okhale *et al.*, 2021). Propolis low commercial exploitation by most Portuguese beekeepers mainly relies on the lack of information and of technical knowledge along with the rooted tradition of honey production, making propolis production practically neglected in the context of national apiculture (FNAP, 2008).

Global Propolis Market is expected to reach USD 60.9 million, with a CAGR of 4 % by 2028 (Market, 2019). Propolis market has as principal target the pharmaceutical industry, which requires larger amounts of propolis of a certain type/ quality. Thus, it is important to call the attention for the value of this beehive product as an additional source of income to the beekeeping sector and simultaneously develop procedures to obtain more and better propolis. With this work we aimed to test the possibility of improving propolis production. For this purpose, different grids with four different pore sizes were placed over beehives for a three-year period and propolis production was recorded yearly.

## 2.2. Materials and Methods

### 2.2.1. Grids preparation

To study the influence of grid pore size on propolis production, metal grids with different pore sizes (0.63 mm, 1.8 mm and 2.8 mm) were selected and cut in 40x40 cm squares to fit in the beehives. The plastic 3 mm pore size grid normally used by the beekeeper was included in the study for comparison purposes (**Figure 2.1**).

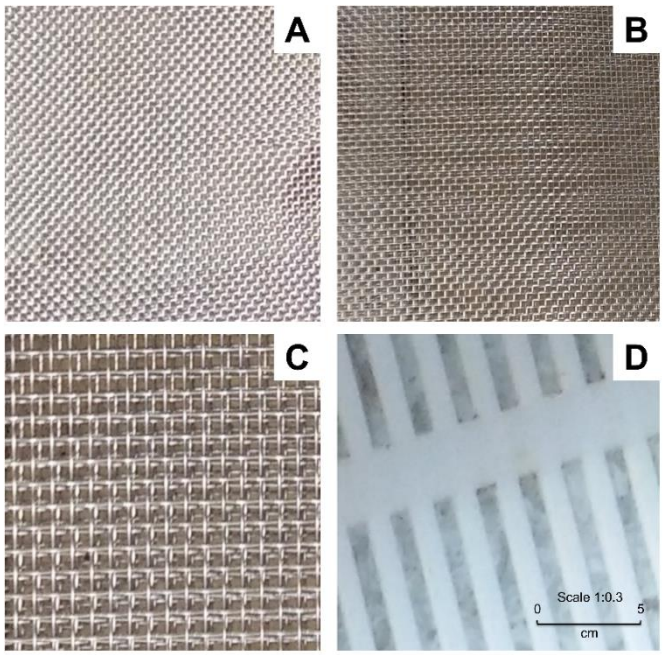


**Figure 2.1**. Types of pore sized grids used in the present study: metal grids with 0.63 mm (**A**), 1.8 mm (**B**) and 2.8 mm (**C**) of pore size and plastic grid with 3 mm (**D**) of pore size.

### 2.2.2. Assessment of grid pore size influence on propolis production

The influence of grid pore size on propolis production was studied weighing and placing different grids ($3 \leq n \leq 6$ of each of the four grid types referred above; **Figure 2.1**) in beehives in an apiary

located near the Cávado River, between the villages of Paradela and Sirvozelo, in Montalegre, Gerês (41º 45'41.62'' N; 7º 58'03.34'' W), during three consecutive years (May 2017, June 2018 and April 2019), where propolis is produced under an organic beekeeping regime. To decrease genetic variability, isogenic queen bees obtained by controlled fertilization were used in the beehives under study. Each year, after propolis production period, grids were collected (August-September 2017, 2018 and 2019) and weighted to calculate the amount of propolis produced in each pore sized grid type.

### 2.2.3. Statistical analysis

Results were expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey test for multiple comparisons were used to assess significance. Differences considered statistically significant ($p < 0.05$) were noted with different letters.

## 2.3. Results and Discussion

The influence of grid pore size on propolis production was studied by placing different pore sized metal grids in beehives and searching for bees´ behavior towards the different pore sizes, reflected on propolis production. After production period, grids (**Figure 2.2**) were collected and weighted.

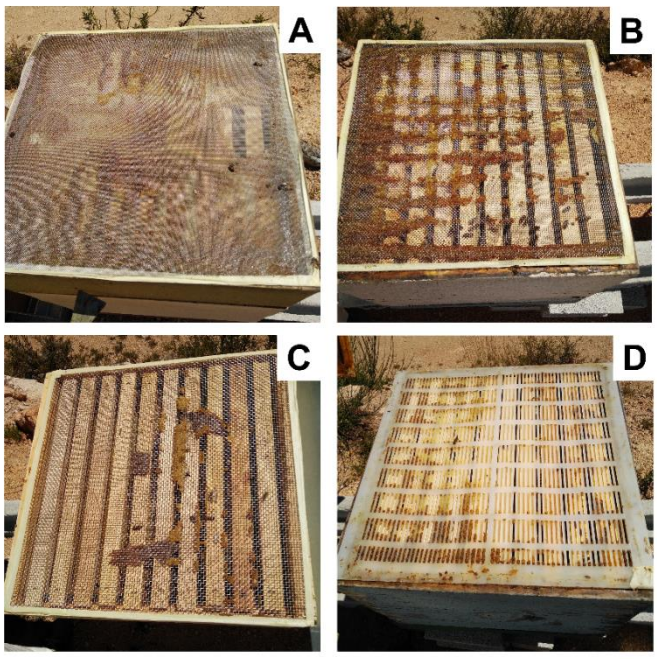


**Figure 2.2**. Examples of different types of grids used in this work at the harvesting time from representative beehives: metallic grids – with 0.63 mm (**A**), 1.8 mm (**B**) and 2.8 mm (**C**) of pore size - and plastic grid with 3 mm (**D**) of pore size.

Quantification of propolis production in 2017 (**Figure 2.3**) showed higher propolis amounts obtained by the smaller pore sized grids (0.63 mm; 25.0±13.2 g/ hive), when compared to the plastic grid (3 mm; 10.0±0.0 g/ hive) used by the beekeeper. However, such tendency was diluted by the lack of significant differences between the propolis mass obtained from different pore sized grids, possibly related to the extremely high temperatures and occurring fires near the apiary location. Also, in that year, some of the isogenic queen bees used in the hives died and were naturally replaced by non-isogenic ones.

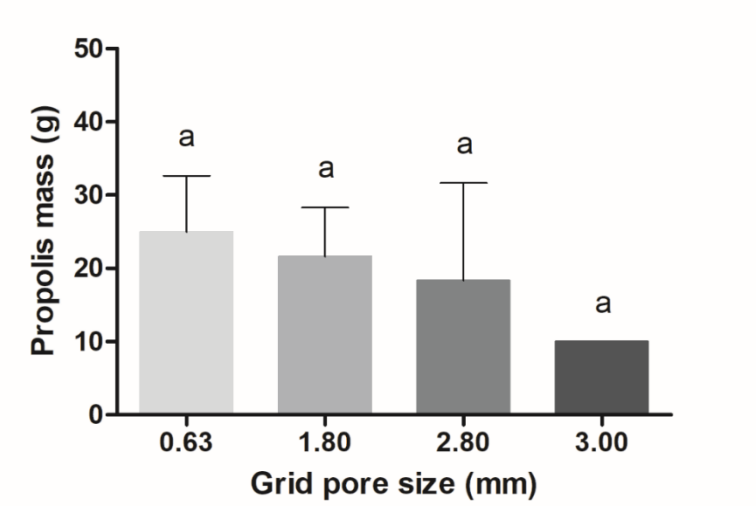


**Figure 2.3**. Propolis harvested from beehives with grids with different pore sizes (0.63, 1.80, 2.80 and 3.00 mm) during the year of 2017. One-way ANOVA was performed, followed by Tukey test to assess significance (n = 3). Same letters mean no statistically significant differences between mean values.

In 2018, grids placement was delayed till late spring due to the rain, reducing propolis production time. Like in 2017, higher amounts of propolis were achieved using grids with the smaller pore size (0.63 mm; 38.0±15.4 g/ hive), with a more pronounced tendency of the inverse relationship between grid pore size and propolis production (**Figure 2.4**).

Quantification of propolis production in 2019 was affected by the elimination of some of the hives due to the presence of the parasite *Varroa destructor*, leading to the need of additional hives to allow the study continuity. As can be seen in **Figure 2.5**, this year was the most affected and it was not possible to observe any tendency in propolis production by varying the pore size of the grids, with the propolis production varying from 25.0±13.0 g/ hive to 27.5±23.6 g/ hive, for the 3.0 mm and 1.8 mm pore sized grids, respectively.

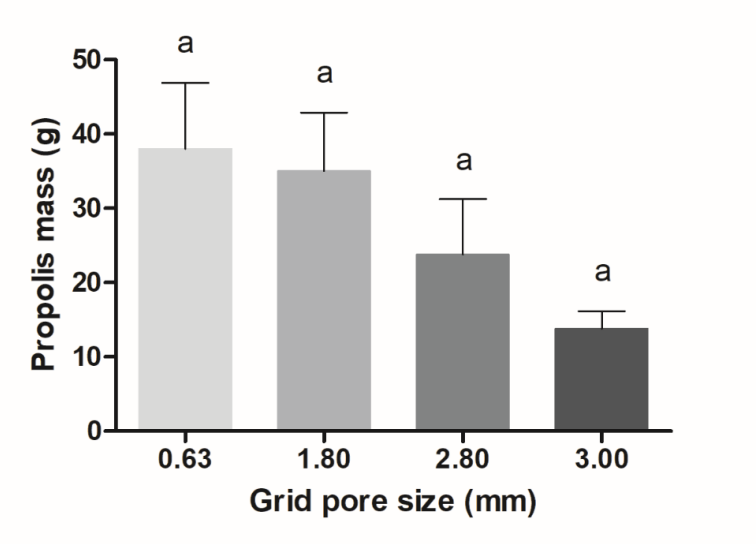


**Figure 2.4**. Propolis harvested from beehives with grids with different pore sizes (0.63, 1.80, 2.80 and 3.00 mm) during the year of 2018. One-way ANOVA was performed, followed by Tukey test to assess significance ($3 \geq n \geq 4$). Same letters mean no statistically significant differences between mean values.

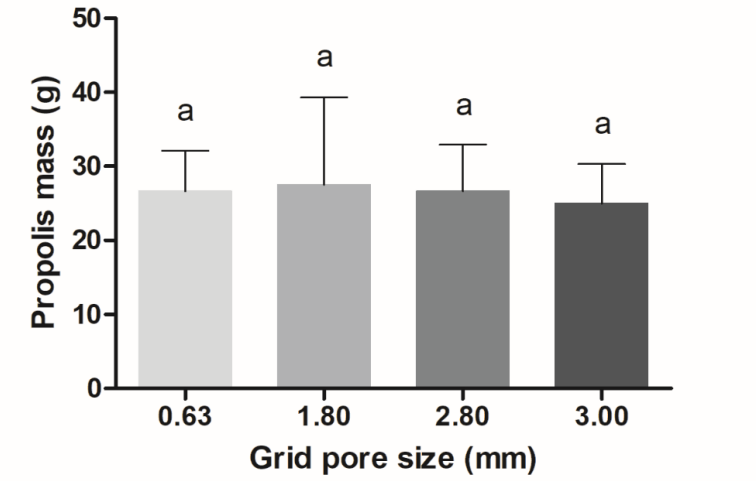


**Figure 2.5**. Propolis harvested from beehives with grids with different pore sizes (0.63, 1.80, 2.80 and 3.00 mm) during the year of 2019. One-way ANOVA was performed, followed by Tukey test to assess significance ($4 \geq n \geq 6$). Same letters mean no statistically significant differences between mean values.

The death of isogenic queens, the elimination of beehives due to the presence of *Varroa destructor* and the variability of the climate conditions which consequently influenced the period of grids placement and time for propolis production, altogether, are factors of inconsistency along the study period that have influenced the results. In fact, these factors can explain the high standard deviation values observed and contribute to the lack of significant differences between the propolis mass obtained from different pore sized grids. Still, when analyzing propolis production over the three years study period, although not statistically significant, the same tendency in which higher propolis amount was obtained from grids with the smallest pore size than from the commonly used 3 mm pore sized grids

(28.1±13.8 g/ hive and 18.8±11.3 g/ hive, respectively) is observed (**Figure 2.6**), suggesting that the smaller the pore size the higher is the propolis amount.

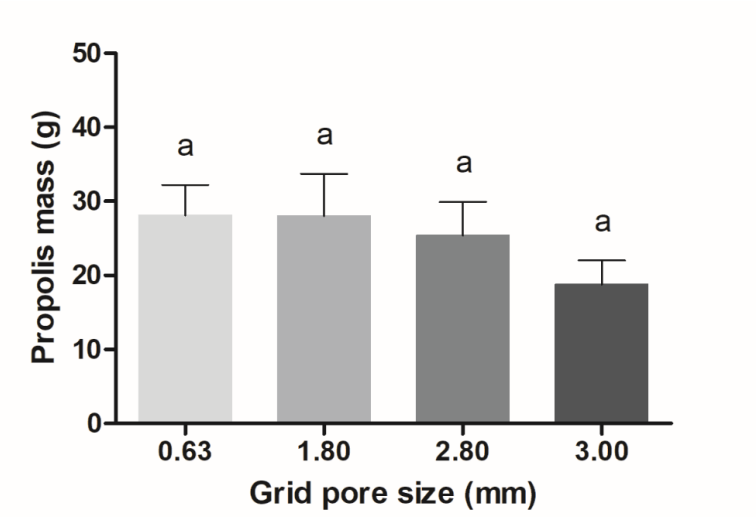


**Figure 2.6**. Propolis harvested from beehives with grids with different pore sizes (0.63, 1.80, 2.80 and 3.00 mm) during three consecutive years, considering propolis production means of 2017 (**Figure 2.3**), 2018 (**Figure 2.4**) and 2019 (**Figure 2.5**). One-way ANOVA was performed, followed by Tukey test to assess significance ($10 \geq n \geq 12$). Same letters mean no statistically significant differences between mean values.

The use of a metallic grid with dimensions close to the dimensions of the plastic grid normally used by the beekeeper made it possible to discard the influence of the grid material on propolis production, since there were no significant differences between the propolis production in the metallic grids of 2.8 mm and in the 3 mm plastic grids.

Unlike for the study of factors impacting on propolis chemical composition and/ or bioactivities, literature regarding factors that influence propolis production is very scarce. A study from Jager *et al.* (2002) on propolis production over a nine-month period (July - March) from an apiary from the Cape region (South Africa), reported ten times higher propolis amount in beehives with grids (361.9±0.18 g/ hive) than in the control group without any grid (36.1±0.18 g/ hive). These propolis production values are also ten times higher than the values obtained in the present study, supporting the hypothesis of the bigger weight of the influence of the environmental factors in the production of this bee product. In the same study, no significant differences were reported for honey production during the same test period (24.7±0.1 Kg/ hive and 26.5±0.1 Kg/ hive for beehives with grids and without grids, respectively; Jager *et al.*, 2002). Also, in the present study, and according to the beekeeper, honey yields were the same independently of using grids or not (Amadeu Fortunas, personal communication) and regardless the year of study. A European hive can produce between 50 to 150 g of propolis per

year (Casaca, 2010), however, different values have been reported. Accordingly, Abu Fares *et al.* (2008) found that the propolis production is very variable but production can be as low as 15.7 g of propolis per hive in the most productive groups. Similarly, Nuru *et al.* (2002) reported a mean production of 24.2±22.5 g/ hive in framed hives. According to the national federation of Portuguese beekeepers FNAP (2010) and considering the traditional method of propolis harvest (scrapping), the annual production of Portuguese propolis is between 100 and 200 g per hive. It was reported that propolis production can reach 500 g/ hive/ year using grids and without quality loss or substantial increased production costs (APINFO, 2019). However, according to the beekeeper who provided the propolis samples used in this work, the annual production of propolis from Gerês is around 27 g/ hive (Amadeu Fortunas, personal communication).

Like in our study (**Figures 2.3-2.6**), Tsagkarakis *et al.* (2017) used different square pore size plastic grids (1 mm, 2 mm, and 4 mm), and the commercial 3 mm rectangular pore sized grid to assess the influence of different pore sized grids on propolis production during two consecutive years. The highest amount of propolis was collected from the 1 mm pore sized grids (29.6±7.6 g/ hive), followed by the 2 mm pore sized (23.7±5.6 g/ hive) and the 3 mm commercial grids (15.6±4.1 g/ hive) though without significant differences. The grid with the largest pore size (4 mm) was the less preferred by bees to produce propolis as judged by the significantly smaller amount of propolis collected (1.2±0.9 g/ hive). In agreement with these findings, the new propolis grids with square openings used nowadays are more effective for propolis production than the ones with rectangular openings like the one used in this work (Sandra Barbosa, personal communication).

Another factor that seems to influence not only propolis production, but its quality as well, is the beehive material (Kiziltas and Erkan, 2020). A higher propolis yield post honey production season was obtained from wooden beehives (17.6±16.2 g/ hive), like the ones used in the present study, followed by styrofoam (8.6±5.1 g/ hive). Plastic beehives were reported as the ones with the lowest propolis production (1.7±1.8 g/ hive) (Kiziltas and Erkan, 2020). Although having slightly different pore sizes and making the collection of propolis more difficult, the metal grids with the largest pore size (2.8 mm) used herein showed higher propolis production than the 3 mm pore sized plastic grids, suggesting that the grid material could also be influencing propolis production.

Seasonality (Mountford-McAuley *et al.*, 2021) and period of propolis production were also pointed as influencing propolis yield and quality. Bees' behavior and work is related to the time of the year, living a semi-rest during winter to ensure survival, consuming honey produced during the previous year. In spring, with the increase of the surrounding temperature, bees become active and begin the

productive tasks, including honey production. Although grids were placed in spring along our three-year study, several factors such as the climate conditions, the vegetation diversity around the hive and the occurrence of suitable conditions have influenced grids placement time each year (May 2017, June 2018 and April 2019), with harvesting being made in early autumn (August-September 2017, 2018 and 2019). However, researchers reported the highest values are achieved in late autumn and early winter (Jager *et al.*, 2002; Abu Fares *et al.*, 2008; Kiziltas and Erkan, 2020), which could explain the low propolis yield along this study, together with other influencing factors. In temperate regions, these seasons are normally followed by a reduction in the average temperature. Temperature fluctuations inside the hive may also encourage propolis production as one of its functions is as thermic isolator of the beehive, helping to prevent steep decreases of temperature inside the hive.

## 2.4. Conclusions

In the past, Portuguese beekeepers neglected propolis production and discarded it while cleaning the hives, disregarding its valuable properties and believing propolis could negatively influence honey production. Today, scientists and beekeepers have been working together encouraging propolis production to help sustain healthy hives. With the increasing interest and research on propolis properties by the scientific community, beekeepers are beginning to look at propolis as an additional income involving low capital and minimum labor inputs. Here we found that small pore sized grids should be used in order to improve propolis production and a new beehive grid could be designed on behalf of the data herein presented. This study presents scientific evidence for the adaptation of apiculture, being a contribution for the valorization of the sector and of propolis.

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# Chapter 3

**Constancy of properties of bee products from Gerês (Portugal): unraveling the impact of beehive relocation**

*The work presented in this chapter has been submitted:*

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*Equal senior authorship

## Abstract

Propolis, a complex mixture of resinous and balsamic materials collected by honeybees from plants and mixed with bees' salivary secretions, is widely known for its variable chemical composition and bioactive profiles, even from the same origin but collected in different years. However, Portuguese propolis from an apiary named Gerês, normally obtained by mixing propolis from three places - Bugalho, Felgueiras and Toutelo - show similar chemical and biological profiles over the years. Recently, a new propolis place - Roca - was added to the apiary to replace Bugalho, lost to the 2017 wildfires, hence questioning the previously claimed constancy of Gerês propolis. To unravel to what extent the beehive relocation affected this particular constancy, we studied different Gerês propolis samples collected in three consecutive years (2017-2019) but composed by different combinations of source places. Two honey samples, collected before (2017) and after (2018) the occurrence of the wildfire, were also investigated. Total phenolics, flavonoids and *ortho*-diphenols contents were determined and the antioxidant and antimicrobial activities were evaluated, using the DPPH· scavenging assay and the agar dilution method, respectively. Both antimicrobial and antioxidant activities were generally in the ranges usually obtained from Gerês propolis samples. However, some variations were detected for the samples containing propolis harvested from the new location when compared to the previous years. This work reinforces the importance of the consistency of a combination of several factors, as the protection and preservation of the flora nearby the hives, providing bee products with more constant chemical and biological profiles over the years.

**Keywords:** Beehive relocation, propolis, honey, antibacterial activity, antioxidant potential.

## 3.1. Introduction

Propolis, or bee glue, is a complex mixture composed by resinous and balsamic materials collected by honeybees from branches, flowers, pollen, buds and exudates of trees which are mixed with salivary enzymes and other compounds resulting from bees' metabolism (Ghisalberti, 1979; Burdock, 1998). Propolis is already recognized as an important source of bioactive compounds with properties for several applications (Moreira *et al.*, 2008; Silva-Carvalho *et al.*, 2015; Pasupuleti *et al.*, 2017). The chemical and biological profiles of this beehive product are usually very variable according to several factors such as the source plant species (Katalinić *et al.*, 2004; Falcão *et al.*, 2010), the climate characteristics (Falcão *et al.*, 2010), the harvesting time (Miguel *et al.*, 2010) and technique, the bee species (Pereira *et al.*, 2002) as well as the solvent and method of extraction (Sheng *et al.*,

2006; Freitas *et al.*, 2022). However, propolis studied in this work is peculiar due to an unusual constancy of its chemical and biological profiles reported for the period between 2011 and 2016, which was linked to its production in an organic mode, a standardized way of collection (from grids) and to the consistency of the vegetation nearby the apiary (Freitas *et al.*, 2019), which is located in a protected area of the Peneda - Gerês National Park.

Plant diversity define different types of propolis, containing numerous but also diverse chemical constituents responsible for its biological properties, which include antimicrobial, antioxidant and antitumor activities, among many others (Fokt *et al.*, 2010; Silva-Carvalho *et al.*, 2015; Anjum *et al.*, 2019). Such biological properties are thought to be connected to the complex chemical composition, involving a mechanism putatively attributed to synergistic effects between their chemical compounds (Montenegro and Mejías, 2013). Among the 850 compounds already identified in different propolis samples (Šturm and Ulrih, 2019; Kasote *et al.*, 2022), phenolics seem the most important. Flavonoids (flavones, flavonols and flavonones), phenolic acids and their esters are the main bioactive compounds found in European propolis (Huang *et al.*, 2014), being the flavonoids pinocembrin, galangin and chrysin and the phenolic acids caffeic acid, ferulic acid and cinnamic acid the most common phenolic compounds in propolis from temperate zones (Bankova *et al.*, 2000; Huang *et al.*, 2014).

Propolis from Gerês is usually composed by a mixture of propolis collected from beehives located at different places in Gerês: Bugalho, Felgueiras, and Toutelo. However, in 2017, wildfires have destroyed one of these source places - Bugalho - and, despite the installation of new beehives at the same location in 2018, allowing to obtain a propolis sample similar to the previous years, the swarms did not resist, and the beehives had to be moved to another location - Roca. Subsequently, in order to search for the constancy usually observed in Gerês propolis (Freitas *et al.*, 2019), questioned by the beehive relocation, we have studied eleven ethanol extracts of propolis sampled from Gerês and collected in three consecutive years (2017-2019) but composed by different combinations of four source places - Bugalho, Felgueiras, Toutelo and Roca. Two honey samples - collected before (2017) and after (2018) the occurrence of the wildfire – were included in the present study too. Pollen analysis as well as the chemical characterization and the antimicrobial and antioxidant activities of propolis and honey were investigated and compared with samples from Gerês collected in previous years.

## 3.2. Materials and Methods

### 3.2.1. Propolis and honey samples and extracts preparation

Propolis and honey samples used in this work were harvested from an apiary located in Gerês (G), in the north of Portugal and near the Cávado River (41°45'41.62'' N; 7°58'03.34'' W). Propolis was collected from the different available places of Gerês apiary – Bugalho (G-b), Felgueiras (G-f), Toutelo (G-t) and Roca (G-r) – over three consecutive years (**Figure S3.1 Supplementary material**). Ethanol extraction was performed as previously described (Freitas *et al.*, 2019) from samples resulting from different combinations of propolis collected each year (**Figure S3.1 Supplementary material**). Briefly, a volume of 80 mL of ethanol was added to approximately 15 g of each propolis sample and the mixtures were placed under orbital agitation (100 rpm), at 24 °C, in the dark, for 24 h. Mixtures were then filtered under vacuum and the filtrates reserved at 4 °C. The solid residues were extracted again with 50 mL of the same solvent. The resulting filtrates were pooled and dried in a Buchi Rotavapor RE 121 (40 rpm, at 40 °C), yielding the dried ethanol extracts of propolis from Gerês (G.EEs): G-r17.EE, G-rt17.EE and G-tf17.EE of 2017; G-r18.EE, G-rt18.EE, G-tf18.EE and G-tfb18.EE (same as G18.EE) of 2018; and G-r19.EE, G-rt19.EE, G-tf19.EE and G-rtf19.EE (same as G19.EE) of 2019 (**Figure S3.1 Supplementary material**). The G.EEs were stored at 4 °C in the dark, for experimental use. Stock solutions of propolis extracts were prepared by dissolving each G.EE in absolute ethanol to obtain the desired working concentrations.

Honey samples (H), harvested in 2017 (H17) and 2018 (H18), were diluted in water to a stock solution of 50 % (v/v) - H17.WE and H18.WE - to be used in the following described assays.

### 3.2.2. Pollen characterization

Pollen samples collected during two consecutive years (2017 and 2018) by the beekeeper were analysed [APISMAIA, Produtos & Serviços] for botanical origin determination (Valdés *et al.*, 1987; Von Der Ohe *et al.*, 2004). Briefly, 10 g of honey were dissolved in 20 mL of distilled water following a centrifugation at 1000 x*g* for 10 min. The supernatant was removed, and the process repeated to obtain the honey sediment, which was placed in a slide over a 22x22 mm marked square and on a heating plate for drying. Glycerine jelly was applied in a cover slip and placed on the slide being the preparation heated for 5 min. The relative frequencies, in percentage, of each pollen type were determined by counting grains of pollen in groups of 100, excluding pollen from nectarless plants, following 5 parallel equidistant lines uniformly distributed, until 500 grains were counted. Pollinic types were attributed according to Valdes *et al.* (1987).

### 3.2.3. Chemical characterization of propolis and honey extracts from Gerês

Chemical characterization of propolis and honey extracts was assessed by three different colorimetric assays: total *ortho*-diphenols, phenolics and flavonoids contents.

#### 3.2.3.1. Determination of total *ortho*-diphenols content (TOC)

The content in *ortho*-diphenol compounds was determined using an adaptation of the colorimetric assay proposed by Mateos *et al.* (2001). In brief, an identical volume of different concentrations of each of the eleven G.EEs (from 25 to 300 μg $mL^{-1}$) or of each honey sample (H.WEs; from 5 to 10 %) was mixed with 5 % (w/v) $Na_2MoO_4$ in ethanol/ water 1:1 (v/v), followed by 15 min incubation at room temperature (RT), in the dark. Control and blank were prepared with similar mixtures but with ethanol replacing G.EEs, H.WEs or $Na_2MoO_4$, respectively. Absorbance of the reaction was measured at 370 nm and results were obtained by interpolation of linear regression using gallic acid as standard (calibration curve with concentrations ranging from 40 to 180 μg $mL^{-1}$, $R^2 = 1$), and expressed in milligrams of gallic acid equivalents (GAE) per gram of propolis or honey extract (mg GAE/ g extract).

#### 3.2.3.2. Determination of total phenolics content (TPC)

The content in phenolic compounds was determined using an adaptation of the Folin-Ciocalteu colorimetric assay (Kumazawa *et al.*, 2004). Briefly, different concentrations (from 1 to 300 μg $mL^{-1}$) of each of the eleven G.EEs or (from 0.1 to 5 %) of each honey sample were mixed with 10 % (v/v) Folin-Ciocalteu reagent and 7.5 % (w/v) $Na_2CO_3$. The control and the blank were prepared with similar mixtures but with ethanol replacing G.EEs, H.WEs or reagents, respectively. Absorbance was measured at 760 nm after 1 h incubation at RT, in the dark. Results were obtained by interpolation of linear regression of a gallic acid calibration curve (1 to 50 μg $mL^{-1}$, $R^2 = 1$), and expressed in milligrams of gallic acid equivalents (GAE) per gram of propolis or honey extract (mg GAE/ g extract).

#### 3.2.3.3. Determination of total flavonoids content (TFC)

The content in flavonoid compounds was determined using an adaptation of the colorimetric assay previously described by Kumazawa *et al.* (2004). In brief, different concentrations (from 200 to 2200 μg $mL^{-1}$) of each of the eleven G.EEs were mixed with 2 % (w/v) $AlCl_3$. Control and blank were prepared with similar mixtures but with ethanol replacing G.EEs or $AlCl_3$, respectively. Absorbance of the reaction was measured at 420 nm after 1 h incubation at RT, in the dark. Results were obtained by

interpolation of linear regression of a quercetin calibration curve (5 to 200 μg $mL^{-1}$, $R^2$ = 0.99), and expressed in milligrams of quercetin equivalents (QE) per gram of propolis extract (mg QE/ g extract).

### 3.2.4. *In vitro* evaluation of the antioxidant potential of propolis and honey extracts from Gerês

DPPH· (2,2-diphenyl-1-picryl-hydrazyl) method was used for *in vitro* evaluation of the antioxidant potential as previously described (Freitas *et al.*, 2019). Succinctly, different concentrations (from 1 to 50 μg $mL^{-1}$) of each G.EE or (from 700 to 45000 μg $mL^{-1}$) of each H.WEs were mixed with 0.004 % (w/v) DPPH· in a proportion of 1:2). The control and the blank were prepared with similar mixtures but with ethanol replacing G.EEs, H.WEs or DPPH·, respectively. Absorbance of the reaction was measured at 517 nm after 20 min incubation at RT, in the dark. Results were expressed in $EC_{50}$ values (μg $mL^{-1}$; concentration that reduces the free radical in 50 %) obtained by interpolation from linear regression analysis of the percentage decrease in absorbance with respect to control values.

### 3.2.5. Antimicrobial properties of propolis and honey extracts from Gerês

Minimum inhibitory concentrations (MIC) were determined using the agar dilution method (Hanson and Martin, 1978) and the following panel of susceptible indicator strains: the Gram-negative bacterium *Escherichia coli* CECT 423, six Gram-positive bacteria - *Bacillus subtilis* 48886, *Bacillus cereus* ATCC 7064, *Bacillus megaterium* 932, (Methicillin Sensitive) *Staphylococcus aureus* ATCC 6538 (MSSA), Methicillin Resistant *Staphylococcus aureus* M746665 (MRSA) and *Propionibacterium acnes* H60803 – as well as two yeast - *Saccharomyces cerevisiae* BY4741 from Euroscarf (http://euroscarf.de/index.php?name=Description) and *Candida albicans* 53B - obtained from the microbial collection of the Department of Biology of the University of Minho.

Bacteria and yeast were cultured, respectively, in LB (Luria-Bertani - 0.5 % w/v yeast extract, 1 % w/v tryptone, 1 % w/v NaCl) and YPD (1 % w/v yeast extract, 2 % w/v peptone, 2 % w/v glucose) broths or in solid media (LBA and YPDA – obtained by adding 2 % w/v agar to each of the previous LB and YPD recipes, respectively). Growth was performed at 200 rpm - at 37 °C for bacteria or 30 °C for yeast - and monitored by optical density at 600 nm ($OD_{600}$). To prepare the cells for the experiments, overnight microbial cultures were diluted with the appropriated fresh medium to an $OD_{600}$ = 0.1 and cultured under the same conditions until $OD_{600}$ = 0.4 - 0.6 (mid-exponential phase).

Drops of 5 μL of exponentially growing microbial cultures were transferred onto LBA and YPDA plates, for bacteria and yeast, respectively, supplemented with different concentrations (0, 10, 50, 100, 200, 500, 750, 1000, 1250, 1500, 1750 or 2000 μg $mL^{-1}$) of each of the eleven G.EEs or different

concentrations (from 2.5 up to 40 %, with increments of 2.5 %) of each of the H.WEs. Plates containing identical volumes of ethanol instead of the respective extract were used as controls. Plates were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h in the case of yeast. MIC values were determined upon observation of the presence/absence of growth.

### 3.2.6. Data analysis - statistics

Chemical profiles by colorimetric assays as well as antioxidant and antimicrobial experiments were done in triplicate and repeated at least three times independently, results being expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's test for multiple comparisons were used to assess treatment effect. Differences considered statistically significant ($p < 0.05$) were noted with different letters.

## 3.3. Results

### 3.3.1. Pollen profiles from Gerês honey samples

The pollen profiles obtained for the Gerês honey samples studied in the present work are presented in **Table S3.1 Supplementary material**. *Erica* spp. was the most predominant pollen in both samples, representing 44 - 55 % of the total pollen composition but while *Castanea sativa* was a secondary pollen for the H17 sample *Frangula alnus* was the secondary pollen for H18. Nevertheless, H17 and H18 can be classified as heather honeys, as they present similar botanical origins according to their pollen analysis (Pires *et al.*, 2009; Manzanares *et al.*, 2017).

### 3.3.2. Total *ortho*-diphenols and phenolics contents from Gerês honey extracts

Total *ortho*-diphenols (TOC) and phenolics (TPC) contents of the honey extracts from Gerês – H17.WE and H18.WE – were assessed. H18.WE showed a significant high content in *ortho*-diphenol compounds (0.535±0.011 mg GAE/ g) when compared to H17.WE (0.455±0.005 mg GAE/ g; $p < 0.001$). On the other hand, H17.WE exhibited a significant higher content in phenolics compounds (3.079±0.145 mg GAE/ g) than H18.WE (2.438±0.035 mg GAE/ g; $p < 0.01$).

### 3.3.3. Antioxidant potential of the honey extracts of Gerês

Antioxidant capacity of the honey extracts from Gerês was evaluated by the DPPH free-radical-scavenging assay and the respective $EC_{50}$ values were calculated. Both H.WEs showed the ability to scavenge the DPPH free radical and, despite being both classified as heather honeys, $EC_{50}$ values were

significantly different – 9662.3 and 5167.5 μg $mL^{-1}$ ($p < 0.0001$), for H17.WE and H18.WE, respectively.

### 3.3.4. Antimicrobial properties of the honey extracts of Gerês

The antimicrobial activity of the honey extracts was evaluated against a panel of susceptibility indicator strains (**Table 3.1**). The Gram-positive bacteria of the genus *Bacillus* were the most susceptible strains to both extracts. H18.WE was the most effective extract against all the tested strains, and the only effective against both tested yeasts, with MIC values of 17.5 and 22.5 % for *Saccharomyces cerevisiae* and *Candida albicans*, respectively.

**Table 3.1.** G.EEs MIC values (%) obtained against the panel of susceptibility indicator strains. Mid-exponential phase cultures were transferred to plates supplemented with increasing concentrations of each of the honey extracts – H17.WE and H18.WE. Plates were observed for the presence/ absence of growth after 24 h incubation at 37 °C for bacteria and 48 h incubation at 30 °C for yeast and the lowest concentrations for which no growth was detected was registered as the MIC.

| Honey extracts | Antimicrobial activity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Bacteria | | | | | | | Yeasts | |
| | *B. subtilis* | *B. cereus* | *B. megaterium* | MSSA | MRSA | *P. acnes* | *E. coli* | *S. cerevisiae* | *C. albicans* |
| | MIC (%) | | | | | | | | |
| **H17.WE** | 20 | 22.5 | 20 | 40 | 27.5 | 22.5 | 27.5 | > 40 | > 40 |
| **H18.WE** | 12.5 | 10 | 15 | 17.5 | 17.5 | 17.5 | 17.5 | 17.5 | 22.5 |

MSSA - Methicillin Sensitive *Staphylococcus aureus*; MRSA - Methicillin Resistant *Staphylococcus aureus*.

### 3.3.5. Total *ortho*-diphenols, phenolics and flavonoids contents from Gerês propolis extracts

**Table 3.2** summarizes the total *ortho*-diphenols (TOC), phenolics (TPC) and flavonoids (TFC) contents in different extracts of propolis samples from Gerês. G-r17.EE, G18.EE and G-r18.EE presented the highest contents in *ortho*-diphenol, phenolic and flavonoid compounds. The highest TOC, TPC and TFC were achieved for extracts of propolis sampled only from Roca place, while the lowest contents were obtained, in general, from extracts without Roca propolis in their composition. The exception is G18.EE with the highest TPC value (185.5±7.1 mg GAE/ g of propolis). Thus, the addition of Roca as a new source place to collect propolis proved to be an asset, enabling to obtain extracts with higher contents in *ortho*-diphenol, phenolic and flavonoid compounds.

**Table 3.2.** Total *ortho*-diphenols (TOC), phenolics (TPC) and flavonoids (TFC) contents of G-r17.EE, G-rt17.EE, G-tf17.EE, G-r18.EE, G-rt18.EE, G-tf18.EE, G18.EE, G-r19.EE, G-rt19.EE, G-tf19.EE and G19.EE. Results are presented as mean ± standard deviation (SD) of mg of gallic acid equivalents per g of extract (mg GAE/ g extract) for TOC and TPC, and as mg of quercetin equivalents per g of extract (mg QE/ g extract) for TFC. Statistical analysis was performed by one-way ANOVA followed by Tukey test for multiple comparisons. For each individual parameter (TOC, TPC and TFC), same letters (a, b, c, d, e or f) mean no statistically significant differences between mean values.

| Propolis extracts | Total *Ortho*-diphenol Content | Total Phenolics Content | Total Flavonoid Content |
|---|---|---|---|
| | mg GAE/ g | mg GAE/ g | mg QE/ g |
| **G-r17.EE** | 467.2±13.1$^{a}$ | 177.9±6.7$^{a}$ | 36.8±2.3$^{a}$ |
| **G-rt17.EE** | 271.0±3.9$^{b}$ | 130.9±9.0$^{b}$ | 22.5±1.8$^{b}$ |
| **G-tf17.EE** | 243.5±6.8$^{b,d}$ | 125.1±5.5$^{b,d}$ | 30.5±1.1$^{c}$ |
| **G-r18.EE** | 373.8±19.3$^{c}$ | 148.4±4.1$^{c}$ | 44.8±2.8$^{d}$ |
| **G-rt18.EE** | 295.7±51.8$^{b}$ | 82.9±5.3$^{e}$ | 29.6±1.6$^{c,f}$ |
| **G-tf18.EE** | 205.6±17.2$^{d,e}$ | 73.7±6.9$^{e}$ | 25.7±1.9$^{b,f}$ |
| **G18.EE (G-tfb18.EE)** | 270.0±15.2$^{b}$ | 185.5±7.1$^{a}$ | 35.8±0.5$^{a,e}$ |
| **G-r19.EE** | 285.9±14.3$^{b}$ | 136.2±5.3$^{b,c}$ | 30.7±0.8$^{c}$ |
| **G-rt19.EE** | 299.3±9.3$^{b}$ | 135.6±9.4$^{b,c}$ | 29.0±0.8$^{c,f}$ |
| **G-tf19.EE** | 247.7±15.9$^{b,e}$ | 110.3±5.9$^{d}$ | 25.6±0.9$^{b,f}$ |
| **G19.EE (G-rtf19.EE)** | 272.3±4.7$^{b}$ | 135.2±8.2$^{b,c}$ | 31.9±1.1$^{c,e}$ |

See Materials and Methods subsection 3.2.1. for elucidation of propolis extracts identification.

### 3.3.6. Antioxidant potential of the ethanol extracts of Gerês propolis samples

Antioxidant capacity of the different propolis extracts from Gerês was evaluated by the DPPH free-radical-scavenging assay and the respective $EC_{50}$ values were calculated (**Table 3.3**). All propolis extracts showed the ability to scavenge the DPPH free radical with $EC_{50}$ values ranging from 3.6 to 30.5 μg $mL^{-1}$, for G-r17.EE and G-r19.EE, respectively. Propolis samples from 2017, particularly those with propolis from Roca place in their composition, exhibited a significantly higher antioxidant potential when compared to the remaining tested samples ($EC_{50}$ = 3.6 μg $mL^{-1}$ for G-r17.EE and $EC_{50}$ = 7.1 μg $mL^{-1}$ for G-rt17.EE). Interestingly, besides exhibiting the highest antioxidant potential, G-r17.EE was the sample with a significantly higher content in *ortho*-diphenol compounds, suggesting that *ortho*-diphenols could promote the antiradical capacity.

**Table 3.3.** Antioxidant potential of G-r17.EE, G-rt17.EE, G-tf17.EE, G-r18.EE, G-rt18.EE, G-tf18.EE, G18.EE, G-r19.EE, G-rt19.EE, G-tf19.EE and G19.EE – measured by the *in vitro* DPPH free-radical-scavenging assay and expressed as a mean of $EC_{50}$ values (μg $mL^{-1}$) and respective standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by Tukey test for multiple comparisons. Same letters (a, b, c, d, e, f, g or h) mean no statistically significant differences between mean values.

| Propolis extracts | DPPH· scavenging activity |
|---|---|
| | $EC_{50}$ (μg $mL^{-1}$) |
| **G-r17.EE** | $3.6\pm0.3^{a}$ |
| **G-rt17.EE** | $7.1\pm0.8^{b}$ |
| **G-tf17.EE** | $20.9\pm0.1^{c}$ |
| **G-r18.EE** | $20.1\pm0.1^{c,e}$ |
| **G-rt18.EE** | $19.0\pm0.5^{e,f}$ |
| **G-tf18.EE** | $18.3\pm0.7^{f}$ |
| **G18.EE (G-tfb18.EE)** | $12.4\pm0.4^{d}$ |
| **G-r19.EE** | $30.5\pm0.3^{g}$ |
| **G-rt19.EE** | $19.5\pm1.3^{c,f}$ |
| **G-tf19.EE** | $21.2\pm0.5^{c}$ |
| **G19.EE (G-rtf19.EE)** | $24.3\pm0.1^{h}$ |

See Materials and Methods subsection 3.2.1. for elucidation of propolis extracts identification.

### 3.3.7. Antimicrobial properties of the ethanol extracts of Gerês propolis samples

The antimicrobial activity of the eleven propolis extracts was evaluated against a panel of susceptibility indicator strains (**Table 3.4**). As reported by several authors (Bankova *et al.*, 2000; Uzel *et al.*, 2005), including our research group (Freitas *et al.*, 2019), the Gram-positive bacteria, such as the ones of the genus *Bacillus*, were the most susceptible strains to all the extracts. G18.EE was the most effective extract against all the tested strains, followed by the extracts from propolis collected in 2017 (G-r17.EE, G-rt17.EE and G-tf17.EE) and G-tf18.EE and G-rt19.EE, which showed to equally affect the growth of the tested bacteria (**Table 3.4**). All the extracts exhibited the same MIC values against the MSSA, MRSA, *Propionibacterium acnes* and *Escherichia coli,* except G-tf17.EE and G18.EE that were the most active against the Gram-positive *Propionibacterium acnes* (MIC = 200 μg $mL^{-1}$) and MSSA

(MIC = 200 μg mL$^{-1}$), respectively. G-tf19.EE was the least active extract, exhibiting the highest MIC values for the *Bacillus* strains (MIC = 200 μg mL$^{-1}$).

**Table 3.4.** G.EEs MIC values (mg L$^{-1}$) obtained against the panel of susceptibility indicator strains. Mid-exponential phase cultures were transferred to plates supplemented with increasing concentrations of each of the eleven G.EEs – G-r17.EE, G-rt17.EE, G-tf17.EE, G-r18.EE, G-rt18.EE, G-tf18.EE, G18.EE, G-r19.EE, G-rt19.EE, G-tf19.EE and G19.EE. Plates were observed for the presence/ absence of growth after 24 h incubation at 37 °C for bacteria and 48 h incubation at 30 °C for yeast and the lowest concentrations for which no growth was detected was registered as the MIC.

| Propolis extracts | Antimicrobial activity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Bacteria | | | | | | | Yeast | |
| | *B. subtilis* | *B. cereus* | *B. megaterium* | MSSA | MRSA | *P. acnes* | *E. coli* | *S. cerevisiae* | *C. albicans* |
| | MIC (μg mL$^{-1}$) | | | | | | | | |
| **G-r17.EE** | 50 | 50 | 50 | 500 | >2000 | 500 | >2000 | 1500 | >2000 |
| **G-rt17.EE** | 50 | 50 | 50 | 500 | >2000 | 500 | >2000 | >2000 | >2000 |
| **G-tf17.EE** | 50 | 50 | 50 | 500 | >2000 | 200 | >2000 | >2000 | >2000 |
| **G-r18.EE** | 100 | 100 | 200 | 500 | >2000 | 500 | >2000 | 2000 | 2000 |
| **G-rt18.EE** | 100 | 100 | 100 | 500 | >2000 | 500 | >2000 | >2000 | 1500 |
| **G-tf18.EE** | 50 | 50 | 50 | 500 | >2000 | 500 | >2000 | >2000 | 2000 |
| **G18.EE (G-tfb18.EE)** | 50 | 50 | 50 | 200 | >2000 | 500 | >2000 | 1500 | 2000 |
| **G-r19.EE** | 100 | 100 | 100 | 500 | >2000 | 500 | >2000 | >2000 | 2000 |
| **G-rt19.EE** | 50 | 50 | 50 | 500 | >2000 | 500 | >2000 | >2000 | 1500 |
| **G-tf19.EE** | 200 | 200 | 200 | 500 | >2000 | 500 | >2000 | >2000 | 2000 |
| **G19.EE (G-rtf19.EE)** | 100 | 100 | 100 | 500 | >2000 | 500 | >2000 | >2000 | 2000 |

MSSA - Methicillin Sensitive *Staphylococcus aureus*; MRSA - Methicillin Resistant *Staphylococcus aureus*;
See Materials and Methods subsection 3.2.1. for elucidation of propolis extracts identification.

The antifungal effect of the eleven extracts was also evaluated (**Table 3.4**). All the extracts showed similar activity against both strains, with G-r17.EE and G18.EE standing out from the others as the most active against *Saccharomyces cerevisiae* (MIC = 1500 μg mL$^{-1}$) and G-rt18.EE and G-rt19.EE as the most active against *Candida albicans* (MIC = 1500 μg mL$^{-1}$).

## 3.4. Discussion

Portuguese beehive products, such as honey and propolis, have been studied by several authors (Cruz *et al.*, 2008; Miguel *et al.*, 2010; Oliveira *et al.*, 2017; Karabagias *et al.*, 2020; Gomes *et al.*, 2021; Peixoto *et al.*, 2021, 2022), particularly in what concerns its phenolic composition and its

antioxidant and antimicrobial activities. The samples from the present study showed high percentage of *Erica* sp. pollens (**Table S3.1 Supplementary material**), giving the classification of Heather honey to both H17 and H18. Heather honey is characterized by its dark brown color and a strong flavor with a little salty taste, which is very common in Portugal (Feás *et al.*, 2010; Feás and Estevinho, 2011; Estevinho *et al.*, 2012).

Honey samples from the North of Portugal have been the major object of study, both from Northeast and Northwest Regions (Estevinho *et al.*, 2008, 2011, 2012; Feás *et al.*, 2010; Gomes *et al.*, 2011; Feás and Estevinho, 2011; Soares *et al.*, 2017). An important aspect of honey is its content in bioactive compounds. To our knowledge, this is the first time *ortho*-diphenols content of honey is determined, having been shown to be an important parameter to analyse considering its potential influence on both honey and propolis bioactivities. Total phenolics content of Portuguese honey samples have been investigated, showing lower TPC values - between 0.744 and 1.910 mg GAE/ g (Estevinho *et al.*, 2012; Karabagias *et al.*, 2020) - than the results obtained in the present work. Antioxidant activity of Portuguese honey has been demonstrated by other authors by the ability to scavenge different free radicals, such as the DPPH, peroxyl and nitric oxide, and by the ability for chelating metal ions (Estevinho *et al.*, 2008; Aazza *et al.*, 2013). Between the two honey samples tested in the present work, H18.WE was the one exhibiting the higher antioxidant potential, suggesting that *ortho*-diphenols could promote the radical scavenging capacity as well. Likewise, H18.WE showed higher antimicrobial activity against both bacteria and fungi strains.

Despite being classified as heather honeys, H18 possess considerably higher percentage of *Frangula alnus* pollen in its composition than H17. *Frangula alnus* extract antioxidant capacity and antimicrobial activity, particularly against *S. aureus*, *E. coli* and *C. albicans* has been demonstrated (Kremer *et al.*, 2012; Sadowska *et al.*, 2014; Brkanac *et al.*, 2015; Elansary *et al.*, 2020). *Frangula alnus* is known to sprout quickly after a fire, becoming more abundant (Catling *et al.*, 2001), which suggests an influence on H18 chemical composition, leading in turn to higher antioxidant and antimicrobial potential.

In line with the present study, other authors found that increasing concentrations of heather honey were able to inhibit growth of the yeast *C. albicans* and *S. cerevisiae* and bacteria like *B. subtilis*, *S. aureus* and *E. coli* (Estevinho *et al.*, 2008; Gomes *et al.*, 2010; Feás and Estevinho, 2011). Additionally, according to the same authors, Gram-positive bacteria seem to be generally more sensitive to honey phenolic compounds extracts, which is also in accordance with our results.

To our knowledge, this work is pioneer in the assessment of TOC of propolis. Studies regarding TPC and TFC of Portuguese propolis, especially concerning the latter parameter (Cruz *et al.*, 2008, 2016; Moreira *et al.*, 2008; Peixoto *et al.*, 2021, 2022) are relatively scarce too. Generally, our results are in accordance with the literature. A Portuguese propolis sample from Côa (Beira Alta) in 2010 showed a value of TFC (30.2 mg QE/ g of propolis) similar to the one obtained in the present study for G-r17.EE (Cruz *et al.*, 2016). Different propolis samples from Pereiro (Peixoto *et al.*, 2022) also showed similar values of both TFC and TPC when compared to the samples tested in this work (**Table 3.2**). Propolis from Bornes, Côa and Fundão presented significant differences between their TPC values (329, 160.4 and 151 mg GAE/ g of propolis, respectively), with Côa (Cruz *et al.*, 2016) and Fundão (Moreira *et al.*, 2008) propolis exhibiting TPC values closer to the ones found for Gerês samples, particularly the ones with Roca propolis in their composition. When compared with samples from the same apiary, TPC and TFC values obtained in the present work are in between the range of those usually observed in Gerês propolis samples. For instance, TPC values ranging from 143.0±1.9 (for G11.EE) and 212.2±9.2 (G12.EE) mg GAE/ g of propolis and TFC values ranging from 31.0±1.4 (for G14.EE) and 51.7±0.9 (for G15.EE) mg QE/ g of propolis were obtained for Gerês propolis samples from previous years (G11.EE-G16.EE; Peixoto *et al.*, 2021). All these results demonstrate that TPC and TFC parameters are typically little affected by geographic origin.

Antioxidant capacity of different propolis samples from Portugal has been demonstrated using the DPPH free-radical-scavenging (Cruz *et al.*, 2008; Miguel *et al.*, 2010; Falcão *et al.*, 2013; Gonçalves, 2017; Freitas *et al.*, 2019). Two propolis samples, Fundão (centre of Portugal; Moreira *et al.*, 2008) and Algarve (south of Portugal; Miguel *et al.*, 2010), presented a significant lower capability in reducing the DPPH radical ($EC_{50}$ of 52 and 40 μg $mL^{-1}$, respectively) when compared to extracts tested in the present work (**Table 3.3**). However, different Portuguese propolis samples – from north (Bornes), centre (Leiria) and south (Algarve) - exhibited higher DPPH radical scavenging activity with $EC_{50}$ values ranging from 6 to 31 μg $mL^{-1}$ (Cruz *et al.*, 2008; Miguel *et al.*, 2010; Falcão *et al.*, 2013), which are in accordance with the values herein obtained. These $EC_{50}$ values are also in line with those obtained in Gerês propolis extracts from previous years (G11.EE-G16.EE) made with a mixture of all places of the apiary (Bugalho, Felgueiras and Toutelo) - values ranging from 5.5 to 25.2 μg $mL^{-1}$ for G16.EE and G13.EE, respectively (Freitas *et al.*, 2019; Peixoto *et al.*, 2021). Additionally, and like what was found for the TOC, TPC and TFC contents, the region does not seem to influence the antioxidant capacity of Portuguese propolis, as different samples from the same region can exhibit distinct DPPH free-radical-scavenging capacity (Falcão *et al.*, 2013).

Antimicrobial activity of propolis has been studied for samples from different Portuguese regions showing considerable activity against several bacterial and fungal strains (Dias *et al.*, 2012; Silva *et al.*, 2012; Gonçalves, 2017; Oliveira *et al.*, 2017; Freitas *et al.*, 2019; Gomes *et al.*, 2021). Propolis samples from Trás-os-Montes Region display similar antimicrobial activity against the MRSA despite their different pollen types, suggesting that this activity is independent from propolis botanical origin (Dias *et al.*, 2012). Despite the lower activity of some extracts (G-r18.EE, G-rt18.EE, G-r19.EE and G-tf19.EE) against bacteria of the genus *Bacillus*, in general, extracts from propolis collected in 2017 (G-r17.EE, G-rt17.EE and G-tf17.EE) and G-tf18.EE and G-rt19.EE exhibited similar activity as the G.EEs of previous years, which were prepared from a mixture of Bugalho, Felgueiras and Toutelo (Gonçalves, 2017; Freitas *et al.*, 2019). However, only G18.EE exhibited exactly the same activity spectrum of those extracts of propolis from Gerês, pointing to the relevance of Bugalho place, in combination with Toutelo and Felgueiras places, to reach higher bioactive potential. So, this work supports the importance of mixing different propolis samples, even from the same apiary, to normalize propolis chemical and biological profiles (Peixoto *et al.*, 2021), which is of upmost importance considering propolis low yield of production and the need of standardization. Still, propolis extracts displayed similar antifungal activity as propolis from the same apiary from previous years (G11.EE-G16.EE), presenting MICs above 2000 μg $mL^{-1}$ (Gonçalves, 2017; Freitas *et al.*, 2019).

## 3.5. Conclusions

Propolis has been subject of intensive studies owing to the long and widely recognized remarkable properties (Ghisalberti, 1979; Burdock, 1998; Silva-Carvalho *et al.*, 2015; Cornara *et al.*, 2017; Anjum *et al.*, 2019). The first study with Portuguese propolis dates only from 2008 (Cruz *et al.*, 2008) but its great potential has been demonstrated over and over. However, Portuguese propolis is still undervalued and often discarded, yet far from getting the due attention from beekeepers and the international scientific community despite its remarkable properties. Once again, propolis from Gerês showed antioxidant and antimicrobial potential regardless of the apiary sources and harvesting years. Some differences were found in the bioactivities of the tested extracts composed by different propolis sources, when compared to the extracts of propolis samples harvested from previous years (G11.EE-G16.EE), made from a mixture of propolis originating from Bugalho, Felgueiras and Toutelo. These results show the significant impact that the relocation of the beehives, as a result of the environmental disaster, can have in the beehive products composition and, consequently, in their bioactivities. Nevertheless, using different sources of propolis allowed to obtain values in the range of those

previously obtained, which highlights the contribute of mixing different propolis samples to the dilution of the differences between samples, helping to overcome propolis low yield of production. In addition, this work highlights the importance of the constancy of a combination of aspects such the preservation of the plant species around the hives, to provide bee products with more constant and reliable chemical and biological profiles over time, making possible to stablish a chemical and biological fingerprint. So, this thorough characterization of propolis samples from a single apiary which operates in an organic mode opens doors to a more global understanding of the chemical and biological properties of hive products from a single producer. Thus, this work is a contribution for future propolis production with more constancy of properties.

## 3.6. Supplementary material

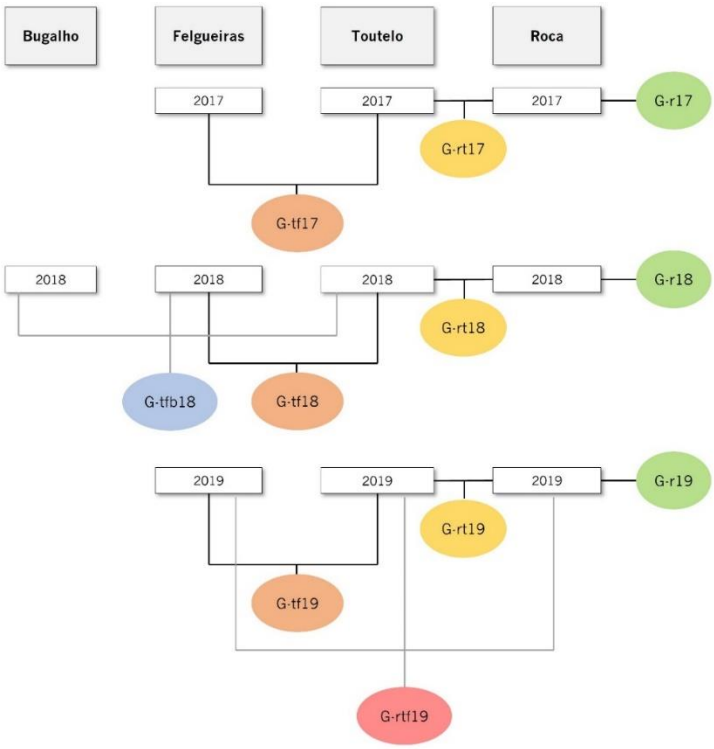


**Figure S3.1.** Propolis was collected from different places of Gerês apiary – Bugalho (G-b), Felgueiras (G-f), Toutelo (G-t), and Roca (G-r) – over three consecutive years (2017-2019). Propolis samples (ovals) resulted from different combinations (connected by lines) of propolis harvested each year: Roca (G-r; green ovals), Roca with Toutelo (G-rt; yellow ovals), Toutelo with Felgueiras (G-tf; orange ovals), Toutelo with Felgueiras and Bugalho (G-tfb; blue oval) and Roca with Toutelo and Felgueiras (G-rtf; red oval). Ethanol extraction of these samples resulted in the following extracts: G-r17.EE, G-rt17.EE and G-tf17.EE (of 2017); G-r18.EE, G-rt18.EE, G-tf18.EE and G-tfb18.EE (same as G18.EE; of 2018); and G-r19.EE, G-rt19.EE, G-tf19.EE and G-rtf19.EE (same as G19.EE; of 2019).

**Table S3.1.** Pollen composition (%) of Gerês honey samples H17 and H18 collected in 2017 and 2018, respectively.

| Species | Pollen composition (%) | |
|---|---|---|
| | H17 | H18 |
| *Erica* spp. | 55 | 44 |
| *Frangula alnus* | 0 | 18 |
| *Castanea sativa* | 20 | 13 |
| Others* | 25 | 25 |

*Sum of pollens with predominance less than 15 %.

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## 3.9. References

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# Chapter 4

**Propolis efficacy: the quest for eco-friendly solvents**

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## Abstract

Propolis, a natural product made by bees with resins and balsams, is known for its complex chemical composition and remarkable bioactivities. In this study, propolis extraction was studied seeking extracts with strong bioactivities using less orthodox solvents, with some derived from apiary products. For that, a propolis sample collected from Gerês apiary in 2018 (G18) was extracted by maceration with six different solvents: absolute ethanol, ethanol/ water (7:3), honey brandy, mead, propylene glycol and water. The solvent influence on the chemical composition and antioxidant and antimicrobial activities of the extracts was investigated. Antioxidant potential was assessed by the DPPH free-radical-scavenging assay and the antimicrobial activity by the agar dilution method. Chemical composition of the extracts was determined *in vitro* by three colorimetric assays: total *ortho*-diphenols, phenolics and flavonoids contents, and by the LC-MS technique. To our knowledge, this is the first time that solvents such as honey brandy and mead have been studied. Honey brandy showed considerable potential to extract propolis active compounds able to inhibit the growth of bacteria such as the methicillin sensitive *Staphylococcus aureus* and *Propionibacterium acnes* (MIC values of 100 and 200 μg $mL^{-1}$, respectively), and the fungi *Candida albicans* and *Saccharomyces cerevisiae* (MIC = 500 μg $mL^{-1}$, for both). Mead extracts displayed high antioxidant capacity ($EC_{50}$ = 1.63±0.27 μg $mL^{-1}$) and great activity against resistant bacteria such as the methicillin resistant *Staphylococcus aureus* and *Escherichia coli* (MIC = 750 μg $mL^{-1}$, for both). The production of such solvents made from beehive products further promotes a diversification of apiary products and the exploration of new applications using eco-friendly solutions.

**Keywords:** Propolis, propolis extraction, propolis extraction solvents, phenolic compounds, antibacterial activity, antioxidant potential.

## 4.1. Introduction

Propolis is a natural product composed by resinous and balsamic material collected by honeybees from different parts of plants and mixed with substances resulting from bees´ metabolism (Fokt *et al.*, 2010). There is a long history of propolis use, which continues today in homemade remedies and personal products, as well as in over-the-counter products, mostly due to its vast list of preparations and uses. The demand for this beehive product is still increasing along with the growing preference for natural products by the consumers. Propolis is commercialized in different parts of the

world, being recognized as an important source of compounds with properties for several applications (Moreira *et al.*, 2008; Pasupuleti *et al.*, 2017).

Most of the marketed propolis-based products require an extraction step, which allows the removal of inert material and the solubilization and preservation of the bioactive portion (Santos-Buelga and González-Paramás, 2017) enabling its introduction in pharmaceutical, food and cosmetic products (Burdock *et al.*, 1998). In addition to the extraction method (Pobiega *et al.*, 2019), the solvent used to extract the bioactive compounds is a determinant factor that influences the chemical composition of the obtained extracts (Sheng *et al.*, 2007). Among the most used solvents - water, methanol, ethanol, chloroform, dichloromethane, ether and acetone - ethanol is considered the solvent of choice for obtaining propolis extracts with a high content of bioactive compounds (Asgharpour *et al.*, 2020). However, ethanol has its drawbacks, such as a strong taste and limited applicability in certain industries, for instance to treat ophthalmological diseases or in pediatric cases (Kubiliene *et al.*, 2015). Different solvents extract distinct compounds (Watanabe *et al.*, 2011), giving each extract particular properties and potentially specific applications. This work aims to highlight the importance of solvent selection, searching for new and more ecological solvents (e.g., honey brandy and mead, made from honey) that could promote a more sustainable beekeeping activity. To this end, a propolis raw sample collected in 2018 from Gerês (G18) was extracted with six different solvents: absolute ethanol, ethanol/ water (7:3), honey brandy, mead, propylene glycol and water. Antioxidant potential and antimicrobial activity of the six extracts were evaluated and the chemical composition was determined. Principal component analysis (PCA) was applied to evaluate possible associations between the chemical composition of propolis and bioactivities, with the aim of finding the main putative compounds responsible for such relevant activities.

## 4.2. Materials and Methods

### 4.2.1. Propolis sample and extracts preparation

Propolis used in this work was harvested in 2018 from an apiary located in Gerês, in the north of Portugal and near the Cávado River (41°45'41.62'' N; 7°58'03.34'' W), a protected area of the Peneda-Gerês National Park (Freitas *et al.*, 2019). Beekeeping is performed under a certified biological mode and propolis is produced and harvested from grids by a standardized method, being of high quality and value.

The sample, named G18, was carefully cleaned of debris and finely fragmented before being extracted by maceration as previously described (Freitas *et al.*, 2019) but using distinct solvents –

absolute ethanol, ethanol/ water (7:3), honey brandy, mead, propylene glycol and water – to achieve different extracts – ethanol extract (EE), ethanol/ water (7:3) extract ($EE_{70}$), honey brandy extract (HBE), mead extract (ME), propylene glycol extract (PGE) and water extract (WE). Home-made honey brandy and mead were obtained using honey from the same region. A volume of 60 mL of each solvent was added to approximately 9 g of propolis and the mixtures were placed under orbital agitation (100 rpm), at 24 °C, in the dark, for 24 h. Mixtures were then filtered under vacuum and the filtrates were reserved at 4 °C. The solid residues were extracted again with 40 mL of the respective previously used solvent. The resulting filtrates were pooled, and the following extracts were obtained: G18.EE, G18.$EE_{70}$, G18.HBE, G18.ME, G18.PGE and G18.WE. At the end of the process, three 500 µL-aliquots of each extract were dried under nitrogen flow for yield analysis.

### 4.2.2. *In vitro* characterization of the chemical composition of extracts of propolis from Gerês harvested in 2018

The six propolis extracts were characterized regarding their phenolic composition, using spectrometric and chromatographic methodologies.

#### 4.2.2.1. Total *ortho*-diphenols content (TOC)

The content in *ortho*-diphenol compounds was determined using an adaptation of a colorimetric assay (Mateos *et al.*, 2001). In brief, an identical volume of different concentrations (from 25 to 300 µg $mL^{-1}$) of each of the six G18 extracts were mixed with 5 % (w/v) sodium molybdate, in ethanol/ water 1:1 (v/v), (following a 4:1 proportion in the reaction mixture), followed by 15 min incubation at room temperature, in the dark. Control and blank were prepared with similar mixtures but with each solvent replacing the extract or sodium molybdate, respectively. Absorbance of the reaction was measured at 370 nm, and results were obtained from linear regression using gallic acid as the standard (calibration curve with concentrations ranging from 40 to 180 µg $mL^{-1}$). TOC is expressed in milligrams of gallic acid equivalents (GAE) per gram of propolis extract (mg GAE/ g extract).

#### 4.2.2.2. Total phenolics content (TPC)

The content in phenolic compounds was determined using an adaptation of the Folin-Ciocalteu colorimetric assay (Kumazawa *et al.*, 2004). Briefly, different concentrations (from 1 to 300 µg $mL^{-1}$) of each of the six G18 extracts were mixed with 10 % (v/v) Folin-Ciocalteu reagent and 7.5 % (w/v) $Na_2CO_3$ (following a 5:5:4 proportion in the reaction mixture). The control and the blank were prepared with

similar mixtures but with each solvent replacing the extract or reagents, respectively. Absorbance was measured at 760 nm after 1 h incubation at room temperature, in the dark and results were obtained from linear regression using gallic acid as the standard (calibration curve with concentrations ranging from 1 to 50 µg $mL^{-1}$). TPC is expressed in milligrams of gallic acid equivalents (GAE) per gram of propolis extract (mg GAE/ g extract).

#### 4.2.2.3. Total flavonoids content (TFC)

The content in flavonoid compounds was determined using an adaptation of the colorimetric assay previously described (Kumazawa *et al.*, 2004). In brief, different concentrations (from 200 to 2200 µg $mL^{-1}$) of each of the six G18 extracts were mixed with 2 % (w/v) $AlCl_3$ (following a 1:1 proportion in the reaction mixture). Control and blank were prepared with similar mixtures but with each solvent replacing the extract or $AlCl_3$, respectively. The absorbance of the reaction was measured at 420 nm after 1 h incubation at room temperature, in the dark, and results were obtained from linear regression using quercetin as the standard (calibration curve with concentrations ranging from 5 to 200 µg $mL^{-1}$). TFC is expressed in milligrams of quercetin equivalents (QE) per gram of propolis extract (mg QE/ g extract).

### 4.2.3. Analysis of G18 extracts phenolic compounds by UHPLC-DAD-ESI/$MS^n$

The phenolic profile of the extracts was elucidated by UHPLC-DAD-ESI/$MS^n$ analysis following the method previously used (Freitas *et al.*, 2019). Analysis was performed on an Ultimate 3000 (Dionex Co., USA) apparatus equipped with an ultimate 3000 Diode Array Detector (Dionex Co., USA) coupled to a mass spectrometer LTQ XL Linear Ion Trap 2D. The chromatographic system comprised a quaternary pump, an autosampler, a photodiode-array detector and an automatic thermostatic column compartment. The mass spectrometer was a Thermo LTQ XL (Thermo Scientific, USA) ion trap MS equipped with an ESI source. Compounds were identified by comparison of the ESI-MS/MS with the data from MS/MS published in the literature (Mena *et al.*, 2012; Sentandreu *et al.*, 2013; Aguilar-Zárate *et al.*, 2017; Dos Santos *et al.*, 2018; Li *et al.*, 2018; Freitas *et al.*, 2019; Quintans-Júnior *et al.*, 2020).

### 4.2.4. *In vitro* evaluation of the antioxidant potential of extracts of propolis from Gerês harvested in 2018

The DPPH· (2,2-diphenyl-1-picryl-hydrazyl) method was used for the *in vitro* evaluation of the antioxidant potential of the propolis extracts as previously described (Freitas *et al.*, 2021). In brief, different concentrations (from 1 to 50 μg $mL^{-1}$) of each of the six G18 extracts were mixed with 0.004 % (w/v) DPPH· in a proportion of 1:2. The control and the blank were prepared with similar mixtures but with each solvent replacing the extract or DPPH·, respectively. Absorbance was measured at 517 nm after 20 min incubation at room temperature in the dark, and results are expressed in $EC_{50}$ values (μg $mL^{-1}$; concentration that reduce the free radical in 50 %) from linear regression of the percentage decrease in absorbance with respect to control values.

### 4.2.5. Antimicrobial properties of G18 extracts - determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of the six G18 extracts were determined using the agar dilution method (Hanson *et al.*, 1978) and the following panel of indicator strains: the Gram-negative bacterium *Escherichia coli* CECT 423, six Gram-positive bacteria - *Bacillus subtilis* 48886, *Bacillus cereus* ATCC 7064, *Bacillus megaterium* 932, (Methicillin Sensitive) *Staphylococcus aureus* ATCC 6538 (MSSA), (Methicillin Resistant) *Staphylococcus aureus* M746665 (MRSA) and *Propionibacterium acnes* H60803 – as well as two yeast strains - *Saccharomyces cerevisiae* BY4741 from Euroscarf (http://euroscarf.de/index.php?name=Description) and *Candida albicans* 53B were obtained from the microbial collection of the Department of Biology of the University of Minho.

Bacteria and yeast were cultured, respectively, in LB [Luria-Bertani - 0.5 % (w/v) yeast extract, 1 % (w/v) tryptone, 1 % (w/v) NaCl]; and YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose] broths or in solid media [LBA and YPDA - by adding 2 % (w/v) agar to the previous recipes of LB and YPD, respectively]. Growth was performed at 200 rpm - at 37 °C for bacteria and 30 °C for yeast - and monitored by optical density at 600 nm ($OD_{600}$). To prepare the cells for the experiments, overnight microbial cultures were diluted with the appropriated fresh medium to an $OD_{600}$ = 0.1 and incubated under the same conditions until $OD_{600}$ reached 0.4 - 0.6 (mid-exponential phase).

Drops of 5 μL of exponentially growing microbial cultures were transferred onto LBA and YPDA plates (for bacteria and yeast, respectively), supplemented with different concentrations of each of the six G18 extracts (0, 10, 50, 100, 200, 500, 750, 1000, 1250, 1500, 1750 or 2000 μg $mL^{-1}$). Plates containing identical volumes of each solvent were used as controls. Plates were incubated at 37 °C for

24 h for bacteria and at 30 °C for 48 h in the case of yeast. MIC values were determined upon observation of the presence/ absence of growth.

### 4.2.6. Principal component analysis

Principal Component Analysis (PCA) was performed for multivariate data analysis using R Studio version 1.4.1106 (Copyright 2009-2021 R Studio, PBC), R Version 4.0.5 (Copyright © 2021 The R Foundation for Statistical Computing). The data matrix is composed by 6 samples (propolis extracts) and 64 variables, including the compounds detected (51 compounds) by UHPLC-DAD-ESI/$MS^n$, the total phenolics (TPC), flavonoids (TFC) and *ortho*-diphenols contents (TOC), the antioxidant potential (DPPH), as well as the antimicrobial activity (MIC values). The correlation between variables and the relationship between samples and biochemical parameters were determined using scores, loadings and biplot graphs.

### 4.2.7. Statistical analysis

Antimicrobial and antioxidant experiments were performed in triplicate, repeated at least three times independently, and results were expressed as mean ± standard deviation (SD). A one-way ANOVA followed by Tukey's test for multiple comparisons were used to assess treatment effect. Differences considered statistically significant ($p < 0.05$) were noted with different letters.

## 4.3. Results and Discussion

### 4.3.1. Extraction yield

Most studies on propolis have used ethanol to extract the bioactive compounds (Santos-Buelga and González-Paramás, 2017; Šuran *et al.*, 2021) and data on the chemical composition and bioactivities of non-ethanol propolis extracts are much more limited. In the present study, the chemical composition and antioxidant and antimicrobial activities of propolis extracts prepared with six different solvents - absolute ethanol, ethanol/ water (7:3), honey brandy, mead, propylene glycol and water - were investigated. To our knowledge, this is the first time propolis is extracted with mead and honey brandy, two sweet-flavored fermented alcoholic drinks made from honey, water and yeast, with 40 % and 15 % of alcohol by volume (ABV), respectively. As presented in **Table 4.1**, the highest yields were obtained for absolute ethanol, ethanol/ water (7:3) and propylene glycol extracts (> 60 %). Extraction with honey brandy result in a significantly lower yield (26.6 %). However, the lowest yields were obtained for extraction with mead and water, the most polar solvent used (6.5 and 6.3 %, respectively).

**Table 4.1.** Yields (%) resulting from the extraction of G18 with different solvents - absolute ethanol (G18.EE), ethanol/ water (7:3) (G18.EE$_{70}$), honey brandy (G18.HBE), mead (G18.ME), propylene glycol (G18.PGE) and water (G18.WE) - expressed as a mean and respective standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by Tukey´s test for multiple comparisons. Mean values with the same letters (a, b or c) are not statistically different.

| Propolis extracts | G18.EE | G18.EE$_{70}$ | G18.HBE | G18.ME | G18.PGE | G18.WE |
|---|---|---|---|---|---|---|
| Yield (%) | 61.6±1.2$^{a}$ | 68.3±0.7$^{a}$ | 26.6±2.8$^{b}$ | 6.5±1.0$^{c}$ | 64.0±5.2$^{a}$ | 6.3±0.7$^{c}$ |

Although the extraction method was distinct from that used in the present work, ethanol was already described as the solvent able to obtain higher yield values, followed by propylene glycol and water (Pujirahayu *et al.*, 2014). However, a yield reported for water extraction was more than double the value obtained in this work, and for ethanol and propylene glycol extraction, it was around four-times lower (Pujirahayu *et al.*, 2014). Similar yield values for propolis water and ethanol extracts - 1.81 and 51.03 %, respectively – were found (Sun *et al.*, 2015). The same study also showed that the higher the alcohol content in the solvent, the higher the yield (Sun *et al.*, 2015).

Yield comparisons between different works is always uncertain since extraction procedures are usually not performed under the exact same conditions. The extraction yield mainly varies with the technique and solvent used, but also depends on a combination of several other factors such as the ratio between the sample and the solvent, the fragmentation state of the sample and the agitation, temperature and time of extraction (Cunha *et al.*, 2004; Mokhtar *et al.*, 2019; Oroian *et al.*, 2020; Stanciauskaite *et al.*, 2021; Aboulghazi *et al.*, 2022). Although there is an increase in yield with increasing extraction time, this increase does not imply a significant increase in the phenolic content (Cunha *et al.*, 2004). Propolis possess compounds with very different affinities to distinct solvents, for instance, compounds with more affinity to ethanol, such as the less polar active compounds, could not be easily extracted with polar solvents such as water, and *vice versa*. Ethanol is, however, a solvent with great affinity for a variety of compounds of different polarity, being consequently able to achieve higher yields (Santos-Buelga and González-Paramás, 2017; Mokhtar *et al.*, 2019).

### 4.3.2. Antimicrobial activity

Antibacterial properties of the six G18 extracts - G18.EE, G18.EE$_{70}$, G18.HBE, G18.ME, G18.PGE or G18.WE - were evaluated to assess the influence of the solvent on this activity (**Table 4.2**). Gram-positive bacteria, such as the bacteria of the genus *Bacillus*, were the most susceptible strains to all the extracts, which is a very common result reported by several authors, including our research group (Freitas *et al.*, 2019; Bankova *et al.*, 2000; Uzel *et al.*, 2005). G18.EE, G18.EE$_{70}$ and G18.PGE were the

most active extracts, exhibiting the same MIC value against all the tested strains, followed by G18.HBE, which was slightly more active against the Gram-positive methicillin-sensitive *Staphylococcus aureus* (MSSA; MIC = 100 μg $mL^{-1}$) and *Propionibacterium acnes* (MIC = 200 μg $mL^{-1}$). G18.ME was, in general, one of the least active extracts, but the only one capable to inhibit the growth of the methicillin-resistant *Staphylococcus aureus* (MRSA; MIC = 750 μg $mL^{-1}$) and the Gram-negative bacterium tested, *Escherichia coli* (MIC = 750 μg $mL^{-1}$), proving to be particularly interesting to be used against these presumptive more resistant bacteria. The least active extract was the G18.WE, exhibiting the higher MIC values for all the tested strains (MIC ≥ 1000 μg $mL^{-1}$). The ethanol extracts, G18.EE, G18.$EE_{70}$, as well as G18.HBE and G18.PGE, showed similar antibacterial activity when compared to ethanol extracts of propolis from Gerês from previous years (Freitas *et al.*, 2019).

**Table 4.2.** MIC values (μg $mL^{-1}$) obtained against the panel of susceptibility indicator bacteria. Mid-exponential phase microbial cultures were transferred to plates supplemented with increasing concentrations of each of the six extracts - G18.EE, G18.$EE_{70}$, G18.HBE, G18.ME, G18.PGE or G18.WE. Plates were observed for the presence/ absence of growth after 24 h incubation at 37 °C, and the lowest concentrations for which no growth was detected were registered as the MIC values.

| Bacteria | G18.EE | G18.$EE_{70}$ | G18.HBE | G18.ME | G18.PGE | G18.WE |
|---|---|---|---|---|---|---|
| | MIC (μg $mL^{-1}$) | | | | | |
| *Bacillus megaterium* | 50 | 50 | 50 | 500 | 50 | 2000 |
| *Bacillus subtilis* | 50 | 50 | 50 | 500 | 50 | 2000 |
| *Bacillus cereus* | 50 | 50 | 50 | 500 | 50 | 1000 |
| MSSA | 200 | 200 | 100 | 750 | 200 | 2000 |
| MRSA | >2000 | >2000 | >2000 | 750 | >2000 | >2000 |
| *Propionibacterium acnes* | 500 | 500 | 200 | 500 | 500 | 2000 |
| *Escherichia coli* | >2000 | >2000 | >2000 | 750 | >2000 | >2000 |

MSSA – Methicillin Sensitive *Staphylococcus aureus*; MRSA – Methicillin Resistant *Staphylococcus aureus*; EE – ethanol extract; $EE_{70}$ – ethanol/water (7:3) extract; HBE – honey brandy extract; ME – mead extract; PGE – propylene glycol extract; WE – water extract.

The antifungal effect of the six G18 extracts - G18.EE, G18.$EE_{70}$, G18.HBE, G18.ME, G18.PGE or G18.WE - was also evaluated to assess the influence of the solvent (**Table 4.3**). All extracts showed similar activity against both strains, with G18.HBE standing out from the others, being the most active against both *Saccharomyces cerevisiae* and *Candida albicans* (MIC = 500 μg $mL^{-1}$). This behavior is unusual for ethanol propolis extracts from Gerês (Peixoto *et al.*, 2021), as the activity against these

yeasts is normally weak, similar to the activity observed for the other extracts in this work. G18.WE was the least active extract against both yeasts (MIC > 2000 μg $mL^{-1}$).

**Table 4.3.** MIC values (μg $mL^{-1}$) obtained against the two-susceptibility indicator yeasts. Mid-exponential phase microbial cultures were transferred to plates supplemented with increasing concentrations of each of the six extracts - G18.EE, G18.$EE_{70}$, G18.HBE, G18.ME, G18.PGE or G18.WE. Plates were observed for the presence/ absence of growth after 48 h incubation at 30 °C and the lowest concentrations for which no growth was detected were registered as the MIC values.

| Yeast | G18.EE | G18.$EE_{70}$ | G18.HBE | G18.ME | G18.PGE | G18.WE |
|---|---|---|---|---|---|---|
| | MIC (μg $mL^{-1}$) | | | | | |
| *Saccharomyces cerevisiae* | 1500 | 1500 | 500 | 1500 | 2000 | >2000 |
| *Candida albicans* | 2000 | 1500 | 500 | 1500 | 1500 | >2000 |

EE – ethanol; $EE_{70}$ – ethanol/ water (7:3) extract; HBE – honey brandy extract; ME – mead extract; PGE – propylene glycol extract; WE – water extract.

Our results are in accordance with findings previously reported (Kubiliene *et al.*, 2015), showing that the propolis $EE_{70}$ had higher activity against the Gram-positive bacteria over the Gram-negative. In addition, similar to our study, the extracts were particularly active against the spore-forming bacteria *Bacillus cereus* and no activity against all the tested strains was found for the propolis WE. Other studies also showed the inefficacy of water extracts of propolis as antimicrobial agents, showing weak or no activity at all against the tested strains, such as *Bacillus subtilis*, *B. cereus*, *S. aureus*, *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa* and *C. albicans* (Ramanauskienė *et al.*, 2013; Kubiliene *et al.*, 2015). On the other hand, ethanol and propylene glycol demonstrated potential as extraction solvents to obtain propolis extracts with great antimicrobial activity, especially against *S. aureus* and *B. subtilis* (Ramanauskienė *et al.*, 2013). Two different propolis extracts – ethanol and water – were tested, and MIC values in the same range as ours were obtained against *S. aureus* (250 and 1000 μg $mL^{-1}$, for propolis EE and WE, respectively) and *E. coli* (1000 and > 1000 μg $mL^{-1}$ for the propolis EE and WE, respectively) (Asgharpour *et al.*, 2020).

### 4.3.3. Antioxidant activity

DPPH free-radical-scavenging activity was determined for the six G18 extracts - G18.EE, G18.$EE_{70}$, G18.HBE, G18.ME, G18.PGE or G18.WE - and the respective $EC_{50}$ values were calculated (**Table 4.4**). G18.EE and G18.$EE_{70}$ showed similar and lower anti-radical activity (10.78±0.43 and 9.91±1.07 μg $mL^{-1}$, respectively) than G18.PGE (8.17±0.14 μg $mL^{-1}$). G18.HBE and G18.WE exhibited similar values of

$EC_{50}$ (4.90±0.10 and 4.54±0.23 μg $mL^{-1}$, respectively) and a high antioxidant potential, but G18.ME displayed the highest antioxidant potential with a particularly low $EC_{50}$ value of 1.63±0.27 μg $mL^{-1}$. Contrary to what happen for the antibacterial activity, where the G18.WE and G18.ME were the weaker extracts against all the tested bacterial strains, these extracts exhibited the higher capability to scavenge DPPH free radicals, suggesting that extracts with lower antibacterial activity usually have higher antioxidant potential.

**Table 4.4.** Antioxidant potential of G18.EE, G18.$EE_{70}$, G18.HBE, G18.ME, G18.PGE or G18.WE – measured by the *in vitro* DPPH free-radical-scavenging assay and expressed as a mean of $EC_{50}$ values (μg $mL^{-1}$) and respective standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by Tukey´s test for multiple comparisons. Mean values with the same letters (a, b, c or d) are not statistically different.

| Propolis extracts | G18.EE | G18.$EE_{70}$ | G18.HBE | G18.ME | G18.PGE | G18.WE |
|---|---|---|---|---|---|---|
| DPPH· scavenging activity $EC_{50}$ (μg $mL^{-1}$) | 10.78±0.43$^{a}$ | 9.91±1.07$^{a}$ | 4.90±0.10$^{c}$ | 1.63±0.27$^{d}$ | 8.17±0.14$^{b}$ | 4.54±0.23$^{c}$ |

EE – ethanol extract; $EE_{70}$ – ethanol/ water (7:3) extract; HBE – honey brandy extract; ME – mead extract; PGE – propylene glycol extract; WE – water extract.

Other authors evaluated the DPPH free-radical-scavenging activity of propolis ethanol and water extracts (Mavri *et al.*, 2012; Kubiliene *et al.*, 2015; Sun *et al.*, 2015; Woźniak *et al.*, 2019; Syed Salleh *et al.*, 2021; Ibrahim and Alqurashi, 2022), reporting their high antioxidant potential. However, the literature does not point to a predominance of EE or WE for higher antioxidant activity, with studies finding higher scavenging ability of WE when compared to the EE of the same propolis sample, similar to this work, whereas others have found EE with greater antioxidant capacity instead ($IC_{50}$ of 695 and 13,798 μg $mL^{-1}$, for the EE and WE respectively) (Kubiliene *et al.*, 2015; Sun *et al.*, 2015; Ibrahim and Alqurashi, 2022). However, the value obtained for the G18.EE fits into the scavenging activity exhibited by the EE from Gerês from previous years, supporting the constancy exhibited by these particular propolis samples (Freitas *et al.*, 2019).

### 4.3.4. Chemical characterization

#### 4.3.4.1. *In vitro* evaluation of total *ortho*-diphenols (TOC), phenolics (TPC) and flavonoids (TFC) contents

**Table 4.5** summarizes the total *ortho*-diphenols (TOC), phenolics (TPC) and flavonoids (TFC) contents of the G18 extracts. TOC ranged from 263.05±15.19 to 1067.79±37.24 mg GAE/ g extract. The lowest value was achieved for absolute ethanol and ethanol/ water (7:3) extracts, while the highest

TOC was achieved by the G18.WE and G18.ME. TPC values ranged from 207.49±8.55 to 1267.57±5.27 mg GAE/ g extract, with G18.PGE exhibiting the lower value, while the highest TPC value was displayed, again, by G18.WE and G18.ME. TFC values ranged from 7.40±0.14 mg QE/ g extract, for G18.WE, to 50.97±0.26 mg QE/ g extract, for G18.HBE. TOC correlated directly to the antioxidant potential as the extracts with higher TOC exhibited higher ability to scavenge DPPH free radicals. On the other hand, TFC seems to be linked, although not directly correlated, to the antibacterial activity, as extracts with higher TFC generally showed stronger antibacterial activity against the different bacterial strains tested.

**Table 4.5.** Total *ortho*-diphenols (TOC), phenolics (TPC) and flavonoids (TFC) contents of G18.EE, G18.EE$_{70}$, G18.HBE, G18.ME, G18.PGE and G18.WE. Results are presented as mean±standard deviation (SD) of mg of gallic acid equivalents per g of extract (mg GAE/ g extract) for TOC and TPC, and as mg of quercetin equivalents per g of extract (mg QE/ g extract) for TFC. Statistical analysis was performed by one-way ANOVA followed by Tukey's test for multiple comparisons. For each variable, mean values followed by the same letter (a, b, c, d, e, or f) are not statistically different.

| Propolis extracts | Total *Ortho*-diphenol Content | Total Phenolics Content | Total Flavonoid Content |
|---|---|---|---|
| | mg GAE/ g | mg GAE/ g | mg QE/ g |
| **G18.EE** | 263.05±15.19$^{a}$ | 224.60±10.86$^{a}$ | 44.74±1.26$^{a}$ |
| **G18.EE$_{70}$** | 367.71±18.86$^{b}$ | 255.30±3.26$^{a}$ | 48.56±0.64$^{b}$ |
| **G18.HBE** | 664.16±24.44$^{c}$ | 581.40±35.51$^{b}$ | 50.97±0.26$^{b}$ |
| **G18.ME** | 1067.79±37.24$^{d}$ | 1267.57±5.27$^{c}$ | 13.26±0.69$^{c}$ |
| **G18.PGE** | 492.30±6.52$^{f}$ | 207.49±8.55$^{a}$ | 34.53±2.43$^{e}$ |
| **G18.WE** | 977.71±51.59$^{e}$ | 1261.11±27.86$^{c}$ | 7.40±0.14$^{d}$ |

EE – ethanol extract; EE$_{70}$ – ethanol/ water (7:3) extract; HBE – honey brandy extract; ME – mead extract; PGE – propylene glycol extract; WE – water extract.

G18.EE showed TPC and TFC values similar to the ones reported for G.EEs from previous years (Peixoto *et al.*, 2021). Our results showed slight differences from what was previously described (Ramanauskienė *et al.*, 2013; Kubiliene *et* al., 2018; Mokhtar *et al.*, 2019; Dönmez *et al.*, 2020), indicating that ethanol extraction leads to higher TPC and TFC values when compared to water extraction. Although both G18.EE and G18.EE$_{70}$ showed higher TFC values than G18.WE, the latter showed a value of TPC around five-times higher than the ethanol extracts. The values of TPC and TFC found in the literature for EE are in the range of the values obtained in the present study. The same is not true regarding WE since different authors have reported distinct values for their extracts. For

instance, some authors have reported TPC values for propolis EE in the range of the values found in our study (152.03 and 231.92 mg GAE/ g extract, respectively) but lower values for the WE (6.68 and 61.92 mg GAE/ g extract, respectively; Sun *et al.*, 2015; Dönmez *et al.*, 2020). The same tendency has been described by other authors who reported TPC values of 321 and 210 mg GAE/ g extract for the propolis EE and WE, respectively (Ibrahim and Alqurashi, 2022), and TPC values of high range for both propolis EE (1207.9 μg $mL^{-1}$) and WE (20791.3 μg $mL^{-1}$) (Kubiliene *et al.*, 2018). Nevertheless, the TPC values found in the literature for WE are generally much lower than this last value and the values obtained in the present work. However, and similar to our work, similar TPC values for both ethanol and propylene glycol extracts have been described and, when compared to the water extract of the same propolis sample, both ethanol and glycol propolis extracts have shown higher TFC (Ramanauskienė *et al.*, 2013; Pujirahayu *et al.*, 2014; Dönmez *et al.*, 2020).

#### 4.3.4.2. UHPLC-DAD-ESI/$MS^n$ Analysis

After liquid chromatography-mass spectrometry (LC-MS) analysis of the different extracts of propolis from Gerês, 51 compounds were detected (**Figure S4.1** and **Table S4.1 Supplementary material**). Regardless the differences found in the total *ortho*-diphenols, phenolics and flavonoids contents, most of the different solvent extracts showed similar phenolic constituents, particularly the extracts with higher alcohol content – G18.EE, $G18.EE_{70}$ and G18.HBE – and G18.PGE. Non-ethanol solvents of glycolic nature, which is the case of propylene glycol and polyethylene glycol, have been shown to be more efficient in extracting the active portion from propolis when compared to ethanol (Kubiliene *et al.*, 2015). Among all compounds, only two, gallic acid and caffeic acid, were detected in all the extracts. Gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, 3,4-dimethyl-caffeic acid (DMCA), quercetin-3-methyl ether, apigenin, chrysin, caffeic acid isoprenyl ester (CAIE), acacetin and pinocembrin were the main compounds found, among many others also observed in the chemical profiles described for European propolis (Freitas *et al.*, 2019), typical for poplar propolis ethanol extracts (Šuran *et al.*, 2021).

Both G18.ME and G18.WE exhibited distinct chemical profiles. The G18.WE was mainly composed by tannins and coumaric acid derivatives, whereas the few phenolic compounds detected in G18.ME consisted of phenolic acids and hydroxycinnamic acids, namely gallic acid, caffeic acid, ellagic acid, *p*-coumaric acid, ferulic acid, 3,4-dimethyl-caffeic acid (DMCA) and *p*-coumaric acid methyl ester. As this extract is characterized by a potent antioxidant capacity ($EC_{50}$ = 1.63±0.27 μg $mL^{-1}$), it is tempting to associate the presence of these phenolic acids to this property, which is in line with what

has already been described in the literature (Šuran *et al.*, 2021). Still, the activity against the MRSA and *E. coli* can also be related to the presence of the same compounds. Thus, it would be interesting to test several combinations of the seven compounds to find the major putative compounds responsible for the activity against these two resistant bacteria.

### 4.3.5. Multivariate analysis

Principal Component Analysis (PCA) was carried out to find possible correlations between the presence of particular groups of compounds and the antioxidant as well as antimicrobial activities. The PC1 and PC2 represented 61.41 and 25.52 % of the total variance, respectively. The biplot graph for the first two PCs is presented in **Figure 4.1**. Considering the similarities of the samples and the variables related to the chemical composition of propolis extracts and their antioxidant and antimicrobial activities, the first ones could be divided into three groups (**Figure 4.1**). The first group is composed by the absolute ethanol (EE), ethanol/ water (7:3; $EE_{70}$) and the mead (ME) extracts; the second group by the honey brandy (HBE) and propylene glycol (PGE) extracts; and the third group by the water extract (WE) alone, as the most differentiated extract.

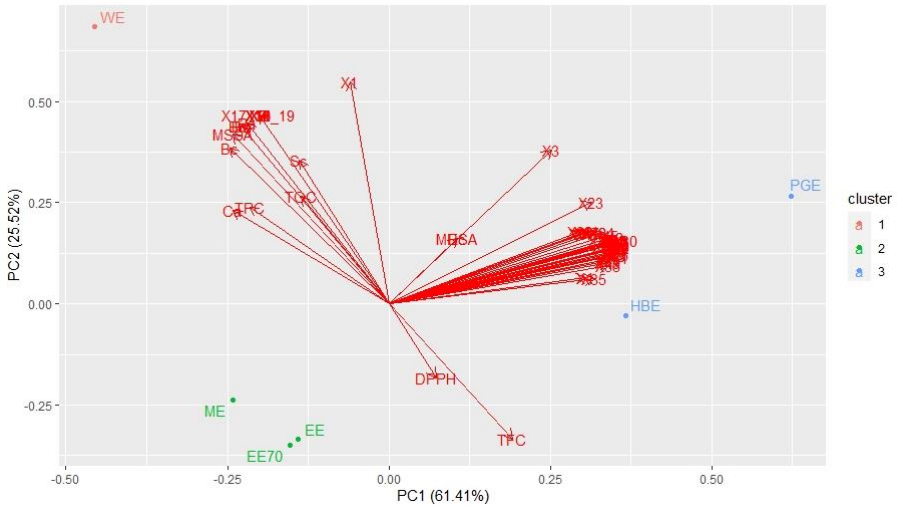


**Figure 4.1.** PC2 *vs.* PC1 biplot graph for the propolis samples and selected biochemical parameters. **EE**: ethanol extract; **$EE_{70}$**: ethanol/ water (7:3) extract; **HBE**: honey brandy extract; **PGE**: propylene glycol extract; **ME**: mead extract; **WE**: water extract; **DPPH**: DPPH free-radical-scavenging activity expressed as a mean of $EC_{50}$ values (μg $mL^{-1}$); **TFC**: total flavonoids content; **TPC**: total phenolics content; **TOC**: total *ortho*-diphenols content; Microorganisms – **Bs**: *Bacillus subtilis*; **Bc**: *Bacillus cereus*; **Bm**: *Bacillus megaterium*; **MSSA**: methicillin sensitive *Staphylococcus aureus*; **MRSA**: methicillin resistant *Staphylococcus aureus*; **Pa**: *Propionibacterium acnes;* **Ec**: *Escherichia coli*; **Sc**: *Saccharomyces cerevisiae*; **Ca**: *Candida albicans* – distributed according to the MIC values; **x1 – x51**: compounds detected by UHPLC-DAD-ESI/$MS^n$ analysis (see **Table S4.1 Supplementary material**).

As depicted in **Figure 4.2**, it is possible to confirm that TOC and TPC are both inversely proportional to DPPH, indicating a correlation between these first ones and antioxidant potential. To our knowledge, this is a new finding since no information was found in the literature concerning TOC analysis of propolis samples and considering that the antioxidant activity is usually positively correlated with the total flavonoids and phenolics contents (Syed Salleh *et al.*, 2021). The TFC is strongly correlated with the antibacterial activity, particularly related to the Gram-positive bacteria - *B. subtilis*, *B. cereus*, *B. megaterium*, MSSA and *P. acnes* - and the antifungal - *S. cerevisiae* and *C. albicans*. On the other hand, the angles between the other two variables, TPC and TOC, and MRSA and the gram-negative bacterium *E. coli* are approximately 90°, indicating correlation coefficients close to 0 (**Figure 4.1**). These results show that TPC and TOC are not correlated with MRSA and *E. coli*, and the response for this last one could not be predicted from propolis TPC and TOC.

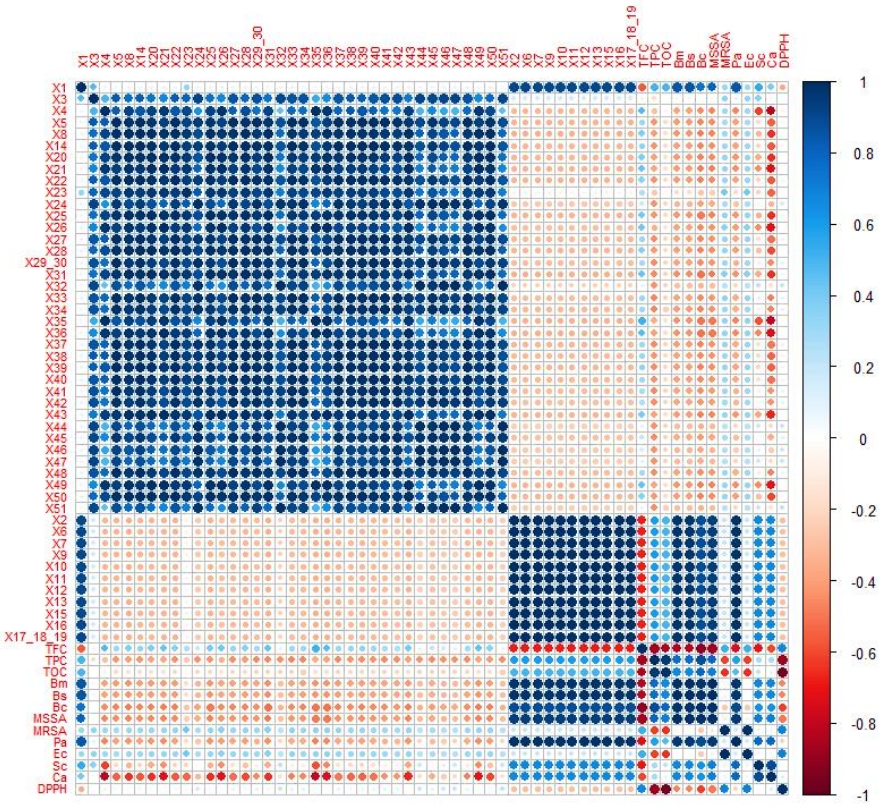


**Figure 4.2.** Correlation plot of the chemical composition and bioactivities of the propolis extracts. Dark blue (positive correlation) and dark red (negative correlation) shows the strength and the direction of the correlations between the variables. **DPPH**: DPPH free-radical-scavenging activity expressed as a mean of $EC_{50}$ values (μg $mL^{-1}$); **TFC**: total flavonoids content; **TPC**: total phenolics content; **TOC**: total *ortho*-diphenols content; Microorganisms – **Bs**: *Bacillus subtilis*; **Bc**: *Bacillus cereus*; **Bm**: *Bacillus megaterium*; **MSSA**: methicillin sensitive *Staphylococcus aureus*; **MRSA**: methicillin resistant *Staphylococcus aureus*; **Pa**: *Propionibacterium acnes;* **Ec**: *Escherichia coli*; **Sc**: *Saccharomyces cerevisiae*; **Ca**: *Candida albicans* – distributed according to the MIC values; **x1 – x51**: compounds detected by UHPLC-DAD-ESI/$MS^n$ analysis (see **Table S4.1 Supplementary material**).

Both antioxidant and antimicrobial activities are universally exhibited by propolis, and the same happens with some of the compounds that are part of this complex mixture, such as gallic acid, ellagic acid, ferulic acid, isorhamnetin, kaempferol, caffeic acid isoprenyl ester (CAIE) caffeic acid phenethyl ester (CAPE) and tannins (Santos *et al.*, 2002; Kumazawa *et al.*, 2004; Orsolić *et al.*, 2006; Chen *et al.*, 2009; Mihai *et al.*, 2011; Yang *et al.*, 2011; Boisard *et al.*, 2014; Daglia *et al.*, 2014; Patel *et al.*, 2014; Boisard *et al.*, 2015; Sun *et al.*, 2015; Kandakumar and Manju, 2017; Li *et al.*, 2017; de Moraes Alves *et al.*, 2018; Adamczak *et al.*, 2019; Tošović and Bren, 2020; Tong *et al.*, 2021). The presence of specific phenolic compounds, such as gallic acid (compound **x1**), present in all the extracts but in different quantities, and the HHDP-hexoside (**x2**), digalloyl hexoside (**x6**), gallotannin (**x7**), di(HHDP-galloylglucose)-pentose (**x9**), tannins (**x10-12, x15**), caffeic acid derivatives (**x13**) and coumaric acid derivatives (**x16-19**), only present in G18.WE, although not individually correlated, could, acting synergistically, be responsible for the strong correlation between the G18.WE and the antioxidant potential (DPPH; **Figures 4.1** and **4.2**). In contrast, the antimicrobial activity, more specifically against the fungi *S. cerevisiae* and *C. albicans*, and the bacteria of the genus *Bacillus* and the *S. aureus* and *P. acnes*, seems to be linked to the presence of other group of compounds, mostly flavonoids, such as the ellagic acid (**x4**), ferulic acid (**x8**), pinobanksin-5-methyl-ether (**x20**), quercetin-3-methyl ether (**x21**), kaempferol (**x25**), isorhamnetin (**x26**), acacetin (**x31**), kaempferide (**x35**), kaempferol-methoxy-methyl ether (**x36**), 3-hydroxy-5-methoxy flavanone (**x43**) and caffeic acid derivatives (**x49**).

## 4.4. Conclusions

In general, ethanol extracts of propolis from Gerês appear to be more suitable for antimicrobial applications, which correlates with their high content in flavonoids, while the mead extract seems to be a more promising choice concerning the antioxidant potential, which correlates with its higher content in phenolic acids. Solvents such as honey brandy and mead must be further explored. Beyond the strong antioxidant potential of the corresponding propolis extracts obtained with such solvents, they possess considerable infections-fighting potential, namely fungal, similar to the infections caused by *C. albicans*, and bacterial, particularly against some resistant bacteria such as MRSA and *E. coli*. As different solvents can extract not only distinct amounts of the same compound but also diverse groups of compounds, this study can contribute to a more conscious choice of the appropriate solvent in order to extract a propolis active portion according to the desired applicability. The increasing health-conscious awareness of consumers regarding the potential toxicity of the chemicals used in their daily life has led to the quest for more natural and sustainable alternatives. Solvents such honey brandy and mead,

made from natural sources such as bee products encompasses bioeconomy, are also an additional source of income for the beekeeping sector as alternative uses for surplus honey production and/ or reuse of products that may not have commercial value to be marketed, as these solvents can be marketed as an eco-friendly alternative to the current solvents in use.

## 4.5. Supplementary material

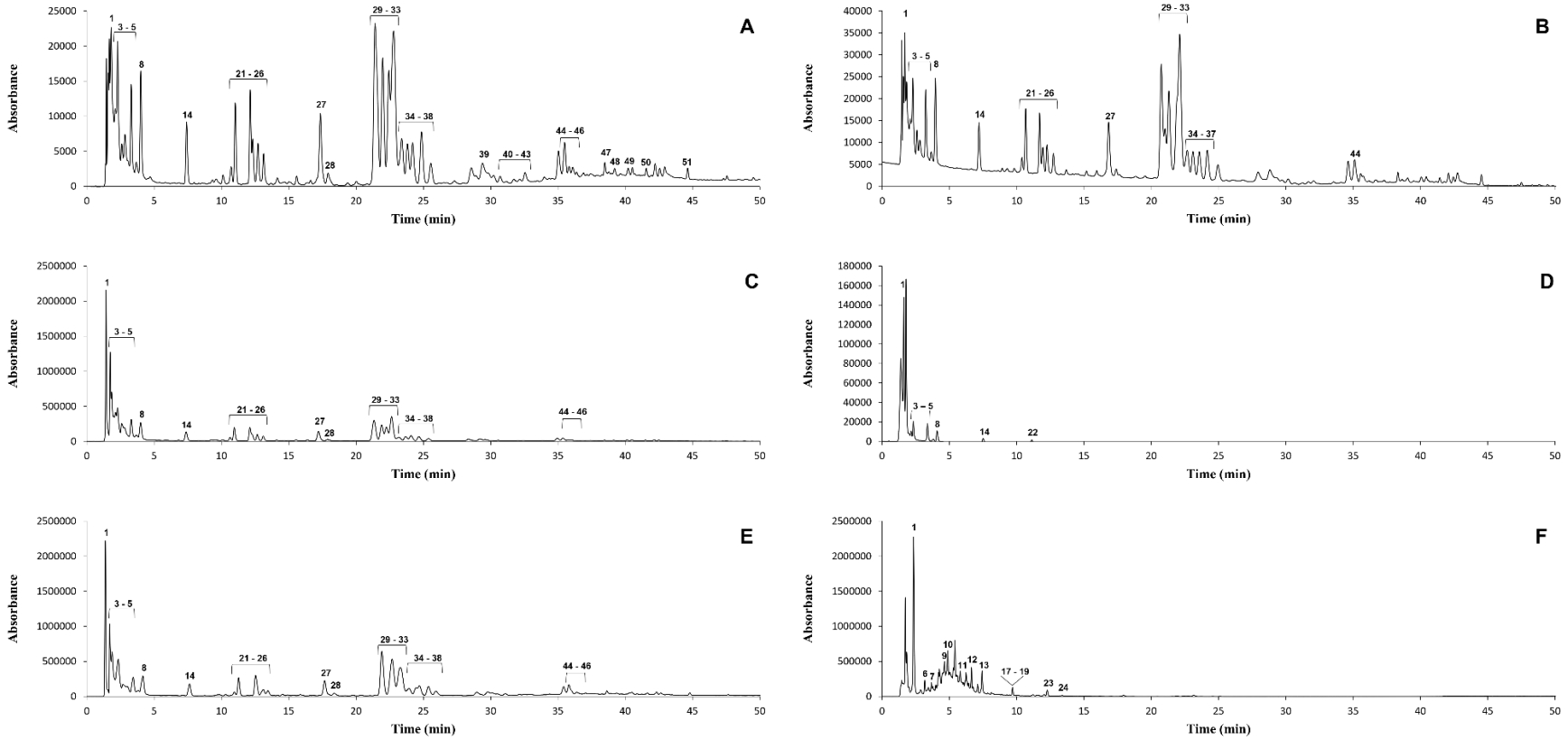


**Figure S4.1**. LC-MS chromatographic profiles of the six G18.Es - G18.EE (**A**), G18.EE$_{70}$ (**B**), G18.HBE (**C**), G18.ME (**D**), G18.PGE (**E**) and G18.WE (**F**) - resulting from the extraction of G18 with different solvents - ethanol extract (EE), ethanol/ water (7:3) extract (EE$_{70}$), honey brandy extract (HBE), mead extract (ME), propylene glycol extract (PGE) and water extract (WE), respectively.

**Table S4.1.** Chemical composition of G18.EE, G18.EE$_{70}$, G18.HBE, G18.PGE, G18.ME and G18.WE according to LC-MS analysis.

| Peaks | $t_R$ (min) | λmax (nm) | $[M - H]^-$ m/z | Main fragments | Compound | EE | EE$_{70}$ | HBE | PGE | ME | WE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.9 | 270 | 169 | 125 | gallic acid | + | + | + | + | + | + |
| 2 | 2.0 | 246, 274, 308 | 481 | 301, 275 | HHDP-hexoside | - | - | - | - | - | + |
| 3 | 2.7 | 292, 322 | 179 | 135, 163 | caffeic acid | + | + | + | + | + | + |
| 4 | 2.9 | 253, 368 | 301 | 257, 229 | ellagic acid | + | + | + | + | + | - |
| 5 | 3.1 | 309 | 163 | 119, 145, 108 | *p*-coumaric acid | + | + | + | + | + | - |
| 6 | 3.2 | 275 | 483 | 331, 313, 271, 169 | digalloyl hexoside | - | - | - | - | - | + |
| 7 | 3.6 | 271 | 803 | 759, 483 | gallotannin | - | - | - | - | - | + |
| 8 | 4.1 | 295, 323 | 193 | 177, 149, 133 | ferulic acid | + | + | + | + | + | - |
| 9 | 4.5 | 275 | 707 | 631, 633 | di(HHDP-galloylglucose)-pentose | - | - | - | - | - | + |
| 10 | 4.6 | 275 | 551 | 542, 451, 301 | tannin | - | - | - | - | - | + |
| 11 | 5.8 | 275 | 551 | 542, 451, 301 | tannin | - | - | - | - | - | + |
| 12 | 6.2 | 272 | 433 | 271, 313, 343, 301, 415 | tannin | - | - | - | - | - | + |
| 13 | 6.6 | 294, 324 | 469 | 451, 425, 393, 289 | caffeic acid derivative | - | - | - | - | - | + |
| 14 | 7.3 | 295, 322 | 207 | 163, 148, 133 | 3,4-dimethyl-caffeic acid (DMCA) | + | + | + | + | + | - |
| 15 | 7.3 | - | 507 | 331, 313 | tannin | - | - | - | - | - | + |
| 16 | 7.4 | - | 349 | 163, 145, 119 | coumaric acid derivative | - | - | - | - | - | + |
| 17 | 9.7 | 308 | 379 | 193, 175, 134 | coumaric acid derivative | - | - | - | - | - | + |
| 18 | 9.7 | 308 | 273 | 255, 193, 163 | coumaric acid derivative | - | - | - | - | - | + |
| 19 | 9.7 | 308 | 439 | 421, 395, 163 | coumaric acid derivative | - | - | - | - | - | + |
| 20 | 10.3 | 288 | 285 | 267, 239, 252 | pinobanksin-5-methyl-ether | + | - | + | + | - | - |

**EE** - ethanol extract; **EE$_{70}$** - ethanol/ water (7:3) extract; **HBE** - honey brandy extract; **PGE** - propylene glycol extract; **ME** - mead extract; **WE** - water extract; + detected; - not detected.

**Table S4.1.** Chemical composition of G18.EE, G18.EE$_{70}$, G18.HBE, G18.PGE, G18.ME and G18.WE according to LC-MS analysis (continued).

| Peaks | $t_R$ (min) | λmax (nm) | $[M - H]^-$ m/z | Main fragments | Compound | EE | EE$_{70}$ | HBE | PGE | ME | WE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **21** | 10.7 | 273, 353 | 315 | 300 | quercetin-3-methyl ether | + | + | + | + | - | - |
| **22** | 11.0 | 308 | 177 | - | *p*-coumaric acid methyl ester | + | + | + | + | + | - |
| **23** | 12.0 | 267, 336 | 269 | 225, 151 | apigenin | + | + | + | + | - | + |
| **24** | 12.2 | 291 | 271 | 253, 225 | pinobanksin | + | + | + | + | - | + |
| **25** | 12.5 | 265, 363 | 285 | 285, 257, 151 | kaempferol | + | + | + | + | - | - |
| **26** | 13.1 | 255, 368 | 315 | 300 | isorhamnetin | + | + | + | + | - | + |
| **27** | 17.1 | 311 | 313 | 298, 269, 257, 241, 179, 153 | unknown | + | + | + | + | - | + |
| **28** | 18.2 | 254, 368 | 329 | - | quercetin-dimethyl-ether | + | - | + | + | - | + |
| **29** | 20.9 | 268, 315 | 253 | 209, 181, 225, 151 | chrysin | + | + | + | + | - | + |
| **30** | 20.9 | 298, 325 | 247 | 179, 135 | caffeic acid isoprenyl ester | + | + | + | + | - | + |
| **31** | 21.9 | 268, 332 | 283 | 268 | acacetin | + | + | + | + | - | + |
| **32** | 22.4 | 299, 325 | 247 | 179, 135 | caffeic acid isoprenyl ester | + | + | + | + | - | + |
| **33** | 22.9 | 289 | 255 | 213, 211, 151 | pinocembrin | + | + | + | + | - | + |
| **34** | 23.7 | 266, 290, 356 | 269 | 223, 169, 249 | galangin | + | + | + | + | - | - |
| **35** | 23.9 | 267, 362 | 299 | 284, 165 | kaempferide | + | + | + | + | - | + |
| **36** | 24.2 | 269, 360 | 329 | 314, 297, 287, 269, 257 | kaempferol-methoxy-methyl ether | + | + | + | + | - | - |
| **37** | 24.9 | 293 | 313 | 253, 271 | pinobanksin-3-*O*-acetate | + | + | + | + | - | - |
| **38** | 25.6 | 298, 325 | 283 | 179, 135 | caffeic acid phenylethyl ester | + | - | + | + | - | - |
| **39** | 29.5 | 311 | 231 | 163, 119 | *p*-coumaric acid isoprenyl ester (isomer) | + | - | + | + | - | - |

**EE** - ethanol extract; **EE$_{70}$** - ethanol/ water (7:3) extract; **HBE** - honey brandy extract; **PGE** - propylene glycol extract; **ME** - mead extract; **WE** - water extract; + detected; - not detected.

**Table S4.1.** Chemical composition of G18.EE, G18.EE$_{70}$, G18.HBE, G18.PGE, G18.ME and G18.WE according to LC-MS analysis (continued).

| Peaks | $t_R$ (min) | λmax (nm) | $[M - H]^-$ m/z | Main fragments | Compound | EE | EE$_{70}$ | HBE | PGE | ME | WE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **40** | 30.8 | 295, 325 | 295 | 178, 134, 251, 211 | caffeic acid cinnamyl ester | + | - | + | + | - | - |
| **41** | 31.4 | 268, 305 | 297<br>403 | 179, 161, 151, 135<br>- | caffeic acid derivative<br>unknown | + | - | + | + | - | - |
| **42** | 31.9 | 293 | 327 | 253, 271 | pinobanksin-3-O-propionate | + | - | + | + | - | - |
| **43** | 32.7 | 340 | 269 | 254, 251, 236, 165 | 3-hydroxy-5-methoxy flavanone | + | - | + | + | - | - |
| **44** | 35.0 | 268, 309 | 387<br>501 | 281, 267, 255, 293<br>457, 439, 247 | unknown<br>unknown | + | + | + | + | - | - |
| **45** | 35.9 | 290 | 417 | - | methylated pinobanksin-3-*O*-phenylpropionate | + | - | + | + | - | - |
| **46** | 36.4 | 293 | 341 | 253 | pinobanksin-3-*O*-butyrate or isobutyrate | + | - | + | + | - | - |
| **47** | 38.6 | 269, 307, 343 | 565 | 283, 269, 281, 417, 455 | *p*-coumaric acid-4-hydroxyphenylethyl ester dimer | + | - | + | + | - | - |
| **48** | 39.3 | 292 | 355 | 253, 271 | pinobanksin-3-*O*-pentenoate or 2-methylbutyrate | + | - | + | + | - | - |
| **49** | 40.2 | 297, 320 | 315 | 179, 135 | caffeic acid derivative | + | - | + | + | - | - |
| **50** | 41.6 | 280 | 293 | 185, 197, 275, 249 | *p*-methoxy-cinnamic acid cinnamyl ester | + | - | + | + | - | - |
| **51** | 44.7 | 310 | 473 | - | *p*-coumaric acid derivative | + | - | + | + | - | - |

**EE** - ethanol extract; **EE$_{70}$** - ethanol/ water (7:3) extract; **HBE** - honey brandy extract; **PGE** - propylene glycol extract; **ME** - mead extract; **WE** - water extract; + detected; - not detected.

## 4.6. Author contributions

Conception and design of the work: Ana Sofia Freitas, Ana Cunha, Rui Oliveira, Cristina Al-meida-Aguiar; experimental work: Ana Sofia Freitas; supervision of the experimental work: Ana Cunha, Rui Oliveira, Cristina Almeida-Aguiar; UHPLC-DAD-ESI/$MS^n$ analysis of propolis extracts and data interpretation: Ana Sofia Freitas, Susana M. Cardoso; Principal Component Analysis and data interpretation: Ana Sofia Freitas, Pier Parpot; drafting the manuscript: Ana Sofia Freitas; re-vision and approval of the manuscript: Ana Sofia Freitas; Ana Cunha, Pier Parpot, Susana M. Cardoso, Rui Oliveira, Cristina Almeida-Aguiar; project supervision: Cristina Almeida-Aguiar.

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# Chapter 5

## Selective cytotoxicity of Portuguese propolis ethyl acetate fraction towards renal cancer cells

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*Equal senior authorship

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## Abstract

Renal cell carcinoma is the most lethal cancer of the urological system due to late diagnosis and treatment resistance. Propolis, a beehive product, is a valuable natural source of compounds with bioactivities and may be a beneficial addition to current anticancer treatments. A Portuguese propolis sample, its fractions (*n*-hexane, ethyl acetate, *n*-butanol and water) and three subfractions (**P1–P3**), were tested for their toxicity on A498, 786-O and Caki-2 renal cell carcinoma cell lines and the non-neoplastic HK2 kidney cells. The ethyl acetate fraction showed the strongest toxicity against A498 ($IC_{50}$ = 0.162 μg $mL^{-1}$) and 786-O ($IC_{50}$ = 0.271 μg $mL^{-1}$) cells. With similar toxicity against 786-O, **P1** ($IC_{50}$ = 3.8 μg $mL^{-1}$) and **P3** ($IC_{50}$ = 3.1 μg $mL^{-1}$) exhibited greater effect when combined ($IC_{50}$ = 2.5 μg $mL^{-1}$). Results support the potential of propolis and its constituents as promising coadjuvants in renal cell carcinoma treatment.

**Keywords:** Propolis, fractionation, phenolic compounds, pectolinarigenin, cytotoxic activity, renal cell carcinoma.

## 5.1. Introduction

According to the GLOBOCAN 2020 database, despite not being one of the most prevalent cancers, renal cell carcinoma (RCC) is the most aggressive and lethal cancer of the urological system. By 2040, its incidence and mortality are expected to increase by approximately 54 and 68 %, respectively (Sung *et al.*, 2021). Metastatic RCC is very frequently identified, mainly due to late diagnosis, and its aggressiveness requires a combination of surgery and systemic treatment, which is associated with important adverse effects (Capitanio and Montorsi, 2016). Therefore, alternative treatment approaches are needed to provide more effective treatments and/ or to reduce the toxicity associated with systemic treatment.

The demand for natural products has been increasing due to their importance in human health and their potential as new drug leads (Yadav *et al.*, 2015). Propolis is a natural product produced by bees as a building material and defensive substance in the hive (Fokt *et al.*, 2010). The chemical composition of propolis is complex and comprises mainly resinous and balsamic material collected from branches, flowers, pollen, buds and exudates of trees mixed with substances resulting from bees' metabolism (Fokt *et al.*, 2010). Propolis is a very popular natural remedy, widely recognised as an important source of compounds that exhibit a range of biological properties, including anticancer activity (Silva-Carvalho *et al.*, 2015), being commonly available as a food supplement with diverse beneficial

effects mostly due to its high flavonoid content (Havsteen, 2002). Several compounds have been identified in different propolis samples, with phenolics being the most important. The flavonoids pinocembrin, galangin and chrysin and phenolic acids such as caffeic acid, ferulic acid and cinnamic acid are the most common phenolic compounds in propolis from temperate zones (Bankova *et al.*, 2000; Huang *et al.*, 2014). Propolis from Gerês has been studied by our research group since 2011, being characterized by the prevalence of the same compounds such as chrysin, caffeic acid isoprenyl ester (CAIE) and pinocembrin, found in abundance, and other compounds such as pinobanksin and phenolic acids derivatives, found in lower amounts (Freitas *et al.*, 2019).

The cytotoxic effect of propolis has been extensively studied against diverse types of cancer cell lines, including breast (Kamiya *et al.*, 2012; Chang *et al.*, 2017), prostate (Li *et al.*, 2007), colon (Ishihara *et al.*, 2009), melanoma and colorectal (Valença *et al.*, 2013; Kubina *et al.*, 2015) cancer cells, as well as on other cell lines (Silva-Carvalho *et al.*, 2014, 2015). However, few studies have explored the potential of Portuguese propolis for the treatment of different types of cancer (Valença *et al.*, 2013; Calhelha *et al.*, 2014; Silva-Carvalho *et al.*, 2014). Propolis activity against RCC has been reported (Valente *et al.*, 2011; Daleprane *et al.*, 2012), but studies on the bioactivity of Portuguese propolis in RCC are scarce (Valente *et al.*, 2011) and lack in the case of propolis-derived fractions, with studies reported to date being only performed on a limited range of cell lines. In the present study, a propolis sample of Portuguese origin was fractionated and its fractions/ subfractions assessed for cytotoxicity on different renal cell lines for the first time.

## 5.2. Materials and Methods

### 5.2.1. Chemicals and reagents

Analytical grade ethanol, *n*-hexane, ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and methanol (MeOH) were obtained from Fisher Scientific, UK. Pre-coated silica gel 60 $F_{254}$ TLC plates (Merck®, Darmstadt, Germany) were used for the Thin-layer Chromatography (TLC) analysis. Sephadex® LH 20 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Silica gel 60 (0.063–0.200 mm; Merck®, Darmstadt, Germany) was used for column chromatography. Silica gel 60 H (Merck®, Darmstadt, Germany) was used for Vacuum Liquid Chromatography (VLC). Dimethyl sulfoxide (DMSO) and DMSO-*d*6 were obtained from Sigma-Aldrich, Portugal.

### 5.2.2. Propolis sample

The propolis sample used in this work (coded as G18) was collected in 2018 from an apiary located near the Cávado River, between the villages of Paradela and Sirvozelo, in Montalegre, Gerês, Portugal (41°45′41.62″ N; 7°58′03.34″ W).

### 5.2.3. Extraction and fractionation of propolis

Propolis G18 was extracted, as previously described (Freitas *et al.*, 2019), and a dried ethanol extract (EE) of propolis was obtained (70 % yield) after solvent evaporation. G18.EE was stored at 4 °C, in the dark, until further use. G18.EE (4 g) was dissolved in ethanol (20 mL) and deionized water (200 mL) and successively partitioned with *n*-hexane, EtOAc and *n*-BuOH (3 × 400 mL each). Organic layers were pooled, dried over sodium anhydrous sulphate, and concentrated under reduced pressure at 40 °C to obtain the G18.EE-*n*-hexane (1.22 g), G18.EE-EtOAc (3.58 g), G18.EE-*n*-BuOH (54.6 mg) and G18.EE-water (5.6 mg) fractions. The partition process was repeated 6 times, with 4 g of G18.EE each time, in order to obtain satisfactory amounts of fractions for further isolation work. All fractions were stored at 4 °C in the dark until further use and after evaporation of the respective solvent.

#### 5.2.3.1. Isolation of compounds from the G18.EE-EtOAc fraction

The total G18.EE-EtOAc fraction obtained (3.58 g) was subjected to VLC, eluting successively with *n*-hexane, *n*-hexane/ EtOAc mixtures of increasing polarity, and finally with mixtures of EtOAc and MeOH (up to 50 % MeOH in EtOAc). A total of 22 subfractions (F1–F22; **Figure 5.1**) were obtained and evaporated to dryness under reduced pressure at 40 °C. Subfractions were pooled according to similar TLC profiles into 13 main subfractions (FA–FM; **Figure 5.1**). The subfraction with the highest yield, FG (311.3 mg), eluted with *n*-hexane/ EtOAc (1:1), was further fractionated using Sephadex LH 20. Elution started with a relatively non-polar solvent system and then the polarity was increased gradually [$CH_2Cl_2$/ *n*-hexane (95:5); $CH_2Cl_2$ (100); $CH_2Cl_2$/ MeOH (95:5); $CH_2Cl_2$/ MeOH (90:10)]. From this subfraction (FG), eight FG-derived subfractions were obtained (FG1–FG8). FG8 was selected for additional fractionation using silica column with the same solvent system [$CH_2Cl_2$/ MeOH (95:5); $CH_2Cl_2$/ MeOH (98:2)], resulting in five FG8-derived subfractions (FG8-1–FG8-5). Based on the TLC profiles, three FG-derived subfractions (FG1, FG5 and FG8-1) displayed a single spot each on TLC plate, suggesting the presence of a single compound and thus being selected for the following experiments. Solvents used in the columns for extraction and the respective yields were: FG1 [**P1**; $CH_2Cl_2$/ *n*-hexane (95:5); 2.2 mg], FG5 [**P2**; $CH_2Cl_2$/ MeOH (95:5); 2.7 mg] and FG8-1 [**P3**; $CH_2Cl_2$/ MeOH (98:2); 4.3 mg] (**Figure 5.1**).

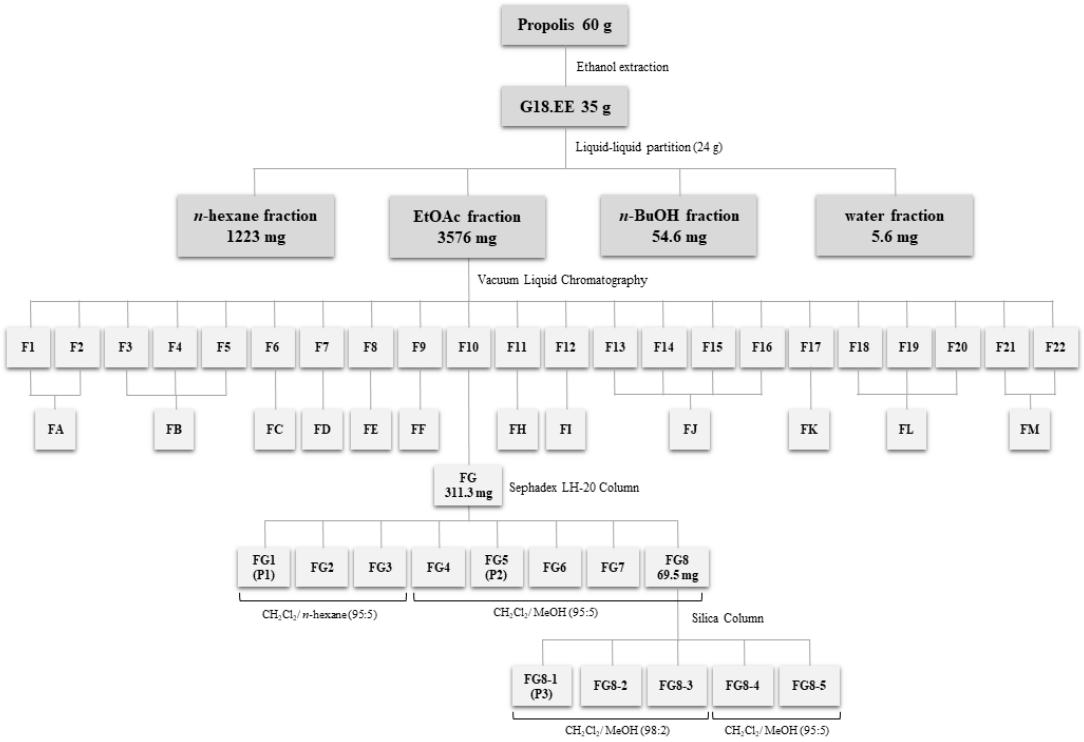


**Figure 5.1.** Fractionation of G18.EE and methodologies used for isolation of subfractions and compounds from the G18.EE-EtOAc fraction of Portuguese propolis. G18.EE–ethanol extract of propolis from Gerês collected in 2018; EtOAc–ethyl acetate; BuOH–butanol; F–fraction.

## 5.2.4. Chemical analysis of the subfractions

### 5.2.4.1. UPLC-DAD-ESI/$MS^n$ analysis

Subfractions **P1**, **P2**, and **P3** were analyzed by UPLC-DAD-ESI/$MS^n$ following a previously described method (Righi *et al.*, 2020). The dried subfractions were dissolved in methanol to a final concentration of 10 mg $mL^{-1}$ and 2 µL were used for injection. The Ultimate 3000 apparatus was equipped with an Ultimate 3000 Diode Array Detector (Dionex Co., Sunnyvale, CA, USA) and coupled to an ion trap mass spectrometer LTQ XL (Thermo Scientific, Waltham, MA, USA) equipped with an ESI source. Analysis was run on a Hypersil Gold (Thermo Scientific, Waltham, MA, USA) C18 column (100 mm length; 2.1 mm i.d.; 1.9 µm particle diameter, end-capped) at 30 °C. The mobile phase was composed of (A) water/ formic acid (99:1; v/v) and (B) acetonitrile/ formic acid (99:1; v/v). The solvent gradient started with 5–40 % B (0–15 min), followed by 40–100 % B (15–19 min), and returning to the initial conditions. The flow rate was 200 µL $min^{-1}$ and UV–Vis spectral data for all peaks were accumulated in the range 200–500 nm. The nitrogen (>99 % purity) gas pressure used for mass spectrometry analysis was 520 kPa (75 psi). The mass spectrometer was operated in negative-ion mode with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 °C. The full scan

covered the mass range from *m*/z 100 to 2000. CID–MS/MS and $MS^n$ experiments were simultaneously acquired for precursor ions using helium as the collision gas with collision energy of 25–35 arbitrary units. Control and data acquisition were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific, Waltham, MA, USA). Compounds in the subfractions (P1–P3) were identified based on a literature comparison of the ESI-MS/MS data (Freitas *et al.*, 2019).

#### 5.2.4.2. NMR analysis

NMR spectra of **P1** were recorded in a Bruker Advance II 400 spectrometer, at 400 MHz ($^1$H) and 100 MHz ($^{13}$C), at 20 °C (**Figures S5.1** and **S5.2 Supplementary material**). The chemical shifts ($\delta$) were recorded in parts per million (ppm) relative to the residual solvent signal (DMSO-*d*6).

5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one (pectolinarigenin; **P1**). Yellow amorphous solid. $^1$H NMR (DMSO-*d*6, 400 MHz): $\delta$ = 3.77 (3H, s, $OCH_3$), 3.85 (3H, s, $OCH_3$), 6.61 (1H, s), 6.86 (1H, s), 7.10 (2H, d, $J$ = 7.2 Hz), 8.03 (2H, d, $J$ = 6.8 Hz), 10.72 (1H, s), 13.02 (1H, s). $^{13}$C NMR (DMSO-*d*6, 100 MHz): $\delta$ = 55.5, 59.9, 94.3, 103.0, 104.1, 114.6 (2C), 122.8, 128.3 (2C), 131.4, 152.4, 152.7, 157.4, 162.3, 163.3, 182.1 (Lim *et al.*, 2008).

### 5.2.5. Cell lines, media and growth conditions

Four human renal cell lines-HK2 (renal non-neoplastic cells) and 786-O, Caki-2 and A498 (RCC cells) were obtained from the American Type Culture Collection (ATCC). The HK2 and 786-O cell lines were cultured in RPMI 2.0 g/ L $NaHCO_3$ without L-glutamine (RPMI 1640, Biochrom GmbH, Berlin, Germany). The Caki-2 cell line was cultured in McCOY's high glucose, L-glutamine, bacto-peptone, and phenol red without sodium pyruvate and HEPES (McCOY's 5A, Biochrom GmbH). The A498 was cultured in MEM with 20 mM HEPES without $NaHCO_3$ and L-glutamine (MEM Earle's, Biochrom GmbH). In addition, 1 % of an antibiotic preparation (penicillin-streptomycin, Gibco) and 10 % heating activated fetal bovine serum (FBS Superior, Biochrom GmbH) were used as supplements for the culture media. Cultures were grown at 37 °C and 5 % $CO_2$ in a humidified incubator.

To prepare cells for the cytotoxicity assay, flasks with sub-confluent cells were washed with phosphate-buffer 1× (PBS Dulbecco, Merck®) and then were detached from the flask with trypsin (TrypLE™ Express, Gibco, New York, USA) at 37 °C. Medium was then added to the flask for trypsin inactivation. Cells were collected into a 15 mL Falcon tube and centrifuged for 5 min at 900 rpm, at 4 °C. Fresh medium was used to resuspend the cells, and 10 µL of the cell suspension were collected for

subsequent addition of 20 µL of trypan blue (Trypan Blue Solution 0.4 %, Gibco) for cell counting (Neubauer chamber).

### 5.2.6. Cytotoxicity assay and selectivity index (SI) calculation

Propolis cytotoxicity was assessed by the sulforhodamine B (SRB) assay as previously described (Pontes *et al.*, 2018), which estimates cell biomass based on cell protein content. Briefly, each cell line was plated at an appropriate density (2000 cells/ well for A498 and 786-O or 3000 cells/ well for Caki-2 and HK2) in 96-well plates for 24 h. The dried extract and subfractions (by solvent evaporation) were dissolved in DMSO for the cells' treatments. Cells were then treated for 72 h with different concentrations of G18.EE (0.005–100 µg $mL^{-1}$), each of the four fractions (*n*-hexane, EtOAc, *n*-BuOH and water fractions; 0.005–30 µg $mL^{-1}$), each of the three subfractions (**P1–P3**; 0.005–30 µg $mL^{-1}$) and two combinations of the subfractions with equal parts of each subfraction (**P1** + **P2** + **P3** and **P1** + **P3**; 0.005–30 µg $mL^{-1}$). Controls were performed with 0.25 % of DMSO (vehicle) for $IC_{50}$ determination (concentration that inhibits cell viability in 50 %). Following this incubation period, the adherent cells were fixed by adding 50 µL of cold trichloroacetic acid 10 % (TCA, PanReac, Barcelona, Spain) and incubating for 60 min, at 4 °C. The plates were then washed with deionized water, dried overnight, and 50 µL of 0.1 % SRB solution (Sigma–Aldrich) in 1 % acetic acid (PanReac) were added to each plate well. After 30 min incubation at room temperature, unbound SRB was removed by washing with 1 % acetic acid and air-dried overnight. Bound SRB was solubilized with 100 µL of 10 mM Tris base (Trizma base, Merck®) and the absorbance was measured at 490 nm in a microplate reader (Tecan Infinite M200, Grödig/Salzburg, Austria). $IC_{50}$ values were determined by transforming the absorbance values into a sigmoidal dose-response curve and extracting the concentration of the extracts/ fractions needed to inhibit the viability of the kidney cells by 50 %, using the GraphPad Prism Software Version 6.0. The selectivity index (SI) was calculated based on the $IC_{50}$ values obtained for the samples against the cell lines, using the following formula:

$$SI = (IC_{50} \text{ HK2 cell line} - IC_{50} \text{ cancer cell line})/IC_{50} \text{ cancer cell line}$$

Cytotoxicity is higher for the cancer cell line than for the non-neoplastic HK2 cell line when SI values >1.

### 5.2.7. Statistical analysis

Experiments were performed in triplicate and repeated at least three times for $IC_{50}$ determination using the GraphPad Prism 5 software for logarithmic transformation after applying a sigmoidal dose-response non-linear regression. For multiple comparisons one-way ANOVA supplemented with post-hoc Tukey test was performed. Different letters mean statistical differences ($p < 0.05$) between mean values.

## 5.3. Results and Discussion

The cytotoxic activity of G18.EE and its fractions was evaluated on three different RCC cell lines-786-O, Caki-2 and A498 (**Table 5.1**). The G18.EE-*n*-hexane fraction showed no effect on the viability of any of the cell lines at the tested concentrations. On the other hand, the G18.EE-water fraction exhibited the strongest effect on cell viability, with the lowest $IC_{50}$ values against all cell lines (**Table 5.1**). However, this fraction had the lowest selectivity indexes (SI), meaning poor selectivity towards cancer cells. G18.EE and the remaining fractions showed lower cytotoxicity for the human non-neoplastic HK2 renal cells ($IC_{50}$ > 30 μg $mL^{-1}$) and Caki-2 cells. The latter was the least sensitive cancer cell line. Considering all the results (**Table 5.1**), the G18.EE-EtOAc fraction was selected for further investigation based on its highest activity, with lower though not statistically significant $IC_{50}$ values than G18.EE-*n*-hexane and G18.EE-*n*-BuOH fractions against both A498 and 786-O cells (0.162 ± 0.000 and 0.271 ± 0.005 μg $mL^{-1}$, respectively) but with higher selectivity indexes.

**Table 5.1.** $IC_{50}$ values and respective selectivity indexes of G18.EE and their fractions against RCC cells (72 h treatment).

| Propolis Fractions | $IC_{50}$ ± SD (μg $mL^{-1}$) * | | | | SI $^{\alpha}$ (vs. HK2) | | |
|---|---|---|---|---|---|---|---|
| | Caki-2 | A498 | 786-O | HK2 (Non-Neoplastic) | Caki-2 | A498 | 786-O |
| *n*-hexane | >30 | >30 | >30 | >30 | 0 | 0 | 0 |
| EtOAc | >30 | 0.162 ± 0.000 $^{c,d}$ | 0.271 ± 0.005 $^{c,d}$ | >30 | 0 | >184.2 | >109.7 |
| *n*-BuOH | >30 | 0.239 ± 0.001 $^{c,d}$ | 0.341 ± 0.003 $^{c,d}$ | >30 | 0 | >124.5 | >87.0 |
| water | 0.573 ± 0.030 $^{c}$ | 0.085 ± 0.001 $^{d}$ | 0.199 ± 0.013 $^{c,d}$ | 0.229 ± 0.000 $^{c,d}$ | −0.6 | 1.7 | 0.2 |
| G18.EE (propolis extract) | 0.765 ± 0.041 $^{c}$ | 0.153 ± 0.004 $^{c,d}$ | 87.170 ± 0.707 $^{a}$ | 49.185 ± 0.106 $^{b}$ | 63.3 | 320.5 | −0.4 |

$^{\alpha}$ SI (Selectivity Index) = ($IC_{50}$ HK2 cell line-$IC_{50}$ cancer cell line)/ $IC_{50}$ cancer cell line; * Experiments were done in triplicate and repeated at least three times. Results are expressed as mean of $IC_{50}$ values ± standard deviation (SD). One-way ANOVA followed by Tukey test was performed to assess significance. Mean values sharing the same superscript letters ($^{a}$, $^{b}$, $^{c}$ or $^{d}$) have no statistically significant differences.

Propolis has been extensively investigated for its anticancer potential (Silva-Carvalho *et al.*, 2015; Chang *et al.*, 2017). Propolis from Poland and Egypt have been investigated on LNCaP and PC3

prostate cancer cells, respectively, and both samples showed cytotoxicity in a concentration-dependent manner against both cell lines (Szliszka *et al.*, 2013; Salim *et al.*, 2015). Ethanol extracts of propolis from China and Brazil showed a dose-dependent reduction of the viability of several colon cancer cell lines (CaCo2, HCT116, HT29 and SW480). The $IC_{50}$ values obtained ranged from 4.4 to 41 μg $mL^{-1}$, CaCo2 cells being the least susceptible to both extracts ($IC_{50}$ > 50 μg $mL^{-1}$) (Ishihara *et al.*, 2009). Other studies revealed the potential of Chinese and Japanese propolis on melanoma (A375) and lung (A549) cancer cells ($IC_{50}$ values of 112 and 12 μg $mL^{-1}$, respectively) (Taira *et al.*, 2016; Zheng *et al.*, 2018). Methanol and water extracts of propolis from Thailand were tested against the SW620 colorectal cancer cell line, the latter extract being the most active, with a 77 % of cell viability reduction when tested at 150 μg $mL^{-1}$ (Umthong *et al.*, 2011). Altogether, these studies provide strong evidence for the anticancer potential of propolis crude extracts against a wide range of different cancer types.

Propolis extracts from other Portuguese regions (Pereiro, Bragança, Mirandela, Coruche, Aljezur, S. Miguel Island from Azores, and Funchal from Madeira Island) have been tested on different cancer cell lines, including breast (MDA-MB-231, MDA-MB-468 and MCF-7), prostate (DU145 and 22RV1) and glioblastoma (SW1088), with $IC_{50}$ values ranging from 9 to 182 μg $mL^{-1}$ (Valença *et al.*, 2013; Calhelha *et al.*, 2014; Silva-Carvalho *et al.*, 2014). A previous study described the effect of methanol extracts of propolis from Bornes and from Fundão (Portugal) on A498 RCC cells ($IC_{50}$ values of 70.8 ± 10.7 and > 100 μg $mL^{-1}$, respectively) (Valente *et al.*, 2011). In the present work, we report the activity of a Portuguese propolis sample with remarkable cytotoxicity against tumoral cell lines. The $IC_{50}$ values obtained are 400 times lower than the values found in the literature.

Different fractions (*n*-hexane, chloroform and ethanol residual) obtained from an ethanol extract of Portuguese propolis from Angra do Heroísmo showed $IC_{50}$ values ranging from 5 to 26 μg $mL^{-1}$ on colorectal cancer HCT-15 cells (Valença *et al.*, 2013). The *n*-hexane fraction of an EE of propolis from Thailand showed high anticancer activity against different cancer cell lines (SW620, BT474, Hep-G2, Chago and Kato-III) (Umthong *et al.*, 2009), unlike the *n*-hexane fraction used in the previous study, which showed no cytotoxic effect.

The molecular mechanism of the anticancer activity of propolis has yet to be fully understood. Propolis has been reported to decrease cell proliferation and migration, down-regulate NF-kB p65 levels, and increase apoptosis by interfering with the caspase pathway in breast cancer cells (Kamiya *et al.*, 2012; Silva-Carvalho *et al.*, 2014; Chang *et al.*, 2017); interfere with the protein expression of cyclins (D1, B1) and cyclin-dependent kinase (CDK) p21, inhibiting proliferation in prostate cancer cells (Li *et al.*, 2007); increase cellular mRNA levels of p21 CIPI and p53 and induce apoptosis in colon cancer

cells (Ishihara *et al.*, 2009); disrupt tumor glycolytic metabolism, inhibiting growth and leading to cell death in melanoma and colorectal cancer cells (Valença *et al.*, 2013; Kubina *et al.*, 2015), among many others (Silva-Carvalho *et al.*, 2015). The exact mechanism of action of propolis against RCC cells is still unknown. Only one study, using endothelial cells and RCC4 cells revealed the inhibitory effect of a red propolis polyphenols (RPP) extract on the vascular endothelial growth factor (VEGF) gene expression through destabilization of the HIF1α protein under hypoxic conditions resulting in reduction of angiogenesis. The authors attributed this destabilization to the ability of RPP to diminish the expression of the Cdc42 protein and consequently increase the expression of the pVHL E3 ubiquitin ligase (Daleprane *et al.*, 2012).

Chemical composition of the G18.EE and its fractions was already investigated and published by our research group (Oliveira *et al.*, 2022; **Figure S5.3** and **Table S5.1 Supplementary material**), revealing the presence of compounds such as apigenin, pinobanksin, pinobanksin 3-O-acetate, chrysin, acacetin, CAIE, and pinocembrin. Partitioning of G18.EE-EtOAc fraction led to the isolation of the compound **P1** and eleven subfractions (FG2–FG7 and FG8-1–FG8-5) composed of a mixture of compounds (**Figure 5.1**). FG1 (**P1**), FG5 (**P2**) and FG8-1 (**P3**) were selected for further chemical investigation, since they appear as a single spot on the TLC, suggesting the presence of a single compound. **P1** was identified as pectolinarigenin, following NMR (as described in Materials and Methods), MS analysis, and by comparison with previously published data (**Table 5.2**; **Figure 5.2**) (Lim *et al.*, 2008). The compounds present in **P2** and **P3** were identified by comparison of the retention times and values of ESI-MS/MS published in the literature (Freitas *et al.*, 2019), with acacetin and caffeic acid isoprenyl ester as the major compounds in **P2** and **P3**, respectively (**Table 5.2**; **Figures 5.3** and **5.4**).

**Table 5.2.** The detection of compounds present in **P1**, **P2** and **P3**, isolated from G18.EE-EtOAc, following LC-MS analysis.

| Subfractions | Compound Code | $t_R$ (min) | λmax (nm) | $[M-H]^-$ m/z | Main Fragments | Compound Detected |
|---|---|---|---|---|---|---|
| P1 | 1 | 20.8 | 275, 330 | 313 | - | Pectolinarigenin |
| P2 | 1 | 11.2 | 309 | 163 | 119, 145, 108 | *p*-Coumaric acid |
| | 2 | 17.9 | 281, 334 | 299 | - | unknown |
| | 3 | 19.1 | 291 | 271 | 253, 225 | Pinobanksin |
| | 4 | 20.1 | 259, 368 | 315 | 300 | Isorhamnetin |
| | 5 | 20.2 | 254, 368 | 329 | 314 | Quercetin-dimethyl ether |
| | 6 | 20.8 | 268, 329 | 283 | *269* | Acacetin |
| P3 | 1 | 20.2 | 253, 368 | 329 | 314 | Quercetin-dimethyl ether |
| | 2 | 20.7 | 298, 325 | 247 | 179, 135 | Caffeic acid isoprenyl ester |
| | 3 | 20.9 | 298, 325 | 283 | 179, 135 | Caffeic acid phenylethyl ester |
| | 4 | 21.2 | 295, 325 | 295 | 178, 134, 251, 211 | Caffeic acid cinnamyl ester |

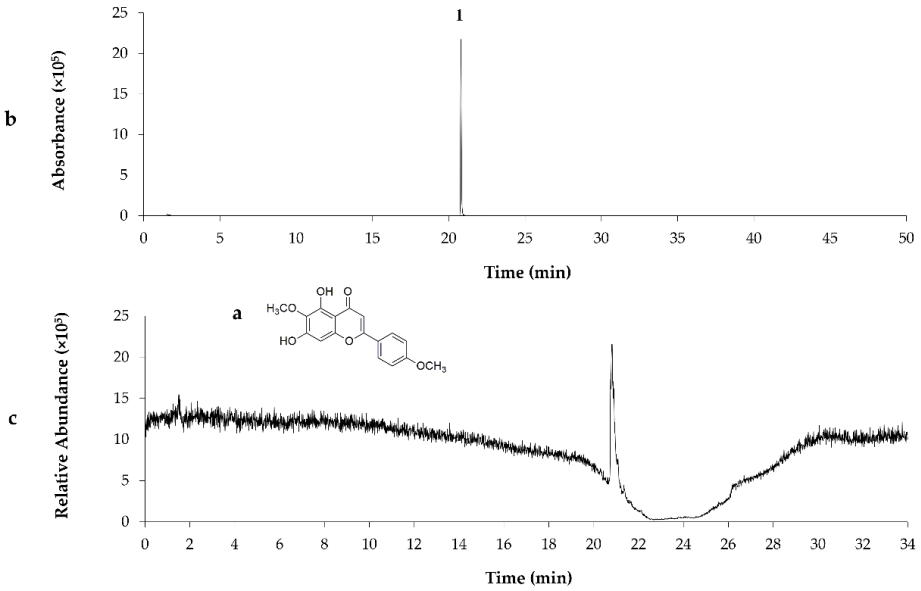


**Figure 5.2.** The chemical structure of pectolinarigenin (**a**); LC-MC chromatogram of **P1** isolated from G18.EE-EtOAc fraction using ESI (**b**); TIC chromatogram in negative ion mode (**c**); Pectolinarigenin (**1**).

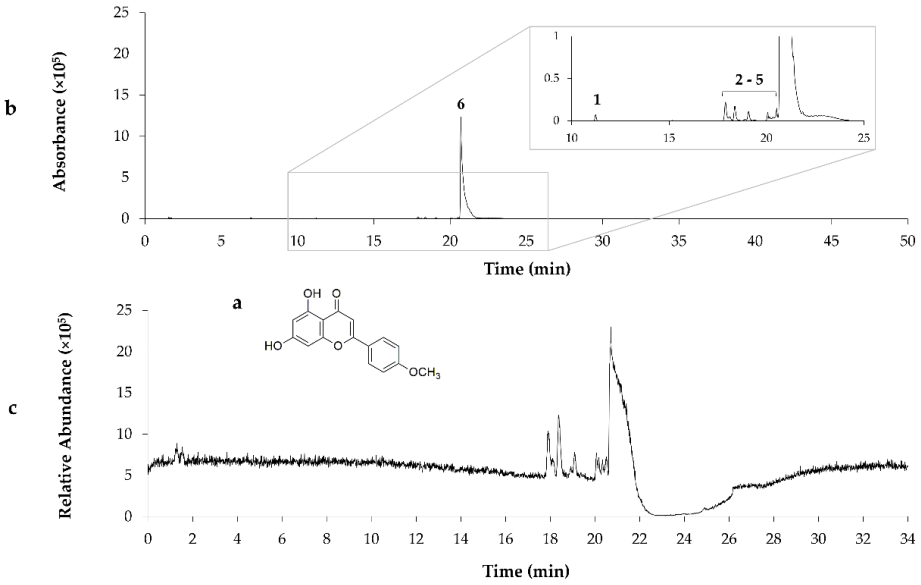


**Figure 5.3.** The chemical structure of acacetin (**a**); LC-MC chromatogram of **P2** isolated from G18.EE-EtOAc fraction using ESI (**b**); TIC chromatogram in negative ion mode (**c**); *p*-Coumaric acid (**1**); unknown (**2**); Pinobanksin (**3**); Isorhamnetin (**4**); Quercetin-dimethyl ether (**5**); Acacetin (**6**).

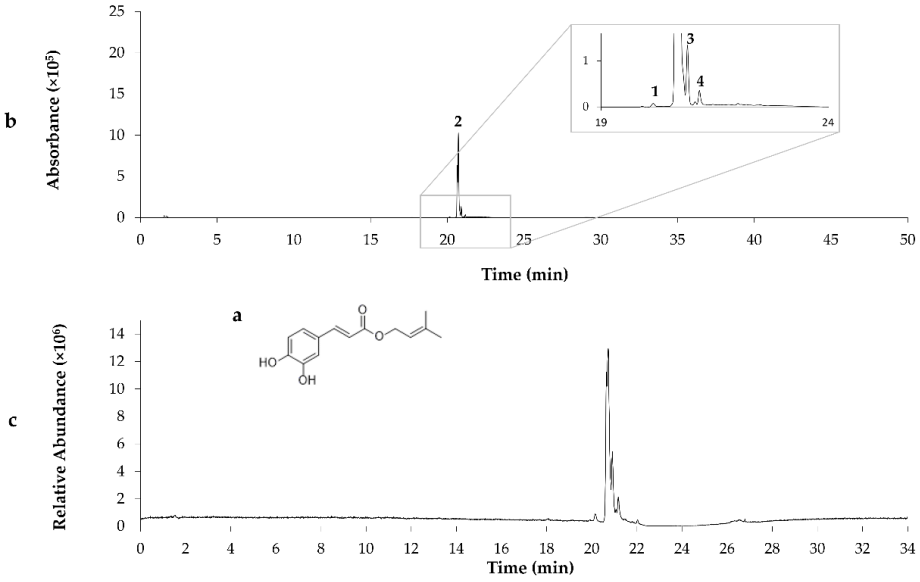


**Figure 5.4.** The chemical structure of caffeic acid isoprenyl ester (**a**); LC-MC chromatogram of **P3** isolated from G18.EE-EtOAc fraction using ESI (**b**); TIC chromatogram in negative ion mode (**c**); Quercetin-dimethyl ether (**1**); Caffeic acid isoprenyl ester (**2**); Caffeic acid phenylethyl ester (**3**); Caffeic acid cinnamyl ester (**4**).

The effect of the subfractions obtained from the G18.EE-EtOAc fraction on the viability of 786-O and A498 cell lines is presented in **Table 5.3**. **P1** and **P3** similarly affected the viability of both cell lines, with **P3** being more selective towards both cancer cell lines. **P2** had no effect on the viability of any of the cell lines at the tested concentrations, and 786-O was the most susceptible RCC cell line to both **P1** ($IC_{50}$ = 3.8 ± 0.2 μg $mL^{-1}$; 12.1 ± 0.6 μM) and **P3** ($IC_{50}$ = 3.1 ± 0.01 μg $mL^{-1}$). All subfractions (**P1–P3**) showed lower cytotoxicity against 786-O and A498 cell lines when compared to their fraction of origin (G18.EE-EtOAc; **Table 5.1**), which is in line with previous observations that the biological properties of propolis can be attributed to not only certain compounds but also to a synergistic effect that may occur between compounds (Garedew *et al.*, 2004).

**Table 5.3.** The $IC_{50}$ values and respective selectivity indexes of subfractions **P1**, **P2** and **P3** isolated from the G18.EE-EtOAc fraction against RCC cells (72 h treatment).

| Subfractions | $IC_{50}$ ± SD (μg $mL^{-1}$/ μM) * | | | SI $^{\alpha}$ (vs. HK2) | |
|---|---|---|---|---|---|
| | 786-O | A498 | HK2 (Non-Neoplastic) | 786-O | A498 |
| **P1** (pectolinarigenin) | 3.8 ± 0.2/ 12.1 ± 0.6 $^{e}$ | 11.8 ± 0.04/ 37.8 ± 0.1 $^{d}$ | 13.2 ± 0.09/ 42.2 ± 0.3 $^{c,d}$ | 2.5 | 0.1 |
| **P2** | >30 | >16 | 29.7 ± 0.3 $^{a}$ | <0 | <0.9 |
| **P3** | 3.1 ± 0.01 $^{e}$ | 11.4 ± 0.1 $^{d}$ | 24.9 ± 0.4 $^{b}$ | 7.1 | 1.2 |

$^{\alpha}$ SI (Selectivity Index) = ($IC_{50}$ HK2 cell line-$IC_{50}$ cancer cell line)/ $IC_{50}$ cancer cell line; * Experiments were done in triplicate and repeated at least three times. Results are expressed as mean of $IC_{50}$ values ± standard deviation (SD). One-way ANOVA followed by Tukey test was performed to assess significance. Mean values sharing the same superscript letters ($^{a}$, $^{b}$, $^{c}$, $^{d}$ or $^{e}$) have no statistically significant differences.

Compounds present in **P1**, **P2** and **P3** have previously been reported in other propolis samples (Garedew *et al.*, 2004; Bankova *et al.*, 2016; Freitas *et al.*, 2019; Dezmirean *et al.*, 2021). Several compounds naturally present in European propolis (Bankova *et al.*, 2016), such as pectolinarigenin, acacetin, caffeic acid phenethyl ester (CAPE), chrysin, galangin, quercetin, apigenin, acacetin, pinobanksin and kaempferol, have demonstrated anticancer activity (Jaganathan and Mandal, 2009; Silva-Carvalho *et al.*, 2015; Patel, 2016; Rejhová *et al.*, 2018; Aumeeruddy and Mohomoodally, 2019; Cheriet *et al.*, 2020; Singh *et al.*, 2020).

Pectolinarigenin was shown to be active against different cancer cell lines (Lee *et al.*, 2018; Somwong and Suttisri, 2018; Wu *et al.*, 2018; Xu *et al.*, 2018; Liu *et al.*, 2020). To date and to our knowledge, only one study described its anticancer potential on RCC cells (ACHN) with an $IC_{50}$ value of 15.2 µM (Bonesi *et al.*, 2008). The effects of pectolinarigenin on the viability of SK-HEP-1, SMMC-7721 and PLC5 liver cancer cells were also reported ($IC_{50}$ values of 10, 11.59 and 11.97 µM, respectively; Wu *et al.*, 2018; Xu *et al.*, 2018; Liu *et al.*, 2020) as well as on A549 and Calu-3 lung cancer cells ($IC_{50}$ values of 21.49 and 22.63 µM, respectively; Xu *et al.*, 2018), SW620 colon cancer cells ($IC_{50}$ = 13.05 µM) and KATO-III, AGS and MKN28 gastric cancer cells ($IC_{50}$ of 24.31, 124.79 and 96.88 µM, respectively; Lee *et al.*, 2018; Somwong and Suttisri, 2018).

Acacetin has demonstrated activity on breast cancer - MDA-MB-231 and MCF-7 - ($IC_{50}$ values of 82.75 and 103.91 µM, respectively; Zhang *et al.*, 2018), liver (SMMC-7721; $IC_{50}$ > 200 µM), lung (A549; $IC_{50}$ = 157.40 µM) and prostate (DU145; $IC_{50}$ = 25 µM) cancer cell lines (Kim *et al.*, 2014). Although acacetin appears to be the lead compound of **P2**, no cytotoxic effect was found against any of the RCC at the tested concentrations in the present study (**Table 5.3**). The $IC_{50}$ values found for acacetin on other cancer cell lines are in general substantially higher than the maximum concentration of **P2** tested herein and **P2** activity cannot be attributed to the activity of acacetin as a single compound, but to the interaction of several compounds present in the subfraction.

To the best of our knowledge, the present work also shows, for the first time, evidence of the anticancer potential of CAIE, identified as the main compound in **P3**. Further studies on the anticancer activity of this pure compound are warranted.

To explore the cytotoxic activity of the combination of different components of the propolis fractions, two different mixtures were prepared (**P1** + **P2** + **P3** and **P1** + **P3**) with equal parts of each subfraction and tested against the 786-O cell line (**Table 5.4**). The **P1** + **P3** combination ($IC_{50}$ = 2.5 ± 0.03 µg $mL^{-1}$) showed no significant differences when compared to each individual subfractions ($p$ > 0.05), contrary to what was observed for the **P1** + **P2** + **P3** combination ($IC_{50}$ = 8.6 ± 0.06 µg $mL^{-1}$; $p \leq$

0.001), with **P1** + **P3** combination showing higher selectivity towards the 786-O cancer cell line. This fact may be explained by the presence of **P2**, for which no activity was detected ($IC_{50}$ > 30 μg $mL^{-1}$). Nevertheless, the effect of these subfractions, individually or mixed, did not surpass the effect shown by the original fraction (EtOAc fraction; **Table 5.1**), which is chemically more complex, suggesting that other constituents of this fraction are acting synergistically.

**Table 5.4.** The $IC_{50}$ values and respective selectivity indexes of the combinations of the subfractions (in equal parts) obtained from the G18.EE-EtOAc fraction (72 h treatment) in RCC cells.

| Mixtures | $IC_{50}$ ± SD (μg $mL^{-1}$) * | | SI[α] (vs. HK2) |
|---|---|---|---|
| | 786-O | HK2 (Non-Neoplastic) | 786-O |
| **P1 + P2 + P3** | 8.6 ± 0.06 [c] | *20.0* ± 0.08 [a] | 1.3 |
| **P1 + P3** | 2.5 ± 0.03 [d] | *15.7* ± 0.11 [b] | 5.4 |

[α] SI (Selectivity Index) = ($IC_{50}$ HK2 cell line-$IC_{50}$ cancer cell line)/ $IC_{50}$ cancer cell line; * Experiments were done in triplicate and repeated at least three times. Results are expressed as mean of $IC_{50}$ values ± standard deviation (SD). One-way ANOVA followed by Tukey test was performed to assess significance. Mean values sharing the same superscript letters ([a], [b], [c] or [d]) have no statistically significant differences.

Interestingly, some combinations of propolis-derived compounds have already been studied for their anticancer potential and additive/ synergistic effects have been observed (Ouhtit *et al.*, 2013). This prompted us to investigate the effect of combining the subfractions isolated in this study on the viability of 786-O cells. The results (**Table 5.4**) reflect the complexity of propolis mixtures which can contain cytotoxic compounds and others without anticancer potential, being antagonistic or just diluting the overall cytotoxic effect of the mixture.

## 5.4. Conclusions

Cancer is one of the leading causes of death worldwide and is expected to cause more than 16 million deaths in 2040 (Sung *et al.*, 2021). Traditional cancer treatments include surgery, radiation, and chemotherapy, which are associated with significant side effects (Capitanio and Montorsi, 2016). Comparisons with the existent literature are difficult to make considering the lack of studies on RCC cell lines, the different methodologies adopted for cytotoxicity assessment, and the variety of treatment conditions implemented by few published studies. Nevertheless, the propolis samples studied in this work show a strong anticancer effect on different RCC cells, presenting very low $IC_{50}$ values, and importantly, very high selectivity indexes, when compared to the data available in the literature. This suggests that Portuguese propolis from Gerês, as well as its chemical constituents, could be used as alternatives or adjuvants to conventional anticancer treatments. They could be also used to help tackle

drug-resistance and toxic effects by reducing the dose of the therapeutic drugs commonly used in RCC treatment.

## 5.5. Supplementary material

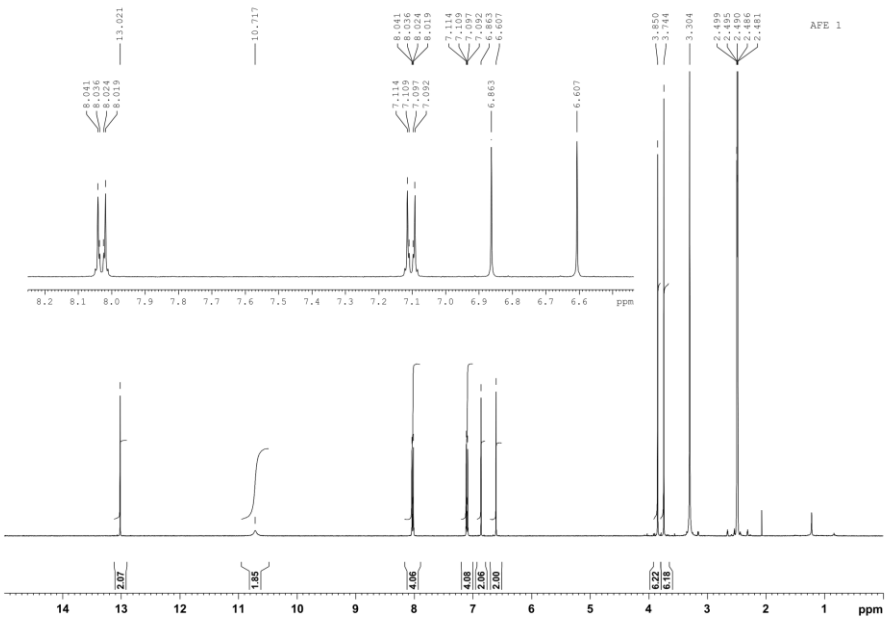


**Figure S5.1.** $^{1}$H NMR spectrum (400 MHz) for **P1** (pectolinarigenin) in DMSO-*d*6.

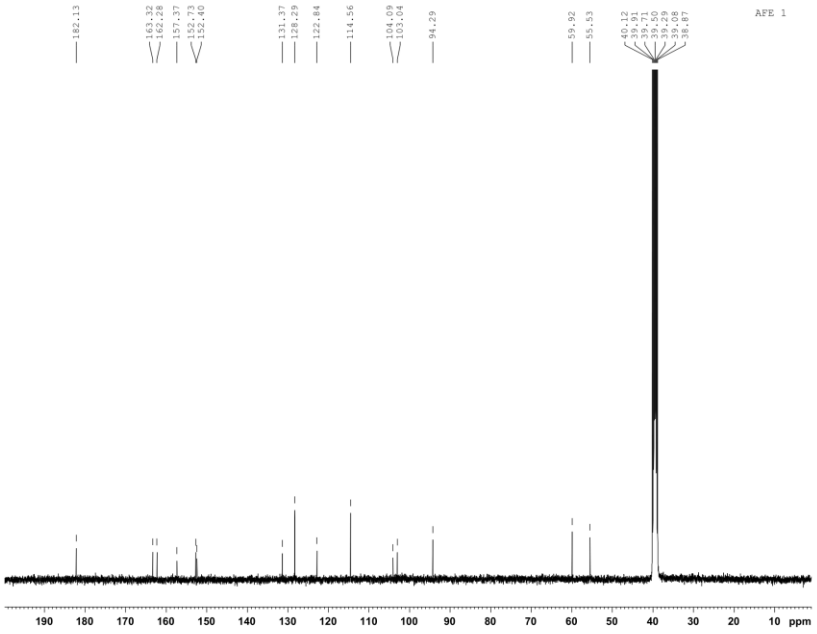


**Figure S5.2.** $^{13}$C NMR spectrum (100 MHz) for **P1** (pectolinarigenin) in DMSO-*d*6.

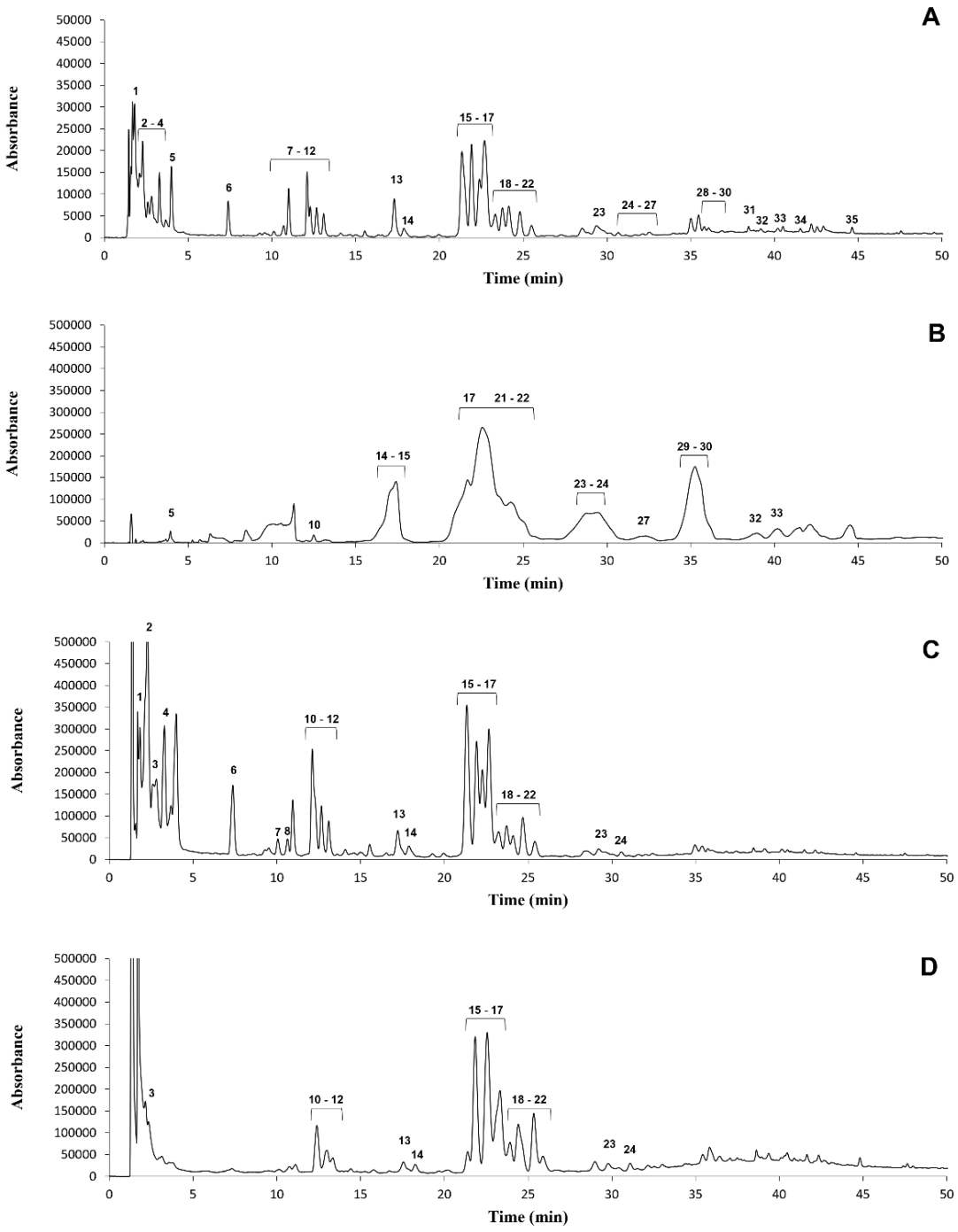


**Figure S5.3 -** Chromatographic profiles of G18.EE (**A**) and its fractions *n*-Hexane (**B**), EtOAc (**C**) and *n*-BuOH (**D**) obtained by LC-MS.

**Table S5.1**. Chemical composition of G18.EE and its fractions *n*-hexane, EtOAc and *n*-BuOH according to LC-MS analysis. ("-"corresponds to a compound not present or not detected). Bold values represent the major phenolic compounds (Composition (%) > 5%).

| Peaks | Time range (min) | λmax (nm) | $[M - H]^-$ m/z | Main fragments | Compound | Composition (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | G18.EE | ***n*-hexane** | EtOAc | ***n*-BuOH** |
| **1** | 1.6 – 2.2 | 270 | 169 | 125 | Gallic acid | 3.39 | - | 1.65 | - |
| **2** | 2.2 – 2.5 | 292sh, 322 | 179 | 135, 163 | Caffeic acid | **7.08** | - | **14.97** | - |
| **3** | 2.3 – 3.6 | 253, 368 | 301 | 257, 229 | Ellagic acid | 2.15 | 0.05 | 0.74 | 2.64 |
| **4** | 3.1 – 3.5 | 309 | 163 | 119, 145, 108 | *p*-Coumaric acid | 3.33 | - | **7.83** | - |
| **5** | 3.8 – 4.2 | 295sh, 323 | 193 | 177, 149, 133 | Ferulic acid | 3.97 | 0.45 | - | - |
| **6** | 7.3 – 7.6 | 295sh, 322 | 207 | 163, 148, 133 | 3,4-Dimethyl-caffeic acid (DMCA) | 2.15 | - | **6.31** | - |
| **7** | 9.9 – 10.3 | 288 | 285 | 267, 239, 252 | Pinobanksin-5-methyl-ether | 0.49 | - | 1.30 | - |
| **8** | 10.5 – 10.8 | 273, 353 | 315 | 300 | Quercetin-3-methyl ether | 0.84 | - | 1.16 | - |
| **9** | 10.8 – 11.2 | 308 | 177 | - | *p*-coumaric acid methyl ester | 2.72 | - | - | - |
| **10** | 11.9 – 12.7 | 267, 336<br>291 | 269<br>271 | 225, 151<br>253, 225 | Apigenin<br>Pinobanksin | **5.03** | 0.64 | **10.14** | **7.39** |
| **11** | 12.5 – 13.2 | 265, 363 | 285 | 285, 257, 151 | Kaempferol | 2.01 | - | 2.87 | 3.04 |
| **12** | 13.0 – 13.6 | 255, 368 | 315 | 300 | Isorhamnetin | 1.32 | - | 2.35 | 1.18 |
| **13** | 16.7 – 17.9 | 311 | 313 | 298, 269, 257, 241, 179, 153 | unknown | 3.30 | 4.16 | 2.47 | 2.66 |
| **14** | 17.3 – 18.5 | 254, 368 | 329 | - | Quercetin-dimethyl-ether | 1.01 | 1.11 | 1.12 | 1.52 |
| **15** | 20.5 – 22.4 | 268, 315<br>298, 325 | 253<br>247 | 209, 181, 225, 151<br>179, 135 | Chrysin<br>Caffeic acid isoprenyl ester | **10.35** | **28.99** | **15.93** | **20.94** |
| **16** | 21.7 – 23.1 | 268, 332<br>299, 325 | 283<br>247 | 268<br>179, 135 | Acacetin<br>Caffeic acid isoprenyl ester | **12.73** | - | **11.48** | **23.43** |
| **17** | 22.5 – 23.6 | 289 | 255 | 213, 211, 151 | Pinocembrin | **13.01** | **20.79** | **8.24** | **5.52** |
| **18** | 23.0 – 24.2 | 266, 290sh, 356 | 269 | 223, 169, 249 | Galangin | 2.69 | - | 1.35 | 2.95 |

**Table S5.1**. Chemical composition of G18.EE and its fractions *n*-hexane, EtOAc and *n*-BuOH according to LC-MS analysis. ("-"corresponds to a compound not present or not detected). Bold values represent the major phenolic compounds (Composition (%) > 5%) (continued).

| Peaks | Time range (min) | λmax (nm) | $[M - H]^-$ m/z | Main fragments | Compound | Composition (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | G18.EE | *n*-hexane | EtOAc | *n*-BuOH |
| **19** | 23.6 – 24.5 | 267, 362 | 299 | 284, 165 | Kaempferide | 2.72 | - | 1.93 | 4.38 |
| **20** | 24.0 – 25.1 | 269, 360 | 329 | 314, 297, 287, 269, 257 | Kaempferol –methoxy-methyl ether | 3.13 | - | 1.02 | 2.29 |
| **21** | 23.9 – 25.8 | 293 | 313 | 253, 271 | Pinobanksin-3-*O*-acetate | 2.56 | 4.38 | 3.82 | **9.75** |
| **22** | 25.2 – 29.7 | 298, 325 | 283 | 179, 135 | Caffeic acid phenylethyl ester | 1.39 | 0.41 | 1.55 | 2.24 |
| **23** | 27.6 – 30.9 | 311 | 231 | 163, 119 | *p*-Coumaric acid isoprenyl ester (isomer) | 2.00 | 3.49 | 0.50 | 1.64 |
| **24** | 30.3 – 31.4 | 295, 325 | 295 | 178, 134, 251, 211 | Caffeic acid cinnamyl ester | 0.58 | 0.53 | 0.39 | 1.34 |
| **25** | 31.5 – 31.8 | 268, 305b | 297<br>403 | 179, 161, 151, 135<br>- | Caffeic acid derivative<br>unknown | 0.29 | - | - | - |
| **26** | 32.3 – 32.8 | 293 | 327 | 253, 271 | Pinobanksin-3-O-propionate | 0.32 | - | - | - |
| **27** | 31.6 – 34.4 | 340 | 269 | 254, 251, 236, 165 | 3-Hydroxy-5-methoxy flavanone | 0.65 | 1.78 | 0.25 | 1.04 |
| **28** | 35.7 – 35.9 | 268, 309b | 387<br>501 | 281, 267, 255, 293<br>457, 439, 247 | unknown<br>unknown | 2.09 | - | - | - |
| **29** | 34.3 – 36.5 | 290 | 417 | - | Methylated Pinobanksin-3-*O*-phenylpropionate | 0.93 | **19.03** | 0.16 | 2.52 |
| **30** | 35.5 – 39.4 | 293 | 341 | 253 | Pinobanksin-3-*O*-butyrate or isobutyrate | 0.48 | **8.11** | 0.04 | 0.95 |
| **31** | 38.5 – 40.3 | 269, 307, 343 | 565 | 283, 269, 281, 417, 455 | *p*-Coumaric acid-4-hydroxyphenylethyl ester dimer | 0.87 | - | - | 1.09 |
| **32** | 38.1 – 41.8 | 292 | 355 | 253, 271 | Pinobanksin-3-*O*-pentenoate or 2-methylbutyrate | 0.89 | 3.24 | 0.21 | 0.97 |
| **33** | 39.6 – 44.8 | 297, 320b | 315 | 179, 135 | Caffeic acid derivative | 0.98 | 2.82 | 0.24 | 0.53 |
| **34** | 41.4 – 41.7 | 280 | 293 | 185, 197, 275, 249 | *p*-Methoxy-cinnamic acid cinnamyl ester | 0.85 | - | - | - |
| **35** | 44.5 – 44.8 | 310 | 473 | - | *p*-Coumaric acid derivative | 0.85 | - | - | - |

## 5.6. Author contributions

Conception and design of the work: A.S.F., M.C., O.P., R.O., F.B., C.A.-A.; experimental work: A.S.F.; supervision of the experimental work: M.C., O.P., F.B., C.A.-A.; compound fractioning and isolation and data interpretation: A.S.F., V.S.; UPLC-DAD-ESI/$MS^n$ analysis of propolis subfractions and data interpretation: A.S.F., S.M.C.; NMR analysis and data interpretation: M.C., F.P.; drafting the manuscript: A.S.F.; revision and approval of the manuscript: A.S.F.; M.C., O.P., V.S., F.P., R.O., F.B., C.A.-A.; project supervision: C.A.-A. All authors have read and agreed to the published version of the manuscript.

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# Chapter 6

## Propolis antibacterial and antioxidant synergisms with gentamicin and honey

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## Abstract

Assess the antibacterial and antioxidant effects of ethanol extracts of Portuguese propolis samples when combined with gentamicin, a widely used aminoglycoside antibiotic, or with honey, collected from the same apiary as propolis. Using the agar dilution method and DPPH free radical scavenging assay, antimicrobial and antioxidant synergistic effects between propolis and gentamicin or honey were assessed. Synergism between propolis and gentamicin was observed for all the tested extracts and against all the susceptibility indicator bacteria, with particular interest against the Methicillin Resistant *Staphylococcus aureus* (MRSA) with a threefold decrease of the gentamicin MIC if mixed with 25 μg $ml^{-1}$ propolis. Likely to propolis and gentamicin, mixtures of sub-MIC concentrations of propolis and honey enhanced the antibacterial action of each individual natural product against the majority of the strains. However, propolis antioxidant capacity decreased along with higher honey content in the mixture. Propolis has a strong synergistic effect when combined with gentamicin, allowing the reduction of the therapeutic dose of this drug. Propolis and honey mixtures also display a stronger antibacterial effect than the activity exhibited by each sample when tested individually, whereas the high antioxidant capacity of propolis seems to be not affected when in combination with honey. When mixed with honey, propolis antioxidant potential is maintained, or just slightly reduced up to 1:1 dilution, and show synergistic antibacterial effects, allowing to optimize the use of this usually scarce natural resource. Also, considering the antibiotic resistance problem, natural beehive products, alone or in the mixture, are promising alternatives to retard the outbreak of microbial resistance.

**Keywords:** Multiresistant bacteria, propolis, honey, gentamicin, antioxidant, synergism.

## 6.1. Introduction

Microbial antibiotic resistance has become a significant major health threat worldwide in recent years. Several factors contribute to this problem, such as the overuse of antibiotics, an inappropriate prescription along with the patients´ misuse, their extensive use in farming practices, as well as the lack of new antibiotics (Ventola, 2015). Data from more than forty countries all over the world have been reporting *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Salmonella* spp. as the most commonly resistant bacteria (WHO, 2018). These and other bacteria are developing new mechanisms of antibiotic resistance, and so they have been spreading around the world, contributing to the escalation of this threat (Mayor, 2018; WHO, 2018). O'Neill (2014) estimated that a continued increase in resistance will result in 10 million premature deaths every year, over the next 30 years. And if microbial drug resistance is not at least attenuated,

the world gross domestic product will decrease between 2 and 3.5 %, causing an economic loss between 60 and 100 trillion USD by 2050 (O'Neill, 2014). Consequently, strategies to overcome microbial resistance as well as new antimicrobial drugs are urgently needed.

Interest in natural products has been increasing due to their spectra of bioactivities that can be applied in food, cosmetic and pharmaceutical industries, in order to replace some commonly used synthetic compounds of potential deleterious side effects. Alone or combined with other antimicrobial agents, natural products are of clinical interest mostly because of their potential for the development of new drugs, both to retard the outbreak of bacterial resistance and to decrease its impacts. Being mixtures of several compounds, natural products are less likely to trigger microbial resistance. Also, their use can contribute to decrease antibiotic overuse by reducing the drug dosage. Propolis, a natural product with promising antibiotic activity, has been attracting an increasing interest for application in antimicrobial therapies and for the development of alternative drugs. Propolis is a complex mixture composed of resinous and balsamic material from branches, flowers and buds, pollen, and exudates of trees collected by honeybees and mixed with salivary enzymes, waxes and other compounds resulting from the insects´ metabolism (Fokt *et al.*, 2010). The chemical composition and bioactivities of this natural product vary with several factors, such as botanic sources and climate characteristics (Falcão *et al.*, 2010), season and technique of harvest (Sales *et al.*, 2006; Tomazzoli *et al.*, 2015), and bee species (Pereira *et al.*, 2002). According to the plant species source, different types of propolis are currently recognized, being characterized by a set of chemical constituents that are responsible for valuable properties (Bankova, 2005; Silva-Carvalho *et al.*, 2015), namely antimicrobial, antioxidant, anti-inflammatory or anti-tumor, among other bioactivities (Falcão *et al.*, 2010; Silva-Carvalho *et al.*, 2015). Although several authors (Kapare *et al.*, 2017; Sadhana *et al.*, 2017) agree with the need to standardize propolis sources in terms of chemical and bioactive profiles, in order to legitimate its use, the extremely valuable bioactivities of this natural product still attract worldwide scientific community and industry for research and innovation. One example is that all propolis samples exhibit some kind of antibacterial activity, although not against all bacteria, which exalts the potential of propolis as a source of alternatives to some of the current commercial drugs. Antimicrobial synergism is also a hot topic for scientists and clinicians as combinations of antimicrobials can be more effective in the treatment of polymicrobial infections, in preventing the prevalence of resistant microorganisms and in reducing a possible dose-related toxicity to humans (Mehta *et al.*, 2014). Several authors have reported propolis synergisms with different types of antibiotics like the ones belonging to the aminoglycoside class that are inhibitors of protein synthesis (**Table 6.1**; Scheller *et al.*, 1999; Stepanović *et al.*, 2003; Fernandes-

**Table 6.1.** Interactions of propolis, from different geographic origins, with aminoglycoside antibiotics.

| Propolis origin | Extraction solvent | Method | Antibiotics | Interaction | References |
|---|---|---|---|---|---|
| Europe | | | | | |
| Poland | Ethanol 95 % | broth dilution<br>disk diffusion | streptomycin<br>tobramycin | synergism | Scheller *et al.*, 1999<br>Wojtyczka *et al.*, 2013 |
| | Ethanol 70 % | broth microdilution | amikacin<br>kanamycin<br>gentamicin | | Grecka *et al.*, 2019 |
| Serbia | Ethanol 95 % | agar dilution/ disc diffusion | amikacin | no synergism | Stepanović *et al.*, 2003 |
| Italy | Ethanol 70 % | agar dilution | gentamicin<br>streptomycin | synergism | Scazzocchio *et al.*, 2006 |
| America | | | | | |
| Brazil | Ethanol 70 % | gradient diffusion | netilmicin<br>gentamicin | synergism | Fernandes Júnior *et al.*, 2005 |
| | Ethanol 54 %<br>Ethanol 100 % | broth microdilution | gentamicin | synergism | Neto *et al.*, 2017<br>de Lima Silva *et al.*, 2015<br>Surek *et al.*, 2021 |
| Asia | | | | | |
| Iraq | Ethanol 100 % | disc agar diffusion | gentamicin | no synergism | AL-safi, 2013 |
| Nepal | Ethanol 70 % | agar dilution | gentamicin<br>amikacin | synergism | Okińczyc *et al.*, 2020 |
| Africa | | | | | |
| Tanzania | Ethanol 100 % | broth microdilution | gentamicin | synergism | Runyoro *et al.*, 2017 |

Júnior *et al.*, 2005; Scazzocchio *et al.*, 2006; AL-safi, 2013; Wojtyczka *et al.*, 2013; de Lima Silva *et al.*, 2015; Neto *et al.*, 2017; Runyoro *et al.*, 2017; Grecka *et al.*, 2019; Okińczyc *et al.*, 2020; Surek *et al.*, 2021), showing propolis potential to be used as adjuvant in antibiotic therapy. However, to the best of our knowledge, such synergisms were never reported for Portuguese propolis. In addition to the standardized harvesting method as well as the constancy of the vegetation found in the region, the beehive products used in this work are of high quality, reliability and value due to a production in a certified biological mode, thus, without any contaminant from pesticides that could interfere with the results. Hereupon, the aim of this work was to investigate possible synergistic effects between Portuguese propolis with other antimicrobial agents. Such antimicrobial agents are either of synthetic or natural origin, respectively: gentamicin, a well-known broad-spectrum antibiotic that inhibits bacterial protein synthesis by irreversibly binding to the 30 S subunit of the ribosome (Morgan, 2014); and honey, a natural mixture produced by honeybees from the nectar collected from flowers and/ or other parts of plants (Montenegro and Mejías, 2013; Jantakee and Tragoolpua, 2015).

## 6.2. Materials and Methods

### 6.2.1. Propolis and honey samples and preparation of extracts

Propolis and honey samples used in this work were harvested from an apiary located near the Cávado River, in Gerês, Portugal (41°45'41.62'' N; 7°58'03.34'' W), a protected area of the Peneda-Gerês National Park and kindly provided by the beekeeper.

Propolis samples, named Gerês (G), were collected in different years – from 2011 (G11) to 2014 (G14) and 2018 (G18) – and extracted with ethanol as previously described (Freitas *et al.*, 2019), giving the ethanol extracts (EEs) - G11.EE, G12.EE, G13.EE, G14.EE and G18.EE. The honey sample, harvested in 2018 (H18), was diluted in water to a stock solution of 50 % (v/v) to be used in the following described assays.

### 6.2.2. Bacterial strains, media and growth conditions

Five Gram-positive bacteria - *Bacillus subtilis* 48886, *Bacillus cereus* ATCC 7064, *Bacillus megaterium* 932, (Methicillin Sensitive) *Staphylococcus aureus* ATCC 6538 (MSSA) and Methicillin Resistant *Staphylococcus aureus* M746665 (MRSA) - and the Gram-negative bacterium *Escherichia coli* CECT 423, all from the microbial collection of the Department of Biology of the University of Minho, were used as indicator strains.

Bacteria were cultured in LB broth (Luria-Bertani - 0.5 % w/v yeast extract, 1 % w/v tryptone, 1 % w/v NaCl) or in solid LB medium (LBA - LB recipe with 2 % w/v agar). Growth was performed at 37 °C, at 200 rpm and monitored by optical density at 600 nm ($OD_{600}$). Overnight cultures diluted with fresh LB medium to an $OD_{600}$ = 0.1 and incubated under the same conditions until $OD_{600}$ between 0.4 - 0.6 (mid-exponential phase) were used for the experiments.

### 6.2.3. Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of G.EEs, gentamicin and H18 were determined against the six bacterial strains using the agar dilution method (Hanson and Martin, 1978). Briefly, 5 µL drops of exponentially growing bacterial cultures were transferred onto LBA plates supplemented with different concentrations of each of the five G.EEs (10, 50, 100, 200, 500, 750, 1000, 1250, 1500, 1750 or 2000 µg $ml^{-1}$), with different concentrations of gentamicin (0.01, 0.05, 0.25, 0.5, 0.75, 1, 1.25 or 1.5 µg $ml^{-1}$) or with different concentrations of H18 (from 2.5 up to 40 %, with increments of 2.5 %).

Antibacterial interactions between G.EEs and gentamicin were assessed on LBA containing mixtures of sub-MIC concentrations of each of the succeeding G.EEs - G11.EE, G12.EE, G13.EE and G14.EE - and gentamicin as follows: 25 µg $ml^{-1}$ of each G.EE with 0.01 or 0.05 µg $ml^{-1}$ gentamicin for *B. megaterium*; 25 µg $ml^{-1}$ of each G.EE with 0.01, 0.05, 0.5, 0.75 or 1 µg $ml^{-1}$ gentamicin for *B. subtilis*, *B. cereus* and MSSA; and 2000 µg $ml^{-1}$ of each G.EE with 1.5 µg $ml^{-1}$ gentamicin was used for MRSA and *E. coli*.

Potential antibacterial interactions between G18.EE and H18 were assessed on LBA plates too but containing mixtures of both samples at sub-MIC concentrations as follows: 5, 10, 50, 100 or 200 µg $ml^{-1}$ G18.EE with 5 %, 10 % and 15 % H18 for all the bacterial strains.

Controls were prepared with LBA containing the solvents used in the same volumes as the samples. Plates were incubated at 37 °C for 24 h. MIC values were determined as the lowest concentrations for which no bacterial growth was detected.

### 6.2.4. *In vitro* evaluation of the antioxidant potential

For *in vitro* evaluation of the antioxidant potential of propolis and honey samples, independently or in mixture, the DPPH· (2,2-diphenyl-1-picryl-hydrazyl) method was used as previously described (Freitas *et al.*, 2019). In brief, G18.EE, H18 or a mixture of both samples at the same stock concentration but in different proportions - 80:20, 50:50 or 20:80 v/v - was mixed with 0.004 % (w/v) DPPH· (1:2), followed by 20 min incubation at room temperature, in the dark. Identical volume of the

solvent was prepared with DPPH· to be used as control. Absorbance of the reaction was measured at 517 nm and results were expressed in $EC_{50}$ values (μg $ml^{-1}$) obtained by interpolation from linear regression analysis of the percentage decrease in absorbance with respect to control values.

### 6.2.5. Statistical analysis

Unless otherwise stated, experiments were performed in triplicate, repeated at least three times independently and results are presented as mean ± standard deviation or as one representative replica. One-way analysis of variance (ANOVA) was used to test treatment effect and post-hoc Tukey test for multiple comparisons between mean values after confirmation of the normal distribution of the variables by Shapiro-Wilk test and the homoscedasticity of the variances by the Bartlett's test. Differences with $p \leq 0.05$ were considered statistically significant. Same letters mean no statistical differences between mean values.

## 6.3. Results

### 6.3.1. Antibacterial interactions between G.EEs and gentamicin

The effects of gentamicin alone or combined with G11.EE, G12.EE, G13.EE or G14.EE were evaluated to assess if propolis can potentiate the action of this antimicrobial drug (**Table 6.2**). *B. megaterium* was the most susceptible strain to the antibiotic (MIC = 0.25 μg $ml^{-1}$) followed by *B. subtilis* and *B. cereus* (MIC = 1.25 μg $ml^{-1}$), MSSA (MIC = 1.5 μg $ml^{-1}$) and by *E. coli* and MRSA (MIC > 1.5 μg $ml^{-1}$), the least susceptible strains to gentamicin. All the G.EEs displayed the same MIC against each of the tested strains (data not shown), which is in line with the reported constancy of the antibacterial activity of propolis from Gerês over the years (Freitas *et al.*, 2019). Yet, as expected, different bacterial susceptibilities were observed to propolis extracts (**Table 6.2**).

To assess possible synergistic effects of propolis from Gerês and gentamicin, a sub-MIC concentration of 2000 μg $ml^{-1}$ of the G.EEs was selected for MRSA and *E. coli* whereas 25 μg $ml^{-1}$ was the G.EEs sub-MIC concentration chosen for the remaining bacterial strains. Similarly, sub-MIC concentrations of gentamicin were used in these assays. Nevertheless, all the strains were tested for all the selected concentration combinations in order to search for other interactions. All the combinations of each G.EE and gentamicin tested displayed the same MIC profile against the bacterial panel when comparing the four different G.EEs (data not shown), supporting once again the antibacterial fingerprint constancy of propolis samples from Gerês.

**Table 6.2.** MIC values (μg ml$^{-1}$) obtained for G.EEs and for gentamicin against the panel of susceptibility indicator bacterial strains. Strains in mid-exponential phase were transferred to LBA plates supplemented with increasing concentrations of each G.EE (G11.EE, G12.EE, G13.EE or G14.EE) or gentamicin. After 24 h incubation at 37 $^{o}$C, plates were observed for the presence of growth. MIC values were determined upon observation of the lowest concentrations for which no growth was detected. As the results were the same between replicates and for the four studied G.EEs, only one column is depicted.

| Strains | MIC (μg ml$^{-1}$) | |
|---|---|---|
| | Gentamicin | G.EEs |
| **Gram-positive** | | |
| *Bacillus megaterium* | 0.25 | 50 |
| *Bacillus subtilis* | 1.25 | 50 |
| *Bacillus cereus* | 1.25 | 50 |
| MSSA[a] | 1.5 | 200 |
| MRSA[b] | > 1.5 | > 2000 |
| **Gram-negative** | | |
| *Escherichia coli* | > 1.5 | > 2000 |

[a]MSSA – Methicillin Sensitive *Staphylococcus aureus*; [b]MRSA – Methicillin Resistant *Staphylococcus aureus*.

As depicted in **Table 6.3**, a synergistic effect was observed for the three *Bacillus* species tested: the MIC of gentamicin combined with each G.EE (25 μg ml$^{-1}$) decreased 96 % relatively to the MIC of the antibiotic when used alone (from 0.25 μg ml$^{-1}$ to 0.01 μg ml$^{-1}$ for *B. megaterium*, and from 1.25 μg ml$^{-1}$ to 0.05 μg ml$^{-1}$ against *B. subtilis* and *B. cereus*). For *E. coli* and for the *Staph. aureus* strains (MSSA and MRSA), the MIC of gentamicin combined with each G.EE (25 μg ml$^{-1}$) decreased at least up to 70 % (from 1.5 μg ml$^{-1}$ to 0.5 μg ml$^{-1}$ for MSSA and from > 1.5 μg ml$^{-1}$ to 0.5 μg ml$^{-1}$ in the case of *E. coli* and MRSA).

**Table 6.3.** MIC values (μg ml$^{-1}$) obtained for the combined effect of sub-MIC concentrations of G.EEs and of gentamicin against the panel of susceptibility indicator bacterial strains. Strains in mid-exponential phase were transferred to LBA plates supplemented with G11.EE, G12.EE, G13.EE or G14.EE and gentamicin (for details see Materials and Methods). After 24 h incubation at 37 $^{o}$C, plates were observed for the presence of growth. MIC values were determined upon observation of the lowest concentrations for which no growth was detected. As the results were the same between replicates and for the four studied G.EEs, only one set is depicted. MIC values of each pair agent-bacteria (Table 1) were herein included for handiness.

| Strains | Agent | MIC (μg ml$^{-1}$) | |
|---|---|---|---|
| **Gram-positive** | | **Alone** | **Combination** |
| *Bacillus megaterium* | G.EEs | 50 | 25 |
| | Gentamicin | 0.25 | 0.01 |
| *Bacillus subtilis* | G.EEs | 50 | 25 |
| | Gentamicin | 1.25 | 0.05 |
| *Bacillus cereus* | G.EEs | 50 | 25 |
| | Gentamicin | 1.25 | 0.05 |
| MSSA[a] | G.EEs | 200 | 25 |
| | Gentamicin | 1.5 | 0.5 |
| MRSA[b] | G.EEs | >2000 | 25 |
| | Gentamicin | >1.5 | 0.5 |
| **Gram-negative** | | | |
| *Escherichia coli* | G.EEs | >2000 | 25 |
| | Gentamicin | >1.5 | 0.5 |

[a]MSSA – Methicillin Sensitive *Staphylococcus aureus*; [b]MRSA – Methicillin Resistant *Staphylococcus aureus*.

Although the MIC of gentamicin has decreased to 0.5 μg ml$^{-1}$ against *E. coli* or MRSA when combined with 25 μg ml$^{-1}$ of any of the G.EEs, both bacteria were able to proliferate in the presence of 1.5 μg ml$^{-1}$ gentamicin combined with 2000 μg ml$^{-1}$ of each G.EE (data not shown). These are both sub-MIC concentrations (**Table 6.2**), but enormously higher than the concentrations tested in the lower range and that have resulted in the absence of bacterial growth.

### 6.3.2. Antibacterial interactions between propolis and honey

Antibacterial activity of G18.EE and H18 was evaluated by determining the MIC values by the agar dilution method against the same panel of bacterial strains (**Table 6.4**). Gram-positive bacteria of the genus *Bacillus* were the most susceptible strains to G18.EE (MIC = 50 μg ml$^{-1}$), followed by MSSA (MIC = 500 μg ml$^{-1}$) and by MRSA and *E. coli* (MIC > 2000 μg ml$^{-1}$), the least susceptible strains to G18.EE, as for G11.EE - G14.EE. Regarding H18 antibacterial activity, *B. cereus* was the most susceptible strain (MIC = 10 %) followed by *B. subtilis* (MIC = 12.5 %) and *B. megaterium* (MIC = 15 %). MSSA, MRSA and *E. coli* were the least susceptible strains to H18 (MIC = 17.5 %).

**Table 6.4.** MIC values obtained for G18.EE (μg ml$^{-1}$) and for H18 (%) against the panel of susceptibility indicator bacterial strains. Strains in mid-exponential phase were transferred to LBA plates supplemented with increasing concentrations of G18.EE or H18. After 24 h incubation at 37 $^{o}$C, plates were observed for the presence of growth. MIC values were determined upon observation of the lowest concentrations of propolis or honey for which no growth was detected.

| Strains | MIC | |
|---|---|---|
| | G18.EE (μg ml$^{-1}$) | H18 (%) |
| **Gram-positive** | | |
| *Bacillus megaterium* | 50 | 15 |
| *Bacillus subtilis* | 50 | 12.5 |
| *Bacillus cereus* | 50 | 10 |
| MSSA[a] | 200 | 17.5 |
| MRSA[b] | > 2000 | 17.5 |
| **Gram-negative** | | |
| *Escherichia coli* | > 2000 | 17.5 |

[a]MSSA – Methicillin Sensitive *Staphylococcus aureus*; [b]MRSA – Methicillin Resistant *Staphylococcus aureus*.

To assess if the interactions between G18.EE and H18 can potentiate the antibacterial action of each individual natural product, sub-MIC concentrations of both samples were mixed, and the resulting mixtures were tested against the same panel of bacterial indicator strains (**Table 6.5**). A synergistic effect was observed for all the tested bacteria with the exception of *B. cereus*. For *B. megaterium* (G18.EE MIC = 50 μg ml$^{-1}$; H18 MIC = 15 %) and *B. subtilis* (G18.EE MIC = 50 μg ml$^{-1}$; H18 MIC = 12.5

%), it was possible to inhibit bacterial growth with only 5 μg $ml^{-1}$ of G18.EE in combination with 5 % and 10 % of H18, respectively. Also, for MRSA and *E. coli* (with a G18.EE MIC > 2000 μg $ml^{-1}$ and a H18 MIC = 17.5 % against both species) no bacterial growth was detected with 5 μg $ml^{-1}$ of G18.EE in combination with 10 % and 15 % of H18, respectively. Using 100 μg $ml^{-1}$ of G18.EE in combination with 10 % of H18 it was possible to inhibit MSSA (G18.EE MIC = 200 μg $ml^{-1}$; H18 MIC = 17.5 %) growth. In short, except for *B. cereus*, all the tested mixtures of sub-MIC concentrations of G18.EE and H18 potentiated the antibacterial action of each individual sample, suggesting that mixtures of propolis and honey are promising alternatives to antibiotics, valuing more than one beehive product.

**Table 6.5.** MIC values obtained for the combined effect of sub-MIC concentrations of G18.EE (μg $ml^{-1}$) and H18 (%) against the panel of susceptibility indicator bacterial strains. Strains in mid-exponential phase were transferred to LBA plates supplemented with G18.EE and H18 (see Materials and Methods for details). After 24 h incubation at 37 $^{o}$C, plates were observed for the presence of growth. MIC values were determined upon observation of the lowest concentrations for which no growth was detected.

| Strains | Agent | MIC | |
|---|---|---|---|
| **Gram-positive** | | **Alone** | **Combination** |
| *Bacillus megaterium* | G18.EE | 50 | 5 |
| | H18 | 15 | 5 |
| *Bacillus subtilis* | G18.EE | 50 | 5 |
| | H18 | 12.5 | 10 |
| *Bacillus cereus* | G18.EE | 50 | 50 |
| | H18 | 10 | 5 |
| MSSA[a] | G18.EE | 200 | 100 |
| | H18 | 17.5 | 10 |
| MRSA[b] | G18.EE | >2000 | 5 |
| | H18 | 17.5 | 10 |
| **Gram-negative** | | | |
| *Escherichia coli* | G18.EE | >2000 | 5 |
| | H18 | 17.5 | 15 |

[a]MSSA – Methicillin Sensitive *Staphylococcus aureus*; [b]MRSA – Methicillin Resistant *Staphylococcus aureus*.

### 6.3.3. Antioxidant interactions between propolis and honey

In this work, the antioxidant potential of different mixtures of G18.EE and H18 was evaluated by the DPPH· assay with determination of the respective $EC_{50}$ (**Table 6.6**). G18.EE exhibits similar anti-radical activity ($EC_{50}$ = 14.11±0.47 μg $ml^{-1}$) to the four G.EEs previously described (14.41±0.56 ≤ $EC_{50}$ ≤ 25.24±2.45 μg $ml^{-1}$; Freitas *et al.*, 2019). When mixed with honey at different proportions, G18.EE antioxidant capacity was increasingly diluted in direct dependence of H18 proportion. However, this decrease in activity was not statistically significant with at least 80 % of G18.EE in the mixture, suggesting that reducing the percentage of G18.EE in the mixtures down to a minimum of 80 % do not affect the antioxidant potential exhibited by G18.EE alone.

**Table 6.6.** DPPH free-radical-scavenging capacity of G18.EE, H18 and mixtures of both (marked with a slash between the designations of the products) was measured *in vitro* and expressed as the mean of $EC_{50}$ values (μg $ml^{-1}$) and respective standard deviation (SD). One-way ANOVA was performed, followed by Tukey test to assess significance. Same letters mean no statistically significant differences between mean values.

| Samples | $EC_{50}$ (μg $ml^{-1}$) |
|---|---|
| **H18** | 5171.06±28.28 |
| **G18** | 12.40±0.43$^{a}$ |
| **G18/ H18 (4:1)** | 14.11±0.47$^{a}$ |
| **G18/ H18 (1:1)** | 16.56±1.27$^{b}$ |
| **G18/ H18 (1:4)** | 46.13±0.15$^{c}$ |

## 6.4. Discussion

### 6.4.1. Antibacterial synergisms between propolis and antibiotics

Synergistic effects between propolis and aminoglycoside antibiotics like gentamicin (**Table 6.7**), but also streptomycin, amikacin, netilmicin, tobramycin and kanamycin (**Table S6.1 Supplementary material**) have been described against a large spectra of both Gram-positive and Gram-negative bacteria, allowing reducing the dose of therapeutic drugs (Scheller *et al.*, 1999; Stepanović *et al.*, 2003; Fernandes Júnior *et al.*, 2005; Scazzocchio *et al.*, 2006; AL-safi, 2013; Wojtyczka *et al.*, 2013; de Lima Silva *et al.*, 2015; Neto *et al.*, 2017; Runyoro *et al.*, 2017; Grecka *et al.*, 2019; Okińczyc *et al.*, 2020; Surek *et al.*, 2021). For instance, tuberculosis bacilli strains resistant to some antibiotics (streptomycin, rifampicin, isoniazide and ethambutol) lost part of their resistance when treated simultaneously with an EE of propolis from Poland (Scheller *et al.*, 1999). Synergistic effect of Brazilian propolis and

**Table 6.7.** Synergistic activity of *Apis mellifera* L. propolis in combination with gentamicin.

| Strains | Inhibitory effect alone | | Inhibitory effect combined | | | References |
|---|---|---|---|---|---|---|
| | Propolis (µg ml$^{-1}$) | Gentamicin (µg ml$^{-1}$) | Propolis (µg ml$^{-1}$) | Gentamicin (µg ml$^{-1}$) | Gentamicin decrease (%) | |
| *E. coli* | 1670 | 0.026 | 0.105 | 0.000025 | 99.9 | Runyoro *et al.*, 2017 |
| *Kl. oxytoca* | 1670 | 0.026 | 0.105 | 0.000025 | 99.9 | |
| MSSA[a] | | | | | | |
| 25 isolates | 0.8 % (v/v) | 0.023 - 256 | 0.2 % (v/v) | 0.047 - 256 | * | Fernandes Júnior *et al.*, 2005 |
| 35 isolates | 620 - 2500 | 0.1 - >200 | sub-MIC | 0.1 - 0.19 | * | Scazzocchio *et al.*, 2006 |
| 42 isolates | 620 - 2500 | 0.05 - 200 | sub-MIC | 0.05 - 50 | * | |
| 70 isolates | 68.7 | 0.2258 | 34.35 | < 0.00695 | >97 | de Lima Silva *et al.*, 2015 |
| 10 | 101.6 | ≈10 | 12.7 | <5 | >50 | Neto *et al.*, 2017 |
| ATCC 6538 | 512 | ≈310 | 64 | <170 | >45 | |
| ATCC 25923; $MLS_B$ P92; ATCC 33591 | 128; 250; 250 | 0.125; 1.0; 3.3 | 32; 125; 62.5 | 0.0312; 0.25; 0.83 | 75 | Grecka *et al.*, 2019; Okińczyc *et al.*, 2020; Surek *et al.*, 2021 |
| ATCC 25923; $MLS_B$ C126 | 250 | 0.5; 1.0 | 125 | 0.25 | 50 | Okińczyc *et al.*, 2020 |
| MRSA[b] | | | | | | |
| C106; P53; B825 | 250 | 0.5; 1.0; 256 | 125 | 0.25; 0.25; 32 | 50; 75; 87.5 | Okińczyc *et al.*, 2020 |
| *Proteus vulgaris* | 3340 | 0.026 | 0.105 | 0.000025 | 99.9 | Runyoro *et al.*, 2017 |
| *Pseudomonas aeruginosa* 03; 24 | 512 | ≈30 | 64 | <20 | ≈33 | Neto *et al.*, 2017 |
| *Staph.* sp. | | | | | | |
| 18 *haemolyticus;* 10 *warnerii;* 7 *hominis;* 4 *capitis;* 3 *auricularis;* 63 *epidermidis* | 620 - 2500 | 0.05 - 200 | sub-MIC | 0.05 - 50 | * | Scazzocchio *et al.*, 2006 |
| *Strep.* sp. | | | | | | |
| 59 *faecalis;* 30 *viridans;* 19 *pneumoniae* | 310 - 2500 | 0.05 - 25 | sub-MIC | 0.05 - 1.56 | * | Scazzocchio *et al.*, 2006 |

[a]MSSA – Methicillin Sensitive *Staphylococcus aureus*; [b]MRSA – Methicillin Resistant *Staphylococcus aureus;* *Values not indexed to each strain individually.

antimicrobial drugs was also found against MSSA (Fernandes Júnior *et al.*, 2005), especially when propolis was combined with agents that interfere with protein synthesis, as is the case of gentamicin. More recently, different combinations of EE of propolis from Brazil and gentamicin showed to decrease the drug dose between 45 and 97 % against different MSSA strains, with higher reductions being observed for propolis extracts with high ethanol content (de Lima Silva *et al.*, 2015; Neto *et al.*, 2017; Surek *et al.*, 2021). Similarly to our work, an European propolis (Poland) combined with gentamicin was able to reduce the antibiotic MIC by 75 % against MSSA (Grecka *et al.*, 2019). Okińczyc *et al.* (2020) tested an EE of propolis from Asia (Nepal), by the same method used in this work, against three MRSA strains (C106, P53 and B825), alone (MIC = 250 μg $ml^{-1}$) and in combination with gentamicin (MIC = 0.5, 1.0 and 256 μg $ml^{-1}$, respectively) and reported that a sub-MIC concentration of propolis (125 μg $ml^{-1}$) decreased by 50 %, 75 % and 87.5 %, respectively, the required amount of the antibiotic when used alone. An EE of propolis from Tanzania (Africa) showed synergistic effect with gentamicin against *E. coli*, allowing a reduction of the drug by 99.9 %, a higher decrease when compared to our work, nevertheless close to that obtained for *Bacillus* strains (96 %; Runyoro *et al.*, 2017).

Phenolics, such as cinnamic acid, and ester derivatives including caffeic acid phenyl ester (CAPE); flavonoids, including quercetin, naringenin (Santos *et al.*, 2002; Boisard *et al.*, 2015), galangin, pinostrobin, pinocembrin, ferulic acid, hydroquinones; and terpenic acids like isopimaric, abietic and dehydroabietic acid (Patel *et al.*, 2014) have been linked to propolis antimicrobial activity. Antibacterial activity of propolis from Gerês can be explained by the presence of some of those compounds, such as pinocembrin, galangin, ferulic acid, quercetin, CAPE and caffeic acid cinnamyl ester, among others, identified in all the G.EEs (Freitas *et al.*, 2019). Although propolis antibacterial mechanism of action is not yet clearly understood, some studies suggest that propolis is able to uncouple the energy-transducing activity of the plasma membrane and to inhibit protein synthesis, RNA-polymerase, motility and proliferation, by preventing cell division (Takaisi *et al.*, 1994; Mirzoeva *et al.*, 1997; Uzel *et al.*, 2005). Furthermore, flavonoids such as galangin, kaempferide, kaempferol, isorhamnetin, apigenin, acacetin, pinocembrin and chrysin, also identified in G.EEs (Freitas *et al.*, 2019), are reported as glycosyltransferase (GTF) inhibitors in bacteria (Koo *et al.*, 2002). Thus, the synergistic effect between propolis and gentamicin can be related either to their ability to inhibit bacterial protein synthesis or to a combination of multiple effects, with compounds possibly acting at different cellular targets and/ or with different mechanisms, complementing the antibacterial action of each other in a highly efficient combination.

Propolis is a very complex mixture where compounds could exhibit both synergistic and/ or antagonistic effects, depending on the concentration and thus having different effects on cell viability, protecting bacteria from antibiotics effect or somehow nullifying their action. Interactions resulting in antagonism were found between a propolis sample from Poland and the antibiotic ethambutol against one of the several mycobacteria strains tested (Scheller *et al.*, 1999). Similarly, Chamandi *et al.* (2015) reported an antagonism between Lebanon propolis and the antibiotics oxacillin, clindamycin and gentamicin against MRSA. The striking loss of synergy we detected at higher concentrations of both propolis and gentamicin can possibly be attributed to the ratio increase between the extract components - now at higher concentrations, particularly those of propolis - that somehow overruled the antimicrobial effect registered at lower concentrations, or by positive effects of propolis components that might contribute to microbial protection allowing growth. To the best of our knowledge, this is the first evidence of such type of interaction. Although no specific compounds or mechanisms were yet proposed for the interactions between propolis and antibiotics, the presence of microbial growth at combined concentrations that are otherwise expected to cause inhibition is of utmost relevance, not only regarding clinical therapeutics with or combined with natural products, due to drug interaction, but also for new prophylactic/ therapeutic approaches, as boosting/ repairing gut microbiome unbalanced by medication.

Our results suggest that the synergism between propolis from Gerês and gentamicin is strong, allowing a decrease of the antibiotic amount initially required. In addition, there is also constancy in such synergism, with all the four propolis samples from Gerês showing the same effect over gentamicin regardless of the harvesting year.

### 6.4.2. Antibacterial synergisms between propolis and honey

There are several studies reporting the antibacterial activity of honey against a large panel of both Gram-positive and Gram-negative bacteria (Hegazi, 2011; Mohapatra *et al.*, 2011), mostly by the agar well diffusion method (Estevinho *et al.*, 2008). Different types of Thailand honey showed antimicrobial activity against MSSA (MIC = 25 %) and MRSA (12.5 < MIC < 25 %). Still, in the same study, a sample of Manuka honey showed strong antimicrobial activity against MSSA and MRSA (MIC = 6.25 %; Jantakee and Tragoolpua, 2015). Honey samples from the North of Portugal have been object of study (Estevinho *et al.*, 2008, 2011, 2012; Feás *et al.*, 2010; Feás and Estevinho, 2011; Gomes *et al.*, 2011; Soares *et al.* 2017) with samples from Trás-os-Montes showing to possess antimicrobial activity against *B. subtilis* (MIC = 4000 μg $ml^{-1}$), MSSA (MIC = 400 μg $ml^{-1}$), *Staph. lentus* (MIC = 40000 μg $ml^{-1}$), *E. coli*

(MIC = 40000 µg $ml^{-1}$) and *Ps. aeruginosa* (MIC = 40000 µg $ml^{-1}$), the Gram-positive bacteria being more susceptible to honey phenolic compounds extracts than the Gram-negative bacteria (Estevinho *et al.*, 2008).

Although the mechanism of honey antibacterial activity is still to be clearly understood, studies trying to unravel this mechanism are increasing and honey effectiveness against bacterial growth has been associated with several properties (Moussa *et al.*, 2012). Such properties include high osmotic pressure, due to the high sugar content; low water content; gluconic acid, which creates an acidic environment; hydrogen peroxide; phytochemical components; among others (Khan *et al.*, 2007; Brudzynski *et al.*, 2012; Miguel *et al.*, 2017).

Studies made on propolis and honey mixtures show an improvement of antibacterial activity too (Noori *et al.*, 2012; Eslami *et al.*, 2016; Osés *et al.*, 2016; Kowalski and Makarewicz, 2017). According to Noori *et al.* (2012), the MIC value of a Saudi Arabian propolis sample was reduced to nearly a half against *E. coli* and MSSA when 15 % of honey from the same origin was added. Iranian propolis mixtures with sub-MIC concentrations of honey decreased the initial propolis MIC value between 12.5 % and 48.75 % against several bacterial strains such as *Strep. mutans*, *Enterococcus faecalis*, MSSA, MRSA, *Staph. epidermidis* and *E. coli*, for which no propolis MIC value was previously found (> 25000 µg $ml^{-1}$). Although, when mixed with approximately 15 % of honey it was obtained a MIC value of 17,390 µg $ml^{-1}$ (Eslami *et al.*, 2016). Other authors showed that mixtures of propolis and honey are advantageous against *E. coli* and MRSA as displaying larger halo inhibition zones than propolis or honey alone (Osés *et al.*, 2016; Kowalski and Makarewicz, 2017). These authors suggest that using honey in combination with propolis strongly influence the bioactive properties of propolis, namely increasing its antimicrobial activity.

Honey effects on the modulation of bacterial resistance may be related to its chemical composition, a characteristic that it has in common with propolis, both containing a combination of components that may act in a synergistic manner, enabling to compromise the resistance (Carnwath *et al.*, 2014; Al-Nahari *et al.*, 2015). Also, when used in combination with antibiotics, such as oxacillin, honey restored MRSA susceptibility (Jenkins and Cooper, 2012) possibly by interfering with the *mecA* gene encoding penicillin-binding protein (Meng *et al.*, 2006), recognized to be involved in biofilm formation (Hamouda *et al.*, 2018).

### 6.4.3. Antioxidant synergisms between propolis and honey

Besides antibacterial activity, antioxidant capacity has come to be one of the most important factors to evaluate the quality and functional properties of honey (Lianda *et al.*, 2012). This bioactivity has been evaluated by several methods in honey, mainly by the free-radical-scavenging activity (Al-Hindi *et al.*, 2011; Aazza *et al.*, 2013; Jantakee and Tragoolpua, 2015; Attanzio *et al.*, 2016; Juan-Borrás *et al.*, 2016; Tahir *et al.*, 2017).

Regarding antioxidant capacity, our results found no synergistic effect between G18.EE and H18. Also, mixtures where propolis is the main component appear to preserve its antioxidant potential. Other authors studied the antioxidant potential of propolis and honey mixtures showing that the use of these two beehive products in combination improves the ability to scavenge free radicals of each beehive product alone and showing that the higher the percentage of propolis in the mixture, the higher the ability to reduce free radicals in a concentration-dependent manner (Osés *et al.*, 2016; Kowalski and Makarewicz, 2017; Habryka *et al.*, 2020).

The main mechanisms of action for honey antioxidant activity are still not very well defined but, like for antimicrobial activity, it may be related to the presence of phenolic compounds. Antioxidant activity is higher in dark honey ($EC_{50}$ = 27,240 μg $ml^{-1}$) from Trás-os-Montes than in clearer sample ($EC_{50}$ = 68,170 μg $ml^{-1}$), probably due to the higher content in phenolic compounds found in dark honey (Estevinho *et al.*, 2008). The antioxidant activity of 30 honey samples from Saudi Arabia was demonstrated through the DPPH free-radical-scavenging activity (50.78 < $EC_{50}$ < 99.52 %) and a linear correlation was found between the presence of phenolic compounds and the antioxidant capacity, with stronger activities exhibited by the samples with higher content in phenolic compounds (Al-Hindi *et al.*, 2011). Additionally to the antioxidant activity of several Sicilian monofloral honey samples evaluated by the DPPH· scavenging activity assay, the antioxidant activity of honey against perferryl-myoglobin radicals, usually formed in red meat was also demonstrated (Attanzio *et al.*, 2016).

## 6.5. Conclusions

In conclusion, propolis samples used in this work show a consistent antibacterial profile regardless of the harvesting year. Still, results suggest that propolis from Gerês, compared to other studied samples, has stronger synergistic effect when combined with either honey or gentamicin. Such combinations allowed a huge reduction of the antibiotic needs, particularly concerning the “super bug” MRSA, whose infection can lead to other associated problems such as pneumonia, chronic wound infection and even to septic conditions, which can, in some cases, lead to death. This outcome is very

interesting considering the worldwide problem of antibiotic resistance and the need to innovate in antibiotic therapy, in particular, if solutions from natural sources, as propolis and honey, are envisaged. Nevertheless, it is crucial to understand not only the complexity of the interactions and underlying mechanisms but also to test other antibiotics and natural products and, most importantly, other combinations in order to optimize synergism conditions and discard possible combinations that may abolish this property.

## 6.6. Supplementary material

**Table S6.1.** Synergistic activity of *Apis mellifera* L. propolis in combination with streptomycin, amikacin, netilmicin, tobramycin and kanamycin.

| Strains | Treatment | Inhibitory effect alone | | Inhibitory effect combined | | References |
|---|---|---|---|---|---|---|
| | | MIC (µg ml$^{-1}$) | Halo (mm) | MIC (µg ml$^{-1}$) | Halo (mm) | |
| *Mycobacterium tuberculosis* $H_{37}Rv$ 2563 333 | propolis | <1800 | - | synergism | - | Scheller *et al.*, 1999 |
| | streptomycin | <4 | | | | |
| MSSA[a] 25 isolates | propolis | 0.8 % | - | 0.2 % | - | Fernandes Júnior *et al.*, 2005 |
| | netilmicin | 0.5 - 24 | | 0.047 - 12 | | |
| 35 isolates | propolis | 620 - 2500 | - | Sub-inhibitory | - | Scazzocchio *et al.*, 2006 |
| | streptomycin | 1.56 - >200 | | 0.05 - 0.39 | | |
| $MLS_B$ C126 $MLS_B$ P92 | propolis | 250 | - | 125 | - | Okińczyc *et al.*, 2020 |
| | amikacin | 4.0 | | 2.0 | | |
| 42 isolates | propolis | 620 - 2500 | - | Sub-inhibitory | - | Scazzocchio *et al.*, 2006 |
| | streptomycin | 0.78 - 50 | | 0.05 - 3.12 | | |

[a]MSSA – Methicillin Sensitive *Staphylococcus aureus*.

**Table S6.1.** Synergistic activity of *Apis mellifera* L. propolis in combination with streptomycin, amikacin, netilmicin, tobramycin and kanamycin (continued).

| Strains | Treatment | Inhibitory effect alone | | Inhibitory effect combined | | References |
|---|---|---|---|---|---|---|
| | | MIC (μg ml$^{-1}$) | Halo (mm) | MIC (μg ml$^{-1}$) | Halo (mm) | |
| MSSA[a] | | | | | | |
| | propolis | 590 | - | 147.5 | - | Wojtyczka *et al.*, 2013 |
| | tobramycin | - | ≈20 | - | ≈30 | |
| | propolis | 128 | - | 16 | - | Grecka *et al.*, 2019 |
| ATCC 25923 | amikacin | 1 | | 0.25 | | |
| | propolis | 128 | - | 32 | - | |
| | kanamycin | 2 | | 0.5 | | |
| | propolis | 250 | - | 125 | - | Okińczyc *et al.*, 2020 |
| | amikacin | 2.0 | | 0.5 | | |
| *Staph.* sp. | | | | | | |
| 18 *haemolyticus;* 10 *warnerii;* 7 *hominis;* 4 *capitis;* 3 *auricularis;* 63 *epidermidis* | propolis | 620 - 2500 | - | Sub-inhibitory | - | Scazzocchio *et al.*, 2006 |
| | streptomycin | 0.78 - 50 | | 0.05 - 3.12 | | |

[a]MSSA – Methicillin Sensitive *Staphylococcus aureus*.

**Table S6.1.** Synergistic activity of *Apis mellifera* L. propolis in combination with streptomycin, amikacin, netilmicin, tobramycin and kanamycin (continued).

| Strains | Treatment | Inhibitory effect alone | | Inhibitory effect combined | | References |
|---|---|---|---|---|---|---|
| | | MIC (μg ml$^{-1}$) | Halo (mm) | MIC (μg ml$^{-1}$) | Halo (mm) | |
| MRSA[b] | | | | | | |
| C106 P53 | propolis | 250 | - | 125 | - | Okińczyc *et al.*, 2020 |
| | amikacin | 2.0 | | 0.5 | | |
| B825 | propolis | 250 | - | 125 | - | |
| | amikacin | 64 | | 8 | | |
| *Strep.* sp. | | | | | | |
| 59 *faecalis* | propolis | 2500 | - | Sub-inhibitory | - | Scazzocchio *et al.*, 2006 |
| | streptomycin | 25 - >200 | | 3.12 - 50 | | |
| 30 *viridans* 19 *pneumoniae* | propolis | 310 - 2500 | - | Sub-inhibitory | - | |
| | streptomycin | 1.56 - 100 | | 0.05 - 6.25 | | |

[b]MRSA – Methicillin Resistant *Staphylococcus aureus*.

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# Chapter 7

## Biofunctional textiles: antioxidant and antibacterial finishings of cotton with propolis and honey

*The work presented in this chapter has been submitted:*

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## Abstract

The growing activity in the textile industry has been demanding the search for new and innovative technologies to meet consumers' needs regarding more sustainable and ecological processes, with functionality getting more attention. Bee products are known for their wide spectra of properties, including the antioxidant and antibacterial activities. Propolis and honey are the most popular and used since ancient times for the most diverse applications due to their health benefits. With the increasing need for safer and more sustainable practices, the use of natural products for the functional finishing process can be a suitable alternative due to their safety and eco-friendly nature. For that, a biosolution, composed by a mixture of propolis and honey in water, was used to perform the functional finishing of cotton knits, both in the presence and in the absence of potassium alum as a chemical mordant. The fastness strength was also evaluated after three washing cycles. Antioxidant potential of the biosolution, assessed with the *in vitro* ABTS scavenging assay, provided textiles with the capacity to reduce more than 90 % of ABTS radical, regardless mordant presence and even after three washing cycles. Furthermore, biofunctional textiles decreased growth of *Bacillus subtilis*, *Propionibacterium acnes*, *Escherichia coli* and particularly *Staphylococcus aureus* cultures after 24 h incubation with an increase of the antibacterial activity when potassium alum was used. These findings show that bee products are promising and effective alternatives to be used in the textile industry to confer antioxidant and antibacterial properties to cotton textiles.

**Keywords:** Biofunctional textiles, cotton, propolis, honey, antioxidant potential, antibacterial activity, washing fastness.

## 7.1. Introduction

Bee products can be used as food, active products for medicinal and cosmetic purposes or inactive products used as bases (Nesheim *et al.*, 2006). Interest in natural products, such as honey and propolis, has been increasing due to their important role in human health and well-being (Yadav *et al.*, 2015). Honey, considered a natural food of high nutritional value and used in human consumption for its organoleptic and therapeutic characteristics over centuries (Montenegro e Mejías, 2013; Juan-Borrás *et al.*, 2016), as well as propolis, both a building and a defensive material of the beehive (Bankova *et al.*, 2014), are the most popular bee products. Both are commercialized in different parts of the world and recognized as important sources of bioactive compounds with properties for several applications (Moreira *et al.*, 2008; Pasupuleti *et al.*, 2017; Sforcin *et al.*, 2017). By 2028, the Global Honey and

Propolis Markets are expected to reach a value of USD 17.34 billion - with a compound annual growth rate (CAGR) of 8.2 % - and USD 60.9 million - with a CAGR of 4 % -, respectively (Market, 2019; Grand View Research, 2021a).

The growing demand for natural products relies on the health-conscious awareness of consumers towards the potential toxicity of the chemicals used in conventional personal care products and the benefits of the properties, especially the antimicrobial and antioxidant, that natural products are known to own (Montenegro and Mejías, 2013). The global natural and organic cosmetics market is projected to reach USD 54.5 billion by 2027 and is expected to grow at a CAGR of 5.2 % between 2018 and 2027, mostly due to the increasing demand for skin and hair products (Future Market Insights, 2019). In 2021, the global natural skin care products market was rated at USD 6.7 billion, growing at a CAGR of 6.6 % from 2022 to 2030 (Grand View Research, 2021b). Bee products, especially honey and propolis, are known for their effects on skin care, mostly due to their antimicrobial, anti-inflammatory, antiviral, regenerative and antiaging properties (Kurek-Górecka *et al.*, 2020). Skin, as the largest human organ, represents the first protection barrier against environmental aggressions and is mostly covered by textile (Morganti *et al.*, 2019). Biofunctional textiles are fabrics with acquired properties which can act like release systems able to deliver substances such as bioactive principles to the skin, having been subject of interest due to their applicability as biomedical devices for skin treatment or cosmetic (Massella *et al.*, 2019). Chemicals used to impart antibacterial activity to textiles are mostly synthetic and usually associated with some level of toxicity, which can be less effective against bacteria due to its extensive use and consequent development of resistance (Balouiri *et al.*, 2016).

The need for the development of new functional products that meet consumers increasing concerns with health and well-being leads to the quest for new and sustainable technologies by the textile industry with less environmental impact and supporting a circular economy (Shahid *et al.*, 2017). Relying on the growing need for safer and sustainable alternatives in textile functionalization, the aim of this work was to investigate the potential of the beehive products propolis and honey for textile (cotton) functionalization, namely with antioxidant and antibacterial activities. Potassium alum mordant capacity to improve textile' chemical affinity and functionalization resistance to the washing process was assessed as well, given the importance of reuse for sustainability.

## 7.2. Materials and Methods

### 7.2.1. Propolis and honey samples and extracts preparation

Propolis and honey samples used in this work were harvested from an apiary located near the Cávado River, in a protected area of the Peneda-Gerês National Park, in the north of Portugal (41°45'41.62'' N; 7°58'03.34'' W), and kindly provided by the beekeeper. Both propolis and honey samples were collected in 2018 and coded as G18 (Gerês 2018) and H18 (Honey 2018), respectively.

Honey was diluted to a stock solution of 50 % (v/v) in sterile water – **$H18_{50}$.** Propolis was extracted with absolute ethanol as previously described (Freitas *et al.*, 2022). Briefly, propolis was extracted twice with absolute ethanol (80 mL and 50 mL, respectively) followed by filtration under vacuum. The resulting filtrates were pooled and dried in a Buchi Rotavapor RE 121 (40 rpm, at 40 °C), yielding the ethanol extract (EE) **G18.EE**.

### 7.2.2. Textile functionalization

#### 7.2.2.1. Preparation of biofunctional solution

Biofunctional solution (**BS**) was prepared by mixing G18.EE and $H18_{50}$ with water to a final concentration of 1000 μg $mL^{-1}$ and 5 % (v/v), respectively. The solutions were stored at 4 ºC, in the dark, until further use.

#### 7.2.2.2. Textile treatments

For textile functionalization, 100 % cotton (**CO**) was chosen, and the adopted technology was the impregnation by exhaustion in a thermostatic bath (Grant OLS200). Two CO textile knits were rinsed with water and placed into different tubes. One tube was filled with the biofunctional solution (**BS**) while the second tube received an identical volume of BS with the chemical mordant (BS_PA), which was prepared with 10 g/ L of potassium alum (**PA**; $KAl(SO_4)_2 \cdot 12H_2O$). A ratio of 1:20 (g/ mL) textile/ BS was used in the bath and incubation was performed at 40 ºC and 100 rpm for 90 min. After textile functionalization, the knits **CO** (used as control without any treatment), **CO+BS** (cotton knit treated with BS) and **CO+BS_PA** (cotton knit cotreated with BS and PA), were dried for 24 h at room temperature.

### 7.2.3. Strength of textile functionalization to washing

BS-treated textiles were washed to evaluate their washing fastness. For that, **CO**, used as control, and the biofunctional knits **CO+BS** and **CO+BS_PA** were divided in two equal parts, one part of each being subject to three washing cycles (**3WC**) in a domestic washing machine (Indesit IWDC 6105). Each

washing cycle was performed at 30 °C for 1 h with addition of Soflan™ (detergent suitable for less abrasive washes). After the 3 washing cycles, textile samples - **$CO_{3WC}$**, **$CO+BS_{3WC}$** and **$CO+BS_PA_{3WC}$** - were dried at room temperature and assessed for antioxidant and antibacterial activities.

### 7.2.4. *In vitro* evaluation of the antioxidant potential

ABTS radical scavenging assay was used for *in vitro* evaluation of the antioxidant potential (Zemljič *et al.*, 2014), with slight modifications adopted according to the samples (BS and BS_PA or textiles) under evaluation. Firstly, an aqueous solution of ABTS (7 mM; Sigma-Aldrich) and potassium persulfate (2.45 mM; Sigma-Aldrich) was kept in the dark, at room temperature for 16 h, and then diluted in phosphate buffer 0.1 M (pH 7.4) until an optical density of 0.7 ± 0.025 (PerkinElmer Lambda 35) was reached at 734 nm ($OD_{734}$). This ABTS solution was used as working solution.

Antioxidant potential of the biofunctional solution was evaluated by adding 30 µL of the BS to 2970 µL of the ABTS working solution (using a 1:99 proportion in the reaction mixture), followed by 6 min incubation at room temperature. The antioxidant potential of textiles, either functionalized (CO+BS, CO+BS_PA, $CO+BS_{3WC}$ and $CO+BS_PA_{3WC}$) or non-functionalized (CO and $CO_{3WC}$), was evaluated by adding 3 mL of ABTS working solution to 0.075 g of the different sample knits, followed by 30 min incubation at room temperature. L-cysteine (0.3 g $L^{-1}$) was used as positive control. Absorbance of the reaction was measured at 734 nm and results were expressed in percentage of ABTS radical reduction according to the following equation:

$$\% \text{ inhibition} = [(A_{control} - A_{sample}) \times 100]/ A_{control}$$

where $A_{sample}$ is the absorbance of the samples and $A_{control}$ the absorbance of the control (ABTS working solution and ethanol, water or a mixture of both, the solvents of G18.EE, $H18_{50}$ or BS respectively, and only ABTS for the textile samples), after 6 or 30 min of reaction, depending on the tested samples.

### 7.2.5. Determination of antibacterial properties

Antibacterial activity was evaluated against the Gram-negative bacterium *Escherichia coli* CECT 423 and the Gram-positive bacteria - *Bacillus subtilis* 48886, *Staphylococcus aureus* ATCC 6538 (MSSA) and *Propionibacterium acnes* H60803 - obtained from the microbial collection of the Department of Biology of the University of Minho. Bacteria were cultured in LB (Luria-Bertani - 0.5 % w/v yeast extract, 1 % w/v tryptone, 1 % w/v NaCl) or in solid medium (LBA by adding 2 % w/v agar to

the LB recipe). Growth was performed at 200 rpm and 37 °C and monitored by optical density measurement at 600 nm ($OD_{600}$).

Antibacterial activity of BS was assessed by the agar dilution method (Hanson and Martin, 1978). Briefly, 5 µL drops of exponentially growing bacterial cultures were transferred onto LBA plates supplemented with 1000 µg $mL^{-1}$ G18.EE and 5 % H18. Control was prepared with LBA containing the solvents used in the same volumes as the sample. Plates were incubated at 37 °C for 24 h. Bacterial susceptibility was determined by observing the presence or absence of growth.

The antibacterial activity of the textiles, either biofunctionalized or non-functionalized, was evaluated according to Nobre (2018) with slight modifications as follows. Firstly, textiles were cut into pieces of 0.1 g and sterilized under UV light for 10 min each side. To prepare the cells for the experiments, overnight microbial cultures were diluted with the appropriated fresh medium to an $OD_{600}$ of 0.05 in 10 mL. A sample of each textile (CO, CO+BS, CO+BS_PA, $CO_{3WC}$, $CO+BS_{3WC}$ and $CO+BS_PA_{3WC}$) was added to the culture and incubated at 200 rpm, at 37 °C, for 24 h. The antibiotic ampicillin (200 µg $mL^{-1}$) was used as positive control. Each bacterial suspension was collected, serially diluted from $10^{-1}$ to $10^{-7}$, and drops of 40 µL of diluted microbial cultures were transferred onto LBA plates. After 24 h incubation at 37 °C, colonies were counted and results expressed in growth reduction percentage relative to controls (CO or $CO_{3WC}$), according to the following equation:

$$\% \text{ reduction} = [(B - A)/ B] \times 100$$

where A is the number of colony-forming units in the biofunctionalized textiles and B is the number of colony-forming units in the non-functionalized textile (CO or $CO_{3WC}$), after 24 h incubation.

### 7.2.6. Statistical analysis

Experiments were done in triplicate and results were expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's test for multiple comparisons were used to assess treatment effect. Differences considered statistically significant ($p < 0.05$) were distinguished with different letters.

## 7.3. Results

### 7.3.1. Antioxidant and antimicrobial properties of the functionalized textiles

According to our previous work on antioxidant and antimicrobial synergisms between propolis and honey (Freitas *et al.*, 2022), where several mixtures of propolis and honey were tested against a wide panel of bacteria, a mixture of 200 µg $mL^{-1}$ of G18.EE with 5 % H18 was able to inhibit the growth of *B. subtilis*, *B. megaterium*, *B. cereus*, *S. aureus* and *P. acnes*, which lead to the selection of this

mixture for textile functionalization. In order to assess if the selected mixture had the desire antioxidant potential, the *in vitro* ABTS scavenging assay was performed before textile functionalization. The mixture exhibited limited antioxidant potential with only 58.8±1.2 % ABTS radical reduction. Therefore, and based on previous results which showed a dilution of the antioxidant potential of the mixture with increasing honey concentration (Freitas *et al.*, 2022), it was decided to increase five times the concentration of propolis (from 200 to 1000 μg $mL^{-1}$). As presented in **Table 7.1**, this mixture of 1000 μg $mL^{-1}$ G18.EE with 5 % H18 showed higher antioxidant potential (95.9±0.7 % ABTS radical reduction), therefore, this mixture was used as BS for textile functionalization.

**Table 7.1.** Antioxidant potential of the biofunctional solution (BS) and textiles measured by the *in vitro* ABTS scavenging assay expressed as % ABTS radical reduction and respective standard deviation (SD). Samples tested were cotton (CO), cotton treated with a biosolution (CO+BS) composed by a mixture of propolis (1000 μg $mL^{-1}$) and honey (5 %) in water, cotton treated simultaneously with BS and potassium alum (CO+BS_PA). L-cysteine (0.3 g $L^{-1}$) was used as positive control. Statistical analysis was performed by one-way ANOVA, followed by Tukey test for significance. Different letters mean statistically significant ($p < 0.05$) differences between mean values.

| Textile samples | ABTS scavenging activity |
|---|---|
| | % ABTS radical reduction |
| **CO** | 27.4±1.3[a] |
| **CO+BS** | 98.4±0.4[b] |
| **CO+BS_PA** | 98.5±0.2[b] |
| **Biofunctional Solution (BS)** | 95.9±0.7[b] |

Antioxidant potential of the biofunctional textiles assessed by the *in vitro* ABTS scavenging assay (Zemljič *et al.*, 2014) show the control sample (CO) with a significantly lower antioxidant capacity value (27.4±1.3 % ABTS radical reduction) when compared to the functionalized samples (>90 % ABTS radical reduction; $p < 0.001$) (**Table 7.1**). Functionalization process did not lead to antioxidant activity loss, as BS and both CO+BS and CO+BS_PA showed no significant differences in their capability to reduce ABTS radical.

Limitations of natural dyeing processes such the lack of uniformity, low yield, poor adhesion and poor fastness properties (Micheal *et al.*, 2003) can be overcome with the use of mordants (Khan *et al.*, 2006), which allow to increase the biocompounds affinity and to enhance the fastness of the functionalized textiles (Jabar *et al.*, 2020). To assess if the antioxidant potential of the biofunctional textiles can be potentiated using a mordant, one textile knit was treated simultaneously with the

biosolution and potassium alum (CO+BS_PA) and tested by the *in vitro* ABTS scavenging assay. The use of potassium alum as mordant did not show improvements in the antioxidant capacity as there were no significant differences when compared to the textiles treated with the biosolution alone (98.5±0.2 % or 98.4±0.4 % ABTS radical reduction in the presence or in the absence of potassium alum, respectively; **Table 7.1**).

Unlike observed for the antioxidant potential, there were significant differences between the antibacterial activity of CO+BS and CO+BS_PA, being the latter more effective in decreasing the growth of *Bacillus subtilis*, *Propionibacterium acnes* and *Escherichia coli* (**Table 7.2**). These results highlight the importance of using a mordant to enhance antibacterial activity of the biofunctional textile though *Staphylococcus aureus* was almost equally affected by both CO+BS and CO+BS_PA. Yet, in mordant absence, there were significant differences between the ability to decrease bacterial growth: *Staphylococcus aureus* was the most affected (94.8±0.8 % growth reduction), followed by *Bacillus subtilis*, *Escherichia coli* and lastly by *Propionibacterium acnes*, the least affected strain (12.3±7.1 % growth reduction; **Table 7.2**). But when potassium alum was used no significant differences were detected between the ability to decrease growth of all the tested strains (**Table 7.2**). Potassium alum, an environmentally friendly compound with no harmful effects on the ecosystems and approved for medical use (Salama, 2015), besides improving the antibacterial activity of textiles may also help to decrease the existing variability between the strains' susceptibility to the treatment.

**Table 7.2.** Antibacterial activity of the biofunctional textiles, assessed by colony-forming units, against four bacterial strains: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Propionibacterium acnes*. Strains in mid-exponential phase were transferred to Erlenmeyers with LB with the respective textile (see Materials and Methods for details). Samples tested were cotton (CO), cotton treated with a biosolution (CO+BS) composed by a mixture of propolis (1000 μg $mL^{-1}$) and honey (5 %) in water, and cotton treated simultaneously with the BS and potassium alum (CO+BS_PA). After 24 h incubation at 37 $^{o}C$, antibacterial activity was determined upon colony-forming units count and results expressed in growth reduction percentage relative to control (CO) and respective standard deviation (SD). Statistical analysis was performed by one-way ANOVA, followed by Tukey test for significance. Different letters mean statistically significant ($p < 0.05$) differences between mean values.

| Strains | Antibacterial activity<br>% Growth reduction | |
|---|---|---|
| | CO+BS | CO+BS_PA |
| **Gram-positive bacteria** | | |
| *Bacillus subtilis* | 55.1±18.1$^{c}$ | 88.2±17.7$^{a,b}$ |
| *Staphylococcus aureus* | 94.8±0.8$^{a}$ | 83.9±2.1$^{a,b}$ |
| *Propionibacterium acnes* | 12.3±7.1$^{d}$ | 78.5±9.8$^{b}$ |
| **Gram-negative bacterium** | | |
| *Escherichia coli* | 21.7±3.2$^{d}$ | 66.6±0.6$^{c,b}$ |

### 7.3.2. Strength of textile functionalization to washing

Functionalized clothing is meant to be worn and washed multiple times. Therefore, it is important to assess the maintenance of textiles functionality upon cycles of washing. Hence, the antioxidant potential of the biofunctional textiles was evaluated after three washing cycles (**Table 7.3**). Control sample ($CO_{3wc}$; 26.5±2.0 % ABTS radical reduction), similarly to the control sample before the washing process (CO; 27.4±1.3 % ABTS radical reduction; **Table 7.1**), showed significantly lower antioxidant capacity than the treated samples (>90 % ABTS radical reduction; $p < 0.001$). Likewise, the use of potassium alum as mordant did not show to improve antioxidant capacity as there were no significant differences when comparing the BS-treated textiles after three washing cycles (93.7±0.6 and 91.8±1.9 % ABTS radical reduction for $CO+BS_{3WC}$ and $CO+BS_PA_{3WC}$, respectively). Despite the slight decrease in the antioxidant capacity of the biofunctional textiles after three washing cycles ($CO+BS_{3WC}$ and $CO+BS_PA_{3WC}$; **Table 7.3**) comparatively to the samples before the washing process (CO+BS and CO+BS_PA; **Table 7.1**), all samples showed a percentage of ABTS radical reduction above 90 %.

**Table 7.3.** Antioxidant potential of the textiles after three washing cycles (3WC) measured by the *in vitro* ABTS scavenging assay and expressed as % ABTS radical reduction and respective standard deviation (SD). Samples tested were cotton ($CO_{3WC}$), cotton treated with a biosolution ($CO+BS_{3WC}$) composed by a mixture of propolis (1000 μg $mL^{-1}$) and honey (5 %) in water, cotton treated simultaneously with BS and potassium alum after three washing cycles ($CO+BS_PA_{3WC}$). L-cysteine (0.3 g $L^{-1}$) was used as positive control. Statistical analysis was performed by one-way ANOVA, followed by Tukey test for significance. Different letters mean statistically significant ($p < 0.05$) differences between mean values.

| Textile samples | ABTS scavenging activity |
|---|---|
| | % ABTS radical reduction |
| **$CO_{3WC}$** | 26.5±2.0[a] |
| **$CO+BS_{3WC}$** | 93.7±0.6[b] |
| **$CO+BS_PA_{3WC}$** | 91.8±1.9[b] |

Beyond antioxidant potential maintenance, antibacterial activity must also persist after the washing process. To assess the influence of the washing process on textiles antibacterial activity, the growth reduction percentage of the control after the three washing cycles ($CO_{3wc}$) was expressed relatively to the control without any washing (CO). As shown in **Figure 7.1**, washed cotton affected bacteria and differently, *Staphylococcus aureus* being the most affected one (63.7±10.0 % growth reduction), followed by *Bacillus subtilis* and *Propionibacterium acnes*, equally affected, and by *Escherichia coli* as the least affected bacterium (2.3±1.3 % growth reduction) by the washing process. These results suggest that the detergent itself possess antibacterial activity, affecting all the tested

strains though differently. Thus, detergent selection is a determining factor as could influence antibacterial activity of the textile after the washing process in a dimension that will depend on the target strain.

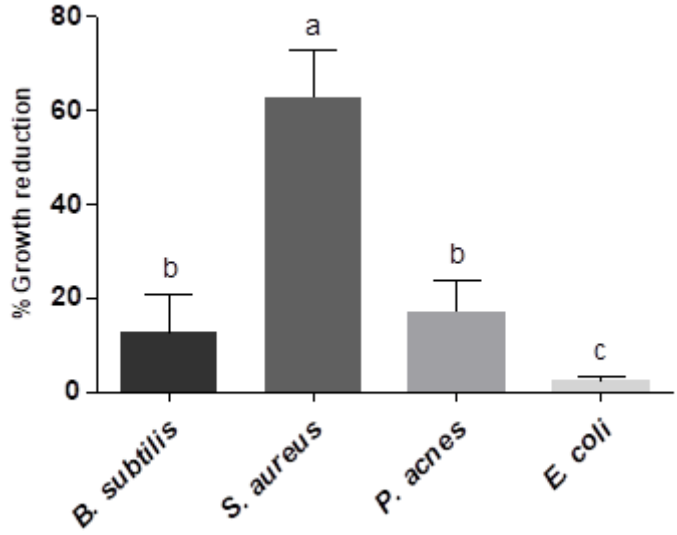


**Figure 7.1**. Assessment of the washing process influence on the antibacterial activity of the textiles against four bacterial strains: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Propionibacterium acnes*. Strains in mid-exponential phase were transferred to Erlenmeyers with LB with the respective textile (see Materials and Methods for details). Samples tested were cotton (CO) and cotton after three washing cycles ($CO_{3WC}$). After 24 h incubation at 37 ºC, antibacterial activity was determined upon colony-forming units count and results were expressed in growth reduction percentage relative to control (CO) and respective standard deviation (SD). Statistical analysis was performed by one-way ANOVA, followed by Tukey test for significance. Different letters mean statistically significant ($p < 0.05$) differences between mean values.

Treated textiles did not lost their function and maintained its antibacterial activity after three washing cycles, except against *Escherichia coli* (**Tables 7.2** and **7.4**). Without the use of the mordant, differences between the ability to decrease growth of the tested strains were less marked than before the washing process (**Table 7.4**), with *Staphylococcus aureus* being the most affected (86.1±9.8 % growth reduction), followed by *Propionibacterium acnes* (56.9±9.4 % growth reduction), *Bacillus subtilis* (47.1±3.5 % growth reduction) and *Escherichia coli* (43.6±0.9 % growth reduction) being the least affected species. The use of potassium alum seems to be less significant in the case of *Staphylococcus aureus* and *Propionibacterium acnes*, or even a disadvantage in the case of *Escherichia coli*. Nevertheless, its use increased the antibacterial activity against *Bacillus subtilis* to the double (from 47.1±3.5 % to 99.8±0.01 % growth reduction; $p < 0.001$; **Table 7.4**). Biofunctional textiles, either with or without simultaneous treatment with potassium alum, exert distinct effects depending on the strain tested, meaning that, depending on the application, several assays with different mordants and several washing cycles are needed to ensure the intended effect on the textile.

**Table 7.4.** Antibacterial activity of the biofunctional textiles measured after three washing cycles (3WC) against four bacterial strains: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Propionibacterium acnes*. Strains in mid-exponential phase were transferred to Erlenmeyers with LB with the respective textile (see Materials and Methods for details). Samples tested were cotton ($CO_{3WC}$), cotton treated with a biosolution ($CO+BS_{3WC}$) composed by a mixture of propolis (1000 μg $mL^{-1}$) and honey (5 %) in water, cotton treated simultaneously with the BS and potassium alum ($CO+BS_PA_{3WC}$), after three washing cycles. After 24 h incubation at 37 °C, antibacterial activity was determined upon colony-forming units count and results were expressed in growth reduction percentage relative to control ($CO_{3WC}$) and respective standard deviation (SD). Statistical analysis was performed by one-way ANOVA, followed by Tukey test for significance. Different letters mean statistically significant ($p < 0.05$) differences between mean values.

| Strains | Antibacterial activity<br>% Growth reduction | |
|---|---|---|
| | $CO+BS_{3WC}$ | $CO+BS_PA_{3WC}$ |
| **Gram-positive bacteria** | | |
| *Bacillus subtilis* | $47.1\pm3.5^{c,d}$ | $99.8\pm0.01^{a}$ |
| *Staphylococcus aureus* | $86.1\pm9.8^{b}$ | $90.1\pm2.7^{a,b}$ |
| *Propionibacterium acnes* | $56.9\pm9.4^{c}$ | $54.8\pm13.3^{c}$ |
| **Gram-negative bacterium** | | |
| *Escherichia coli* | $43.6\pm0.9^{d}$ | $13.6\pm2.1^{e}$ |

## 7.4. Discussion

Several studies have been showing that natural products, in addition to coloring textiles, give them particular functional properties such as the antioxidant and antimicrobial activities (Abramiuc *et al.*, 2013; Sharaf *et al.*, 2013; Oh and Na, 2014; Pucciarini *et al.*, 2019; Suneeta *et al.*, 2021; Verma *et al.*, 2021). However, just a few authors have investigated propolis potential as functional finishing agent to be applied in the textile industry (Abramiuc *et al.*, 2013; Sharaf *et al.*, 2013; Arikan and Aygun, 2017; Rogina-Car *et al.*, 2018; Yan *et al.*, 2018; Rogina-Car and Bogović, 2019; Cardoso *et al.*, 2021). According to our knowledge, this study is the first one exploring the combined capability of propolis and honey to provide smart textiles with antioxidant and antibacterial activities and the first one to test the action of biofunctional textiles against *Propionibacterium acnes*, the most abundant bacterial species found on human skin and involved in the development of various inflammatory skin conditions, like acne (Mollerup *et al.*, 2016).

Also, according to the best of our knowledge, investigation on the antioxidant potential of textiles dyed with propolis is lacking. Within our investigation group, pioneers in the matter Cardoso *et al.* (2021) showed the great antioxidant potential of cotton dyed with propolis from Gerês (88.7±0.4 %

ABTS radical reduction). In the present study we used 10 times less the amount of propolis used by Cardoso *et al*. (2021) but in combination with 5 % of honey, achieving a higher antioxidant capacity (98.4±0.4 and 98.5±0.2 % ABTS radical reduction for CO+BS and CO+BS_PA, respectively; **Table 7.1**).

Textile direct contact with skin, habitat to millions of microorganisms, can lead to dermal infection, allergies, unpleasant odor and damage of the textile properties due to the proliferation of pathogenic microorganisms, mainly bacteria, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (Khan *et al*., 2011; Bonaldi, 2018). Therefore, antibacterial activity of textile, particularly provided by natural agents, is an important parameter to consider for textiles which are in direct contact with skin. Sharaf *et al*. (2013) studied for the first time the antibacterial effect of propolis-dyed cotton against *Staphylococcus aureus* and *Escherichia coli*. The finishing of the textile was carried out using different mordants, such as glyoxal, arcofix, glutraldehyde and 1,2,3,4 butanetetracarboxylic acid (BTCA) for propolis fixation, showing the importance of the use of a crosslinking agent to achieve higher interaction between propolis and the textile surface and to consequently increase the antibacterial activity. Unlike our results, the untreated-cotton showed no differences when compared to the propolis-treated cotton (Sharaf *et al*. 2013). In our study the BS-treatment itself provided substantial antibacterial activity when compared to the untreated cotton sample. Abramiuc *et al*. (2013) evaluated the antibacterial activity of cotton treated with Romanian propolis against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus β haemolytic,* showing higher activity against *S. aureus* and *E. coli*. Activity was enhanced with simultaneous treatment with chitosan, a natural polysaccharide widely used as finishing agent for surface modification due to its antimicrobial activity and eco-friendly nature (Simoncic and Tomsic, 2010).

The use of bee products such as propolis, honey and beeswax as natural antibiotics in the medical fields by providing antibacterial activity to non-woven fabrics like cotton gauze has proven effectiveness against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* with or without a crosslinking agent such as chitosan (Rogina-Car *et al*., 2018; Rogina-Car and Bogović, 2019; Unango and Ramasamy, 2019). Propolis in combination with nanostructures such as polylactic acid (PLA; a plant-derived thermoplastic) and polyvinyl alcohol (PVA; a water-soluble synthetic polymer) was found to be active against bacteria by inhibiting *Staphylococcus aureus* proliferation, revealing potential to be used in medical textiles for burns, mouth ulcers and human diabetes wounds (Arikan and Aygun, 2017; Yan *et al*., 2018).

Sharaf *et al.* (2013) and Cardoso *et al.* (2021) also evaluated the fastness to the washing process, showing excellent durability of the treatments to the washing process up to twenty washing cycles and three washing cycles, respectively. The importance of using potassium alum (pre-treatment) was demonstrated as well by Cardoso *et al.* (2021), after five washing cycles, by preventing the antioxidant activity decrease shown by the sample treated only with propolis (from 88.7±0.4 to 62.8±8.7 % ABTS radical reduction after five washing cycles). In the present study, the washing fastness was evaluated till three washing cycles and no loss of function was registered for the textiles. On the other hand, simultaneous treatment with potassium alum did not have a significant impact on the antioxidant and antimicrobial properties of the functionalized textiles. This is a significant outcome considering the additional costs and negative impacts of the use of chemicals by the textile industry.

In short, biofunctional textiles with propolis and honey mixture showed antioxidant and antibacterial activity even after three washing cycles, which is important for the lifespan of the textile. The prevalence of these activities after textile washing help to ensure the durability of the functionalization and consequently reducing the waste. In general, the use of potassium alum as chemical mordant showed to be advantageous by maintaining or improving the overall antibacterial activity of the textiles with more or less impact depending on the tested strain, with the exception for *E. coli* where such use revealed to be a disadvantage after the washing process. Nevertheless, the assessment of the potential of other mordants would be an asset for the investigation.

## 7.5. Conclusions

Synthetic chemicals commonly used in the textile dyeing process are usually associated with adverse side effects to the environment and health. Global demand for safe, effective and natural products has been increasing in parallel with consumers´ concerns about personal and environmental health. Beehive products, known for their complex chemical composition linked with their wide range of bioactivities, have been shown great potential to be used by the textile industry as natural dyeing products with the ability to improve the performance of textiles for specific purposes such as antioxidant and antibacterial finishes. In this sense, beehive products, such as propolis and honey, proved to be promising natural products to be introduced in human daily personal care, for instance, in face masks due to its antioxidant and antimicrobial effects, with the possibility of several reuses.

## 7.6. Author contributions

Conception and design of the work: A.S.F., R.O., A.R., C.A.-A.; experimental work: A.S.F.; supervision of the experimental work: R.O., A.R., C.A.-A.; drafting the manuscript: A.S.F.; revision and approval of the manuscript: A.S.F., R.O., A.R., C.A.-A.; project supervision: C.A.-A. All authors have read and agreed to the published version of the manuscript.

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# Chapter 8

General discussion, conclusions and future perspectives

## 8.1. General discussion

Portuguese propolis has been investigated by our research group at the Department of Biology of the University of Minho (Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Cruz *et al.*, 2016; Freitas *et al.*, 2019; Peixoto *et al.*, 2021, 2022; Araújo *et al.*, 2022; Oliveira *et al.*, 2022; Pereira *et al.*, 2022), however, there is still much to do to prove the real importance of this high value bee product. In this way, as stated in **Chapter 1**, this work was fundamentally focused to overcome some of the barriers that are blocking propolis acceptance and introduction in some industries: the low productivity and the chemical complexity of this natural product.

According to data from the beekeeper who provided the propolis samples investigated in this work, the annual propolis production is around 27 g per hive (Amadeu Fortunas, personal communication), a value yet far from meeting the production needs of a propolis-based product at an industrial level. Thus, as described in **Chapter 2**, we investigated the impact of the grid pore size on propolis production using different metal grids with varied pore sizes (0.63 mm, 1.8 mm, 2.8 mm) and the normally plastic grid used by the beekeeper (3 mm). This study was conducted over three consecutive years (2017-2019), placing the grids in the hives every Spring and observing the resulting annual propolis production. In 2017 (**Figure 2.3**), smaller pore-sized grids (0.63 mm) yielded higher propolis amounts compared to the plastic grid (3 mm) commonly used by the beekeeper. However, the high temperatures and the wildfire occurred in that same year likely affected the results, and the replacement of isogenic queen bees with non-isogenic ones may have also influenced the outcomes. In 2018 (**Figure 2.4**), propolis production time was reduced due to delayed grid placement caused by the rain. Similarly to what happened in 2017, smaller pore-sized grids (0.63 mm) resulted in higher propolis amounts, and there was a more noticeable inverse relationship between the grid pore size and the propolis production. The quantification of propolis production in 2019 (**Figure 2.5**) was affected by the elimination of some hives due to *Varroa destructor* parasite infestation. Additional hives were introduced to ensure the continuity of the study. This year showed no clear tendency in propolis production based on grid pore size. Overall, considering propolis production over the three years studied period, higher propolis yield was obtained from the smaller pore sized grids, with no detected influence on the honey production.

The literature on factors influencing propolis production is scarce, but a study from Jager *et al.* (2022) reported significantly higher propolis amounts in hives with grids compared to control groups without grids, with no significant differences in honey production between hives with and without grids. The material of the beehive and seasonality were identified as additional factors affecting propolis

production (Kiziltas and Erkan, 2020; Mountford-McAuley *et al.*, 2021). The present study faced inconsistent factors throughout the three years, such as the death of queens, hive eliminations due to wildfires, and variable climate conditions. These factors have most probably contributed to the lack of significant differences between propolis yields obtained from different pore-sized grids. Nevertheless, the study highlights the complex interplay of various factors in propolis production and overall suggests that smaller pore sizes may result in higher propolis amount without affecting honey production.

Usually, propolis from Gerês is composed by a mixture of different source places from Gerês (Bugalho, Toutelo and Felgueiras) and is characterized by an unusual constancy of its chemical and biological profiles (Freitas *et al.*, 2019). However, the wildfires from 2017 lead to the destruction of one of the source places (Bugalho) making us question to what extent this stated constancy was affected. Thus, as described in **Chapter 3**, several propolis ethanol extracts made from mixtures of propolis samples harvested from different source places as well as two honey samples from the same apiary were evaluated for their antioxidant and antimicrobial activities to understand the implications of the above-mentioned changes. Some variations in pollen composition, antioxidant and antimicrobial activities were found, maybe related to the wildfire occurred in 2017 and the consequent change in flora and hive relocation. But, overall, the results of this study align with previous research on Portuguese beehive products, including honey and propolis, in terms of their phenolic composition, antioxidant capacity, and antimicrobial activity. Additionally, the study provides valuable insights into the botanical origins and biological properties of honey and propolis samples from Gerês (Portugal), contributing to the existing knowledge about these natural products and their potential health-promoting properties. Also, this study helped us to choose the propolis sample to proceed for further investigation (**Chapters 3-7**), G18.EE.

In recent years, consumers are becoming increasingly health-conscious when it comes to consumption of food and medicines, seeking alternative solutions that offer a more natural and sustainable approach to wellness. Among the factors that influence propolis chemical composition and, consequently, its biological prolife, is the solvent used to extract its bioactive portion (Sheng *et al.*, 2007). Ethanol, the most used solvent (Asgharpour *et al.*, 2020), has some limitations in what concerns its applicability in some industries. Thus, we searched for new and ecological solvents in order to unravel their potential to provide propolis extracts with distinct applications. For that, and as stated in **Chapter 4**, absolute ethanol, ethanol/water (7:3), honey brandy, mead, propylene glycol and water were used for propolis extraction. To our knowledge, mead and honey brandy were never used for such purpose, presenting interesting sustainable alternatives, favoring the circular economy and a greater

use/ profitability of the beehive's resources. The study evaluated the yields, the antioxidant potential and antimicrobial activities as well as the chemical composition of the six obtained extracts. The highest extraction yields were obtained with absolute ethanol, ethanol/water (7:3), and propylene glycol extracts, while honey brandy and water extracts had the lowest yields (**Table 4.1**). The results showed that the extraction yield varied depending on the solvent used; however, and in accordance with what is described in the literature, ethanol was found to have a higher affinity for extracting phenolic compounds from propolis compared to polar solvents like water (Santos-Buelga and González-Paramás, 2017; Mokhtar *et al.*, 2019). In terms of antimicrobial activity (**Tables 4.2** and **4.3**), the extracts were more active against Gram-positive bacteria being the ethanol extracts the ones with larger antimicrobial spectrum. Interestingly, the mead extract showed potential against the methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*, being the only one capable to inhibit their growth at the range of concentrations tested. The water extract was generally less active against the tested bacterial strains. As expected, by previous results of propolis samples from Gerês harvest from previous years (Freitas *et al.*, 2019), all the extracts showed similar and weak activity against the tested yeasts. However, honey brandy extract exhibited higher activity against *Saccharomyces cerevisiae* and *Candida albicans* compared to the other extracts. Generally, the results obtained for the antimicrobial activity of these extracts are in accordance with the literature (Ramanauskienė *et al.*, 2013; Kubiliene *et al.*, 2015; Asgharpour *et al.*, 2020) namely in what concerns a more expressive antibacterial than antifungal activity and regarding the higher susceptibility of Gram-positive bacteria when compared to the Gram-negative. DPPH free-radical-scavenging activity was also determined for the extracts (**Table 4.4**): the mead extract displayed the highest antioxidant potential, followed by the honey brandy and water extracts. Ethanol and propylene glycol extracts showed similar or lower antioxidant activity when compared to the remaining extracts.

The total *ortho*-diphenols (TOC), phenolics (TPC), and flavonoids (TFC) contents of the extracts were also analyzed (**Table 4.5**). The extracts with higher TOC exhibited higher DPPH scavenging ability, while higher TFC values were generally associated with stronger antibacterial activity. Through the chemical analysis of the extracts using LC-MS was possible to identify 51 compounds (**Figure S4.1** and **Table S4.1 Supplementary material**), with most of the solvents showing similar phenolic constituents. Gallic acid, caffeic acid, *p*-coumaric acid, and quercetin-3-methyl ether were among the main compounds found in the extracts. Principal Component Analysis (PCA) was applied to explore correlations between different compounds present in propolis extracts and their antioxidant and antimicrobial activities. The analysis revealed three distinct groups of propolis extracts based on their

chemical composition and activity profiles (**Figure 4.1**): a first group consisting of the ethanol and mead extracts, a second one comprising honey brandy and propylene glycol extracts and a third group represented by the water extract, which was the most differentiated extract. The analysis (**Figure 4.2**) showed that the TOC and TPC were correlated to the antioxidant activity, indicating a correlation between these variables and antioxidant potential. This finding is noteworthy since previous literature has primarily associated antioxidant activity with flavonoid and phenolic contents, being the first time TOC was determined for propolis samples. Thus we propose to include TOC determination in the context of the characterization of propolis extracts. The TFC was strongly correlated with antibacterial activity, particularly against Gram-positive bacteria (*B. subtilis*, *B. cereus*, *B. megaterium*, methicillin-sensitive *Staphylococcus aureus* (MSSA), and *Propionibacterium acnes*) and antifungal activity against *S. cerevisiae* and *C. albicans*. However, there was no significant correlation observed between TPC, TOC, and the Gram-negative bacterium *E. coli*, as well as with MRSA. The presence of certain phenolic compounds, such as gallic acid and various tannins, appeared to contribute to the antioxidant potential of the water extract. In contrast, the antimicrobial activity against specific fungi and bacteria seemed to be linked to the presence of flavonoids like ellagic acid, ferulic acid, pinobanksin-5-methyl-ether, quercetin-3-methyl ether, kaempferol, isorhamnetin, acacetin, kaempferide, kaempferol-methoxy-methyl ether, 3-hydroxy-5-methoxy flavanone, and caffeic acid derivatives.

The study concludes that ethanol extracts of propolis from Gerês are more suitable for antimicrobial applications, which may be related with the high flavonoid content. On the other hand, mead extract shows promise in terms of antioxidant potential, attributed to its higher content of phenolic acids. Solvents like honey brandy and mead are interesting as well due to their strong antioxidant potential and infections-fighting capability against fungal infections caused by *C. albicans*, as well as bacterial infections including MRSA and *E. coli*.

The complex and variable chemical composition of propolis, along with its low productivity, has hindered its industrial-scale production and commercialization in cosmetic and pharmaceutical products (Bankova *et al.*, 2000; Falcão *et al.*, 2010; Sforcin, 2017; Cornara *et al.*, 2017). To address this barrier, as described in **Chapter 5** and **Appendix 1**, compound fractioning and isolation from a propolis sample from Gerês in 2018 was performed to search for the main bioactive compounds, establishing a chemical fingerprint for this Portuguese propolis. Four different fractions (*n*-hexane, ethyl acetate, *n*-butanol, and water) were obtained through liquid-liquid partitioning and different chromatographic techniques were used to isolate compounds from these fractions (**Figures 5.1-5.4** and **Table 5.2**). Some compounds were isolated from the *n*-hexane (**Appendix 1**) and ethyl acetate fractions. The ethanol

extract, the four fractions and the resulting sub-fractions and isolated compounds from the ethyl acetate fraction were then tested for their activity on renal cell carcinoma (RCC) cell lines (A498, 786-O, and Caki-2) and non-neoplastic kidney cells (HK2) to assess their toxicity (**Table 5.1**). The results showed that the water fraction of the propolis extract had the strongest effect on cell viability, however also presented lower selectivity towards cancer cells. The ethyl acetate fraction (G18.EE-EtOAc) exhibited high activity with lower $IC_{50}$ values against RCC cells compared to the other fractions. Previous research has shown the anticancer potential of propolis from various regions against different cancer types, including prostate, colon, melanoma, lung, breast, and glioblastoma (Li *et al.*, 2007; Ishihara *et al.*, 2009; Kamiya *et al.*, 2012; Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014, 2015; Kubina *et al.*, 2015; Chang *et al.*, 2017), however few studies (Valente *et al.*, 2011; Daleprane *et al.*, 2012) have explored the potential of propolis for the treatment of RCC. The subfractions obtained from the G18.EE-EtOAc fraction (P1, P2 and P3) were tested for their cytotoxic effects on RCC cell lines alone (**Table 5.3**) and combined (**Table 5.4**). The original G18.EE-EtOAc fraction still exhibited stronger cytotoxicity when compared to the activity exhibited by the subfractions alone or even combined, indicating the contribution of other constituents, acting synergistically. Overall, despite the complexity of propolis mixtures suggesting the presence of synergistic effects and the involvement of multiple compounds in the observed cytotoxicity, propolis fractions and their compounds have demonstrated potential as anticancer agents.

The overuse and misuse of antibiotics, their use in farming practices, and the lack of new antibiotics are contributing factors to the growing problem of microbial antibiotic resistance worldwide. To combat this issue, there is a need for strategies to overcome resistance and the development of new antimicrobial drugs (Ventola, 2015) and interest in natural products as alternatives to synthetic compounds has been increasing. Natural products, such as propolis, are attracting attention for their antibiotic activity and potential to the development of alternative drugs. Aiming to explore propolis as an adjuvant in antibiotic therapy, Portuguese propolis from Gerês was used to investigate possible synergistic effects between gentamicin (a broad-spectrum antibiotic), and honey (**Chapter 6**). The results showed that all the tested ethanol extracts of propolis from Gerês (G.EEs) had the same minimum inhibitory concentration (MIC) against the tested strains (**Table 6.2**). The most susceptible strain to gentamicin alone was *Bacillus megaterium*, followed by *Bacillus subtilis*, *Bacillus cereus*, MSSA, *E. coli*, and MRSA; **Table 6.2**). When gentamicin was combined with each G.EE, a synergistic effect was observed against the *Bacillus* species, MSSA, *E. coli*, and MRSA. The MIC of gentamicin decreased by up to 96% when combined with each G.EE (**Table 6.3**). However, at higher concentrations

of gentamicin and propolis, bacterial growth was observed, suggesting a loss of synergy and possible antagonistic effects. The combination of a propolis extract G18.EE and the honey extract H18.WE also showed a synergistic effect against all tested bacteria (**Table 6.5**), except for *B. cereus*, with lower concentrations of G18.EE and H18.EE being more effective in inhibiting bacterial growth when combined. The study suggests that propolis from Gerês can enhance the antimicrobial activity of gentamicin, allowing a reduction in the required antibiotic dosage, particularly against MRSA, resistant to some antibiotics, whose infection can lead to septic conditions and even to death. Also, the potential of propolis in combination with honey as alternative to antibiotics was demonstrated. This outcome is valuable considering the global issue of antibiotic resistance and the importance of exploring natural sources like propolis and honey for innovative antimicrobial therapies.

The skin, the largest organ, mostly covered by textile, is the first line of defence against environmental aggressions and has garnered interest in terms of skincare (Morganti *et al.*, 2019). Biofunctional textiles, which possess acquired properties acting as release systems for releasing bioactive substances to the skin, are being explored as biomedical for skin treatment or cosmetic application (Massella *et al.*, 2019). However, chemicals used to provide antibacterial activity to textiles are often synthetic and associated with toxicity (Balouiri *et al.*, 2016). Moreover, their effectiveness against bacteria is reduced due to a widespread use and the development of resistance (Balouiri *et al.*, 2016). The textile industry, driven by the need for functional products that align with consumer concerns regarding health and sustainability, is exploring alternatives with reduced environmental impacts as the use of bee products, such as honey and propolis, known for their valuable biological properties. The study described in **Chapter 7** specifically focuses on the potential of honey and propolis for functionalizing cotton textiles, providing antioxidant and antibacterial benefits.

Previous work on antioxidant and antimicrobial synergisms between propolis and honey (**Chapter 6**) indicated that a specific mixture of propolis and honey exhibited potent antibacterial activity against various bacteria. Biofunctional textiles treated with this propolis and honey mixture (biosolution) exhibited significantly higher antioxidant capacity compared to the control sample (**Table 7.1**). Additionally, the use of potassium alum as a mordant to enhance the properties of the functionalized textiles was also explored and showed to not improve the antioxidant capacity compared to the textiles treated with the biosolution alone at the tested concentration (**Table 7.1**). In terms of antibacterial activity, there were significant differences between the biosolution-treated textiles and those treated with the same biosolution along with potassium alum (**Table 7.2**), the latter showing enhanced antibacterial activity against *B. subtilis*, *P. acnes*, and *E. coli* whereas *S. aureus* was equally affected by both

treatments. Overall, the use of potassium alum as a mordant helps to reduce the variability in bacterial susceptibility to the treatment. To assess the maintenance of functionality after the washing process, the antioxidant potential and antibacterial activity of the biofunctional textiles were evaluated after three washing cycles (**Tables 7.3** and **7.4**). The functionalized textiles maintained their antioxidant capacity, although a slight decrease was observed. Similarly, the antibacterial activity persisted after washing, except against *E. coli*.

Natural products have been used to functionalize textiles and provide functional properties (Abramiuc *et al.*, 2013; Sharaf *et al.*, 2013; Oh and Na, 2014; Pucciarini *et al.*, 2019; Suneeta *et al.*, 2021; Verma *et al.*, 2021), but, to our knowledge, the combined potential of propolis and honey for textile functionalization is still unexplored, as the effectiveness of functional textile against *P. acnes*, a bacterial species commonly found on human skin and associated with skin conditions like acne. Previous research has demonstrated the antioxidant potential of textiles dyed with propolis, showing its ability to reduce ABTS radicals (Cardoso *et al.*, 2021). Other studies have explored the antibacterial effects of propolis-dyed cotton (Sharaf *et al.*, 2013) but contrarily to what was found in the present study, where biofunctional textiles without crosslinking agents already exhibited substantial antibacterial activity against various strains of bacteria, no differences were found between the untreated and the propolis-treated cotton. Overall, we demonstrated that the propolis and honey mixture effectively functionalized the textiles with antioxidant and antibacterial properties and that this functionality is maintained after three washing cycles, which contributes to the longevity of functionalization, reducing waste.

All the investigations described in the present work provide a better understanding of propolis complexity, placing the academic communing a step forward in the path to overcome the problems that hinders propolis use and acceptance in some industries, namely propolis scarcity and standardization. This work also represents a contribution to propolis characterization and highlights propolis potential in different applications, enhancing the value of this beehive product.

## 8.2. Conclusions and final remarks

Acknowledging the main hindrances that are challenging propolis acceptance and use by some industries, we have guided this research to find ways to overcome those barriers, namely by valuing the beehive products, particularly propolis, while unraveling its chemical and biological profiles, envisaging potential applications.

Regarding propolis scarcity, we studied the influence of the grid pore size on propolis production over a 3-year period and observed a preference of bees for smaller pore sized grids. Thus, a different grid allowing a better production of propolis could be designed based on the results of the present study. Also, as no significant influence was detected on honey yield, one of the major beekeepers' concerns if hives are used both for honey and propolis production, propolis potential as an additional source of income in the beekeeping sector is reinforced.

Propolis from Gerês has long been recognized by an unusual consistency of its chemical and biological profiles. Mixing propolis samples from the same apiary (Gerês) harvested in different years, showed to be a valuable strategy to cope with some constrains of propolis, particularly in situations where such constancy may be threatened. Nature is in constant changing and as climatic conditions can change bees' activity, natural disasters too, and in an even more devastating way. Wildfires from 2017 have destroyed one part of the beehives from Gerês and the attempts to replace those hives with new ones were unsuccessful, hence propolis samples were never like the ones harvested previously. Several ethanol extracts of propolis sampled from the different apiary locations of Gerês were investigated for their chemical profiles and antioxidant and antimicrobial activities to find a representative sample of the apiary and to evaluate the constancy and influence of the replacement of hives. The ethanol extract of propolis harvested from Gerês in 2018 - G18.EE - showed to be the one that comes closest to this criterion, displaying high antioxidant potential and antimicrobial activity, with particular interest against several spore forming bacteria of the genus *Bacillus*, MSSA and the yeast *S. cerevisiae* and *C. albicans*, being promising for further investigations and use in different industries.

Despite the inherent factors that impact on propolis chemical composition, the solvent has high influence in the phenolic composition of the resulting extract. In fact, the use of different solvents - absolute ethanol, ethanol/ water (7:3), honey brandy, mead, water and propylene glycol - showed extracts with higher alcohol content as the ones exhibiting higher antimicrobial activity. Honey brandy and mead extracts showed considerable potential to fight fungal infections, such as the ones caused by *C. albicans*, and resistant bacteria such as MRSA and *E. coli*, with the additional benefit of being two alternatives to the commonly used solvents to extract propolis bioactive portion, with less environmental impact and supporting a circular economy by reusing mead and honey brandy. The findings emphasize the importance of selecting the appropriate solvent for propolis extraction and contributes to a more informed choice of solvent based on the desired application of propolis. Additionally, the use of natural solvents derived from bee products, such as honey brandy and mead, aligns with the growing consumer demand for natural and sustainable alternatives to chemical-based products. These solvents not only

offer eco-friendly alternatives but also provide additional economic benefits to the beekeeping sector by utilizing surplus honey production and repurposing products that may not have commercial value.

The fractioning of propolis from Gerês resulted in four different fractions (*n*-hexane, ethyl acetate, *n*-butanol and water fractions) as well as some isolated compounds from the *n*-hexane fraction and some sub-fractions and also an isolated compound (pectolinarigenin) from the ethyl acetate fraction. Further investigation of the cytotoxic activity of propolis and its fractions on renal cell carcinoma showed the ethyl acetate fraction as the one with the strongest cytotoxicity against A498 and 786-O cells. Pectolinarigenin and a sub-fraction (mixture of quercetin-dimethyl ester and caffeic acid derivatives), both obtained from the ethyl acetate fraction, displayed similar cytotoxic activity against 786-O but a greater effect when combined, showing the importance of the synergistic effect between phenolic compounds. Results obtained support the potential of propolis and its constituents as promising agents for the treatment of renal cell carcinoma, either to be used as alternative or in combination with conventional anticancer treatments.

A synergistic effect was observed between propolis from Gerês and gentamicin against several susceptibility indicator bacterial strains, with particular interest against MRSA for which a threefold decrease of the gentamicin MIC was observed if mixed with a sub-MIC concentration of propolis, suggesting the possibility of reducing the therapeutic dose of this antibiotic. Also, mixtures of sub-MIC concentrations of propolis and honey display stronger antibacterial effect than the activity exhibited by each natural product individually, which is an important finding considering propolis scarcity that could be partially solved by mixing with honey.

The projection of a bioactive product using beehive resources, namely propolis and honey, was a major purpose of this work. Considering the results of the mixture of propolis and honey and its properties, the potential of this mixture in dyeing and functionalization was investigated. The biofunctional textiles showed great antioxidant potential and particular promising activity against *P. acnes*, a Gram-positive bacterium commonly found in human skin and involved in the pathogenesis of acne, even after 3 washing cycles.

Considering the problem of antibiotic resistance and the adverse side effects to both the environment and health associated to the use of synthetic chemicals, natural beehive products, alone or in mixture, proved to be promising safer and eco-friendly alternatives. To be introduced in the pharmaceutical industry as coadjutants in cancer treatment, or in the textile industry providing the textile with antioxidant and antimicrobial properties with good fastness to the washing process, an

important feature of sustainability, propolis and honey are two natural products worth of attention and further investigation.

To conclude, this work is the first in-depth characterization of a single apiary, approaching several aspects such as the optimization of propolis production, the extensive chemical and biological characterization of propolis sampled from different places at the apiary, all these over a 3-year period. Yet, for the propolis sample chosen and more deeply studied (G18.EE), some potential applications in several industries were explored. Altogether, the approaches in this work aimed to be a turning point in the Portuguese mindsets, being an inspiration for new researchers to further explore this and other aspects involving the outstanding beehive products, particularly propolis, while simultaneously contributing to the valorization of the beekeeping sector.

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# Appendix 1

Compound Isolation from the n-hexane fraction of the ethanol extract of propolis from Gerês harvested in 2018 (G18.EE-*n*-hexane)

## A1.1. Extraction and fractionation of propolis

Propolis G18 was extracted as previously described (Freitas *et al.*, 2019) and 4 g of the ethanol extract obtained (G18.EE) was dissolved in 20 mL ethanol (Fisher Scientific, UK) and deionized water (200 mL) and successively partitioned with *n*-hexane, EtOAc and *n*-BuOH (Fisher Scientific, UK; 3 x 400 mL each) as previously described (Freitas *et al.*, 2022).

## A1.2. Isolation of compounds from the G18.EE-*n*-hexane fraction

G18.EE-*n*-hexane fraction (1.22 g) showed several spots on pre-coated silica gel 60 $F_{254}$ TLC plates (Merck®) indicating the presence of secondary metabolites, which justified further investigation by Sephadex LH-20 (Sigma-Aldrich, UK) Column. Elution started with a relatively non-polar solvent system and then the polarity was increased gradually [$CH_2Cl_2$ (Fisher Scientific, UK)/ *n*-hexane (95:5); $CH_2Cl_2$ (100); $CH_2Cl_2$/ EtOAc (95:5)]. Similar organic layers obtained were pooled according to similar TLC profiles into a total of **15** sub-fractions (**F1 – F15**; **Figures A1.1 and A1.2**).

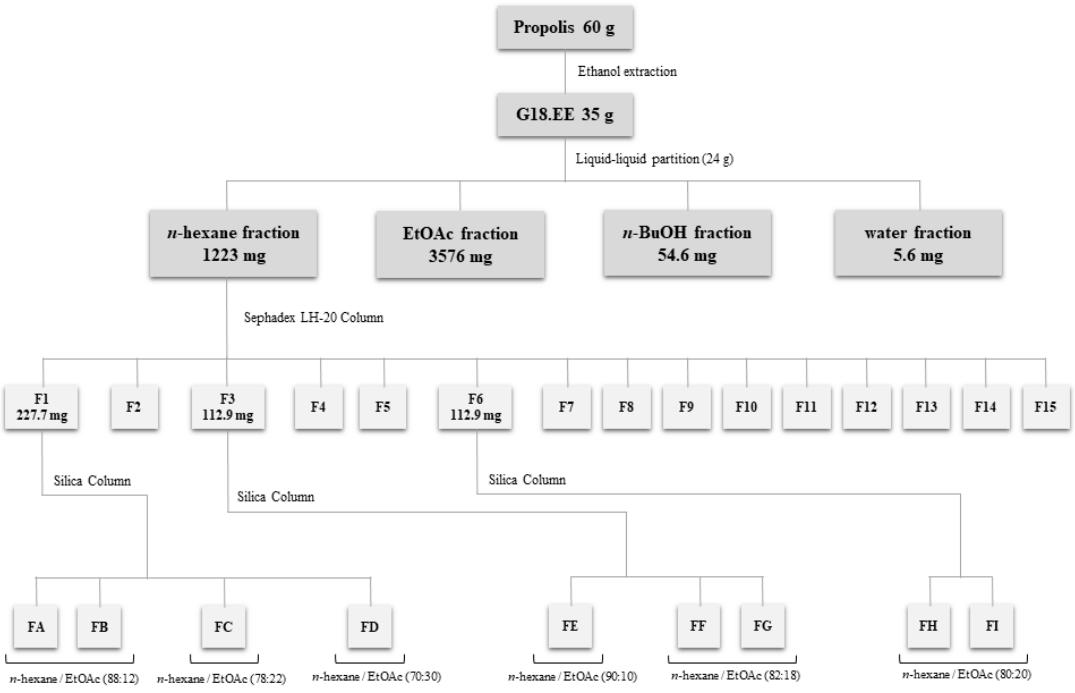


**Figure A1.1.** Fractionation of G18.EE and methodologies used for isolation of subfractions and compounds from the G18.EE-*n*-hexane fraction of the studied Portuguese propolis from Gerês. G18.EE – ethanol extract of propolis from Gerês collected in 2018; EtOAc – ethyl acetate; BuOH – butanol; F – fraction.

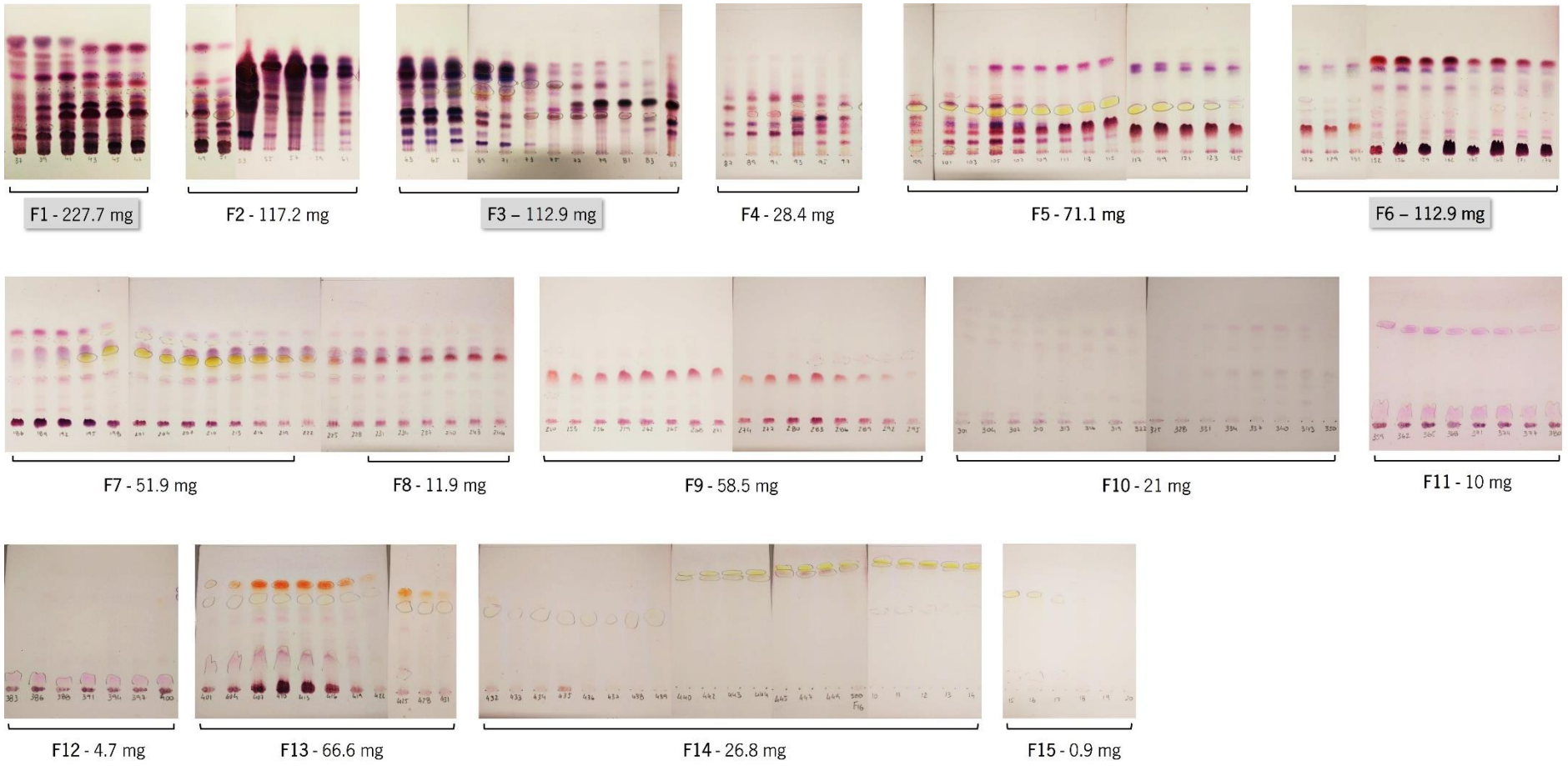


**Figure A1.2.** TLC analysis of the G18.EE-*n*-hexane fraction. Solvents used in column for extraction of the 15 resulting sub-fractions (F) were: $CH_2Cl_2$/ *n*-hexane (95:5) for **F1** - **F9**, $CH_2Cl_2$ (100) for **F10** and $CH_2Cl_2$/ EtOAc (95:5) for **F11** - **F15**.

Solvents used in column for extraction of the 15 sub-fractions were: **F1 - F9** [$CH_2Cl_2$/ *n*-hexane (95:5)], **F10** [$CH_2Cl_2$ (100)], **F11 - F15** [$CH_2Cl_2$/ EtOAc (95:5)]. Three sub-fractions with the highest yields - **F1** (227.7 mg), **F3** (112.9 mg) and **F6** (112.9 mg), eluted with $CH_2Cl_2$/ *n*-hexane (95:5) - were further fractionated using Silica (VWR International, UK) Column (**Table A1.1**).

**Table A1.1.** Solvents used in Sephadex LH-20 Column for the fractioning of the G18.EE-*n*-hexane fraction to obtain the 15 resulting sub-fractions (**F1 – F15**) and respective yields (mg).

| **Sephadex LH-20 Column of G18.EE-*n*-hexane fraction Solvent System** | **Sub-fractions Code name** | **Yield (mg)** |
|---|---|---|
| $CH_2Cl_2$ 95 % / *n*-hexane 5 % | **F1** | 227.7 |
| | F2 | 117.2 |
| | **F3** | 112.9 |
| | F4 | 28.4 |
| | F5 | 71.1 |
| | **F6** | 112.9 |
| | F7 | 51.9 |
| | F8 | 11.9 |
| | F9 | 58.5 |
| $CH_2Cl_2$ 100 % | F10 | 21 |
| $CH_2Cl_2$ 95 % / EtOAc 5 % | F11 | 10 |
| | F12 | 4.7 |
| | F13 | 66.6 |
| | F14 | 26.8 |
| | F15 | 0.9 |

Elution of **F1**, **F3** and **F6** started with *n*-hexane 100 % and followed with EtOAc mixtures of increasing polarity (up to 50 % EtOAc in *n*-hexane). Four compounds were isolated from **F1** - namely **FA, FB, FC** and **FD (Figure A1.3)** - three compounds from **F3** – namely **FE**, **FF** and **FG** (**Figure A1.4**) - and two compounds from **F6** – namely **FH** and **FI (Figure A1.5).**

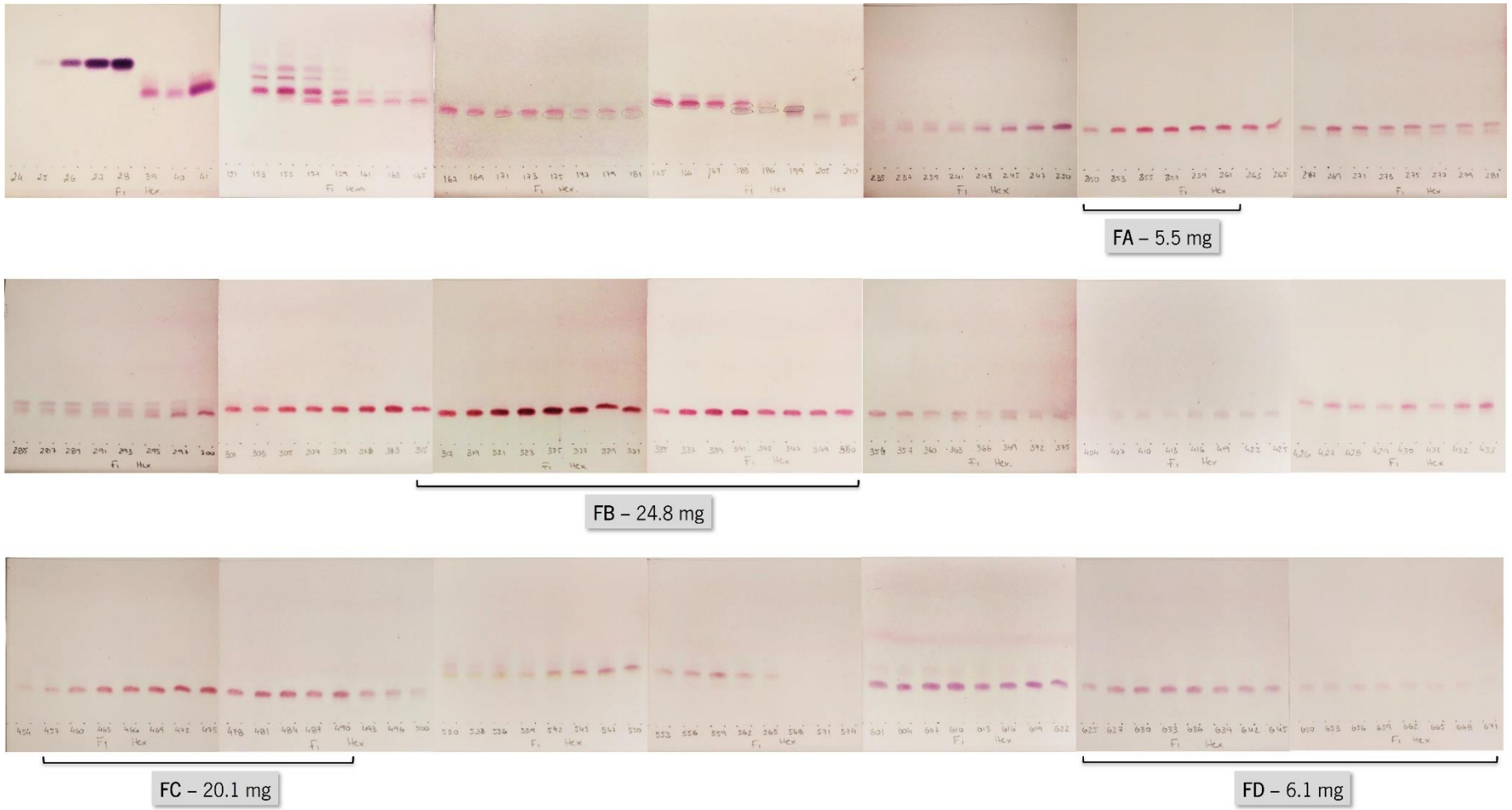


**Figure A1.3.** TLC analysis of the sub-fraction **F1**. Solvents used in the column for extraction of the 4 resulting isolated compounds and respective yields were: *n*-hexane/ EtOAc (88:12) for **FA** (5.5 mg) and **FB** (24.8 mg) *n*-hexane/ EtOAc (78:22) for **FC** (20.1 mg) and *n*-hexane/ EtOAc (70:30) for **FD** (6.1 mg).

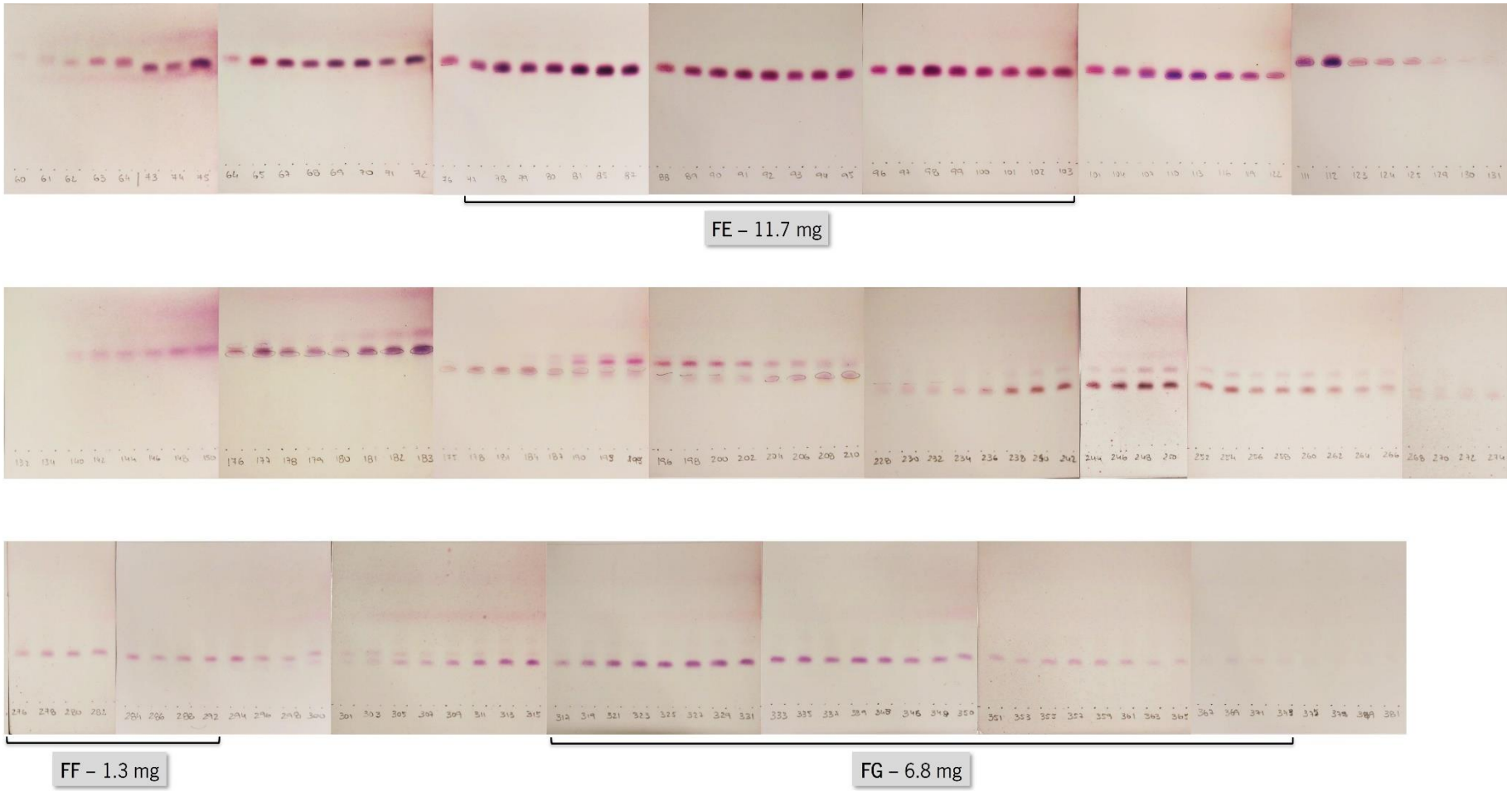


**Figure A1.4.** TLC analysis of the sub-fraction **F2**. Solvents used in the column for extraction of the 3 resulting isolated compounds and respective yields were: *n*-hexane/ EtOAc (90:10) for **FE** (11.7 mg) and *n*-hexane/ EtOAc (82:18) for **FF** (1.3 mg) and **FG** (6.8 mg).

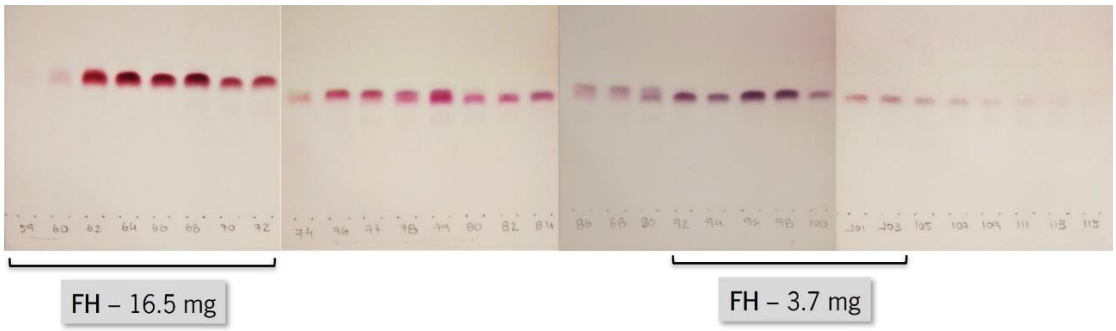


**Figure A1.5.** TLC analysis of the sub-fraction **F3**. Solvents used in the column for extraction of the 2 resulting isolated compounds and respective yields were: *n*-hexane/ EtOAc (80:20) for **FH** (16.5 mg) and **FI** (3.7 mg).

Solvents used in the columns for extraction and respective yields (**Table A1.2**) were: **F1** [**FA** and **FB**: *n*-hexane/ EtOAc (88:12); 5.5 and 24.8 mg, respectively; **FC**: *n*-hexane/ EtOAc (78:22); 20.1 mg; **FD**: *n*-hexane/ EtOAc (70:30); 6.1 mg], **F3** [**FE**: *n*-hexane/ EtOAc (90:10); 11.7 mg; **FF** and **FG**: *n*-hexane/ EtOAc (82:18); 1.3 and 6.8 mg, respectively] and **F6** [**FH** and **FI**: *n*-hexane/ EtOAc (80:20); 16.5 and 3.7 mg, respectively].

### A1.3. NMR analysis of the isolated compounds

Nuclear magnetic resonance (NMR) spectra of (**FA – FI; Table A1.3**) were recorded in a Bruker Advance III AV500 spectrometer, at 500 MHz ($^{1}$H, HMBC, HSQC) and 100 MHz ($^{13}$C), at 20 °C. The chemical shifts ($\delta$) were recorded in parts per million (ppm) relative to the residual solvent signal ($CDCl_3$). All samples dissolved in $CDCl_3$ (Sigma-Aldrich, UK) were transferred to Norell NMR tubes (5 mm; Sigma-Aldrich, UK). Samples in low amounts (< 5 mg) were transferred to Shigemi microbore tubes. MestReNova software (version 14) was used for data processing.

Further analysis of the isolated compounds was performed by NMR but, unfortunately, the compounds suffered degradation and were not able to identify. Despite this outcome, the developed protocol can be applied to other G.EE samples with predictable isolation of the same compounds (given sample constancy of chemical composition), which is of particular interest to reproduce since they are thought to be terpenes, compounds not analyzed in this thesis but to which some properties of propolis have also been associated (Popova *et al.*, 2009; Masyita *et al.*, 2022; Zullkiflee *et al.*, 2022).

**Table A1.2.** Solvents used in Silica Column for the fractioning of the 3 selected sub-fractions (**F1**, **F3** and **F6**) and the 9 resulting isolated compounds (**FA** – **FI**); respective yields (mg) and solvents used for TLC analysis.

| G18.EE-*n*-hexane | Code name | Silica column | Color | UV active | Yield (mg) | TLC |
|---|---|---|---|---|---|---|
| Sub-fractions | Isolated compounds | Solvent system | | | | Solvent System |
| F1 | FA | *n*-hexane 88 % / EtOAc 12 % | pink | no | 5.5 | *n*-hexane 85 % / EtOAc 15 % |
| | FB | | pink | no | 24.8 | |
| | FC | *n*-hexane 78 % / EtOAc 22 % | pink | no | 20.1 | *n*-hexane 75 % / EtOAc 25 % |
| | FD | *n*-hexane 70 % / EtOAc 30 % | pink | no | 6.1 | *n*-hexane 60 % / EtOAc 40% |
| F3 | FE | *n*-hexane 90 % / EtOAc 10 % | purple | no | 11.7 | *n*-hexane 50 % / EtOAc 50 % |
| | FF | *n*-hexane 82 % / EtOAc 18 % | pink | no | 1.3 | |
| | FG | | purple | no | 6.8 | |
| F6 | FH | *n*-hexane 80 % / EtOAc 20 % | grey pink | no | 16.5 | *n*-hexane 50 % / EtOAc 50 % |
| | FI | | purple blue | no | 3.7 | |

**Table A1.3.** NMR spectra of the 9 resulting isolated compounds (**FA** – **FI**).

| Isolated compounds | NMR spectra | | | |
|---|---|---|---|---|
| | $^{1}H$ | $^{13}C$ | HMBC | HSQC |
| **FA** | Figure A1.6 | Figure A1.7 | - | - |
| **FB** | Figure A1.8 | Figure A1.9 | - | - |
| **FC** | Figure A1.10 | Figure A1.11 | Figure A1.12 | Figure A1.13 |
| **FD** | Figure A1.14 | - | - | - |
| **FE** | Figure A1.15 | - | Figure A1.16 | Figure A1.17 |
| **FF** | Figure A1.18 | - | - | - |
| **FG** | Figure A1.19 | - | Figure A1.20 | - |
| **FH** | Figure A1.21 | Figure A1.22 | - | - |
| **FI** | Figure A1.23 | Figure A1.24 | - | - |

- not performed

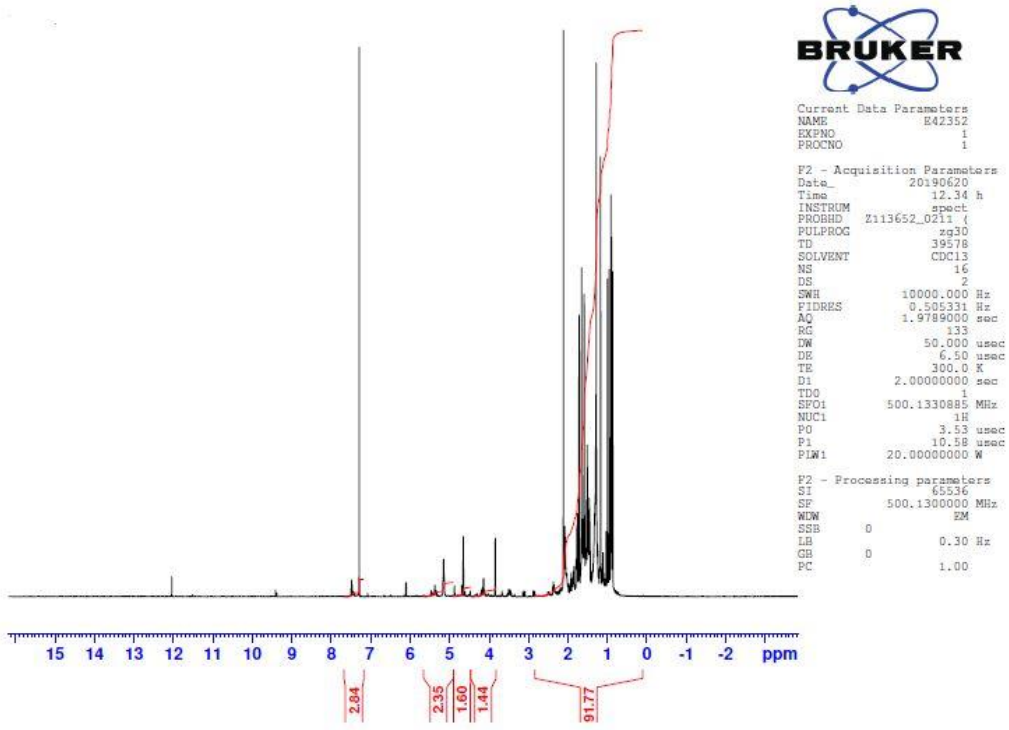


**Figure A1.6.** $^{1}H$ NMR spectrum of **FA** in $CDCl_3$ (500 MHz).

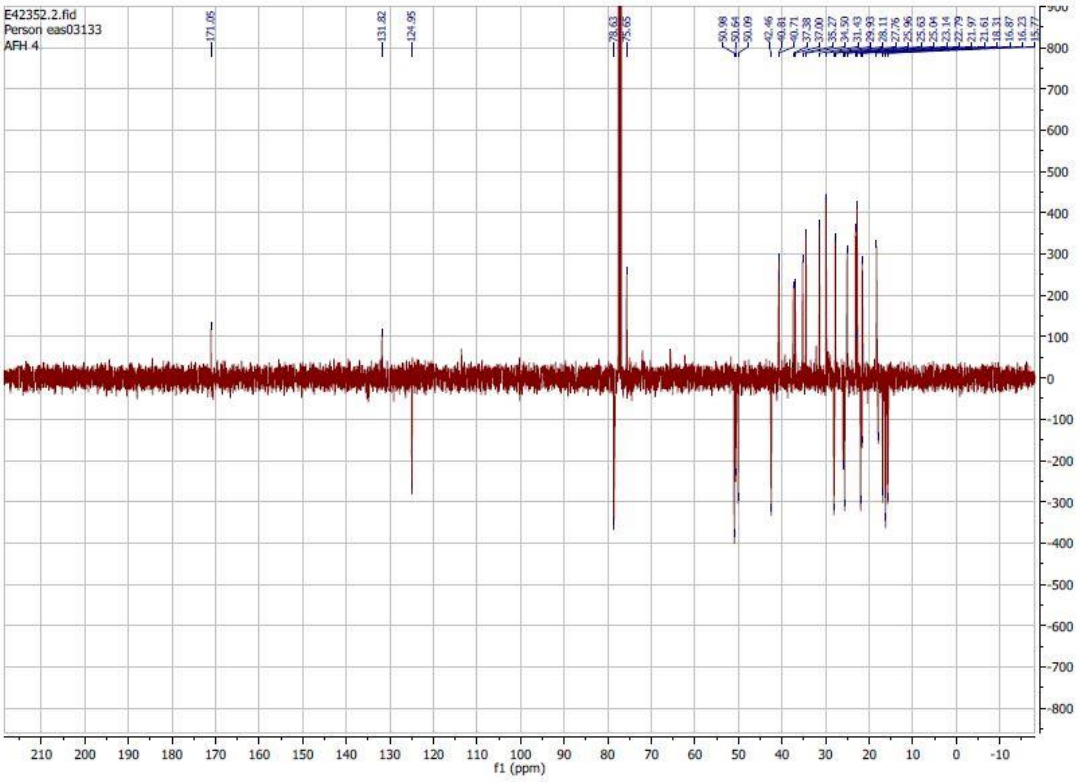


**Figure A1.7.** $^{13}C$ NMR spectrum of **FA** in $CDCl_3$ (100 MHz).

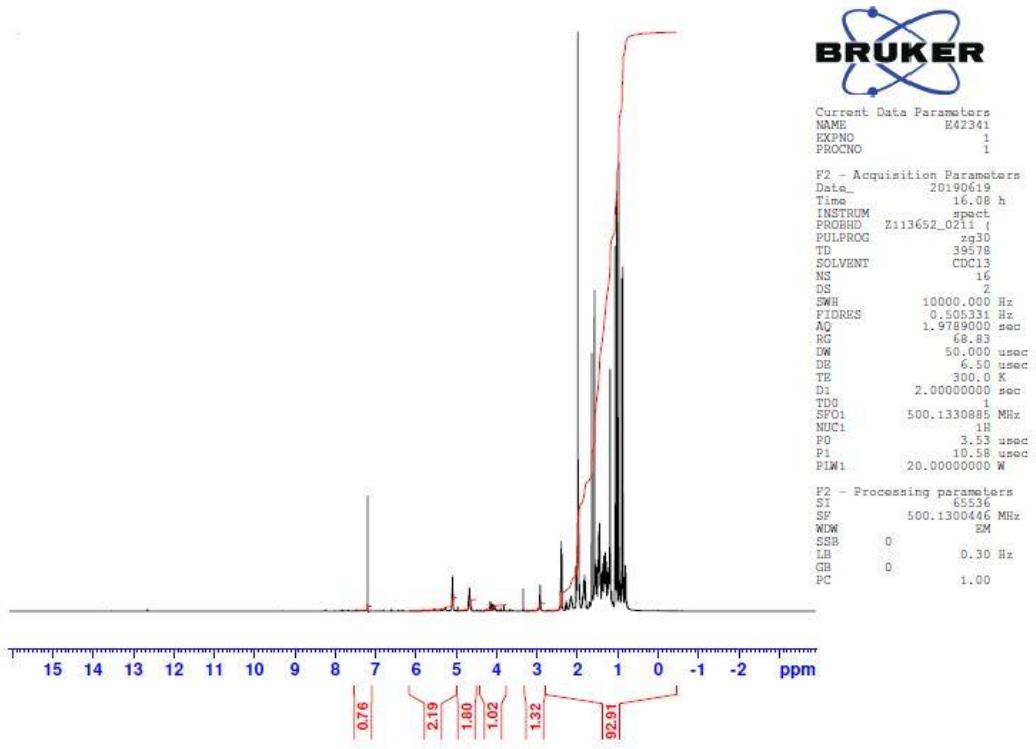


**Figure A1.8.** $^{1}H$ NMR spectrum of **FB** in $CDCl_3$ (500 MHz).

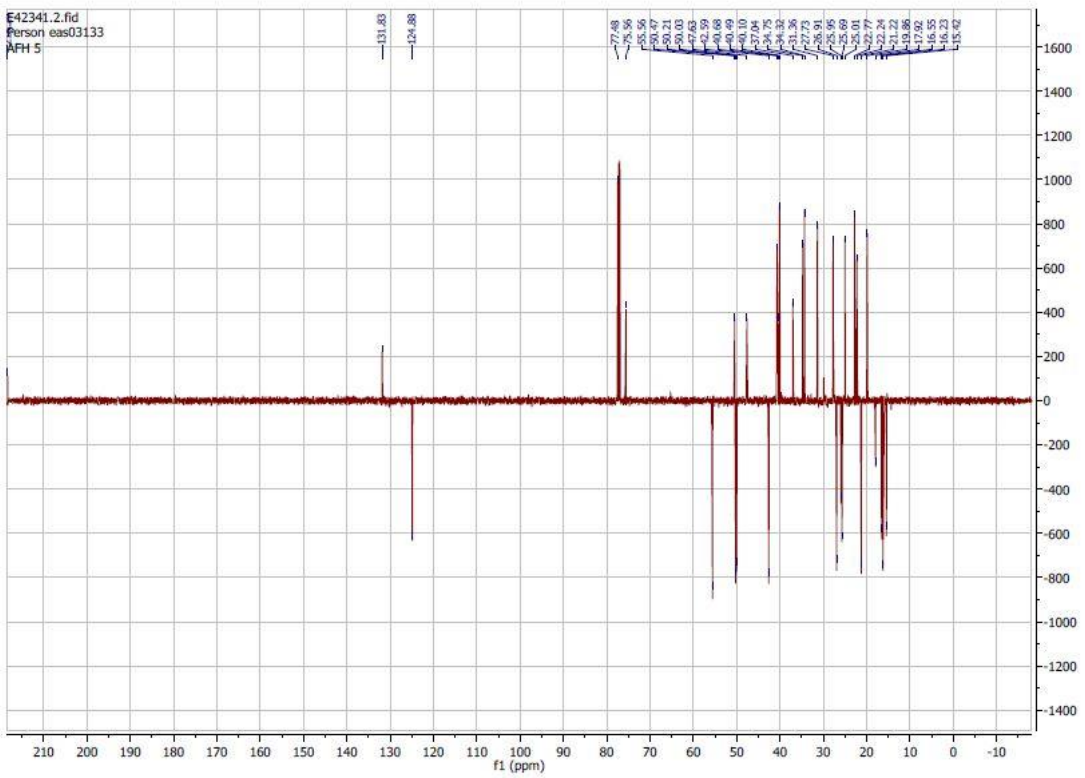


**Figure A1.9.** $^{13}C$ NMR spectrum of **FB** in $CDCl_3$ (100 MHz).

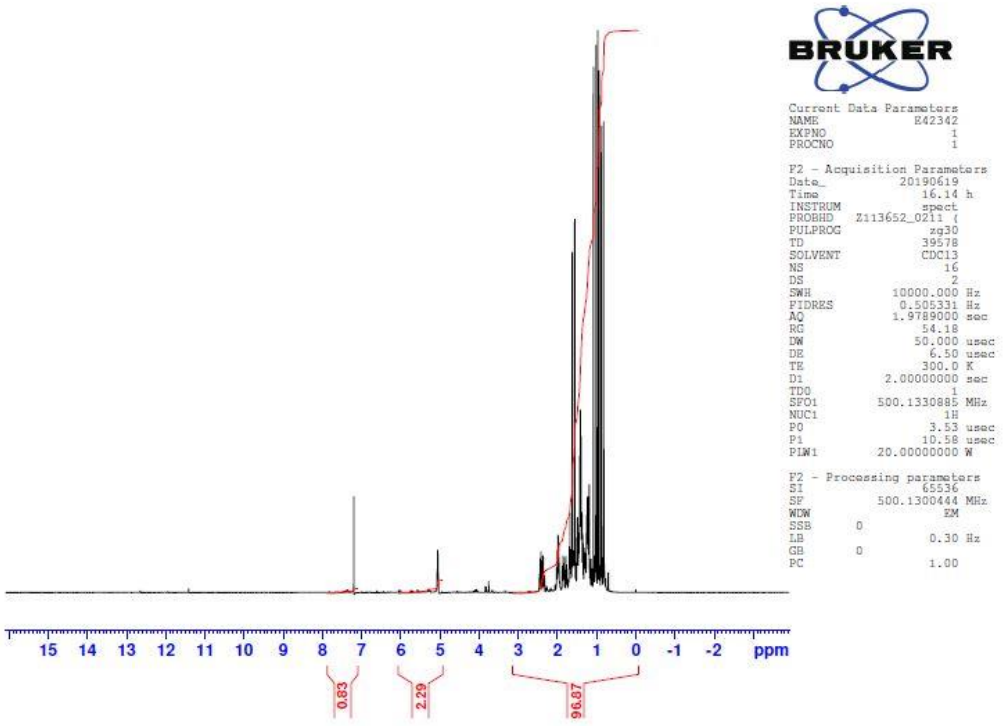


**Figure A1.10.** $^{1}H$ NMR spectrum of **FC** in $CDCl_3$ (500 MHz).

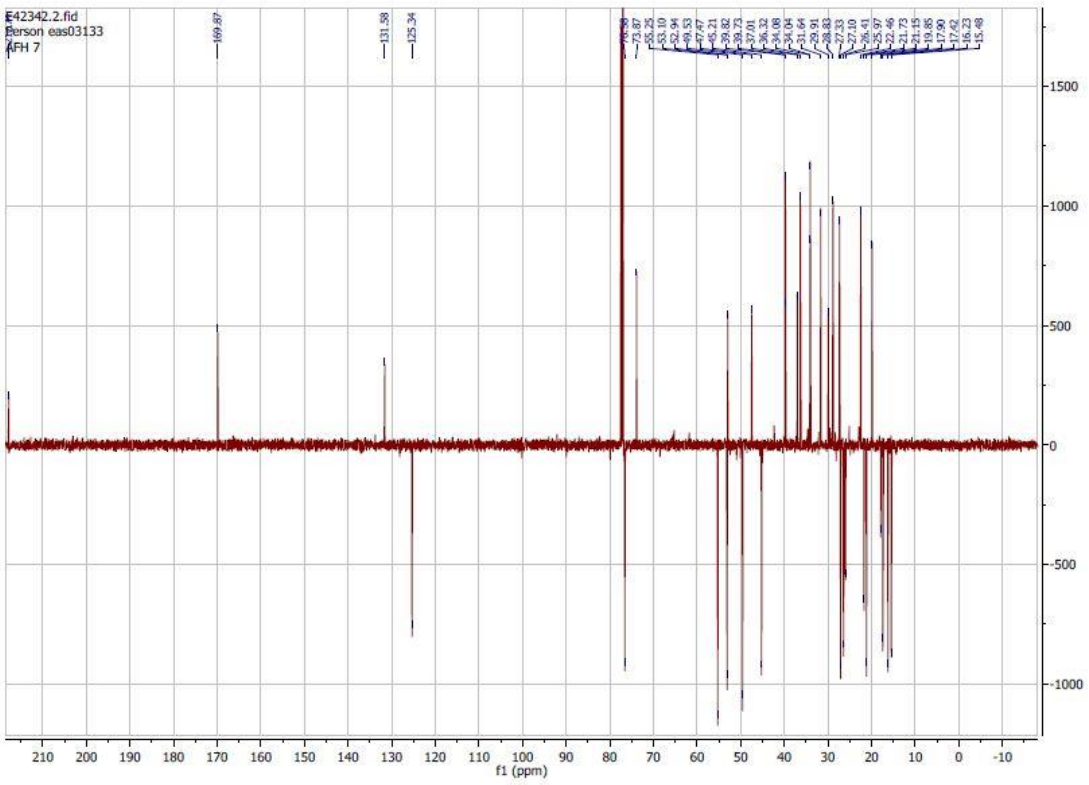


**Figure A1.11.** $^{13}C$ NMR spectrum of **FC** in $CDCl_3$ (100 MHz).

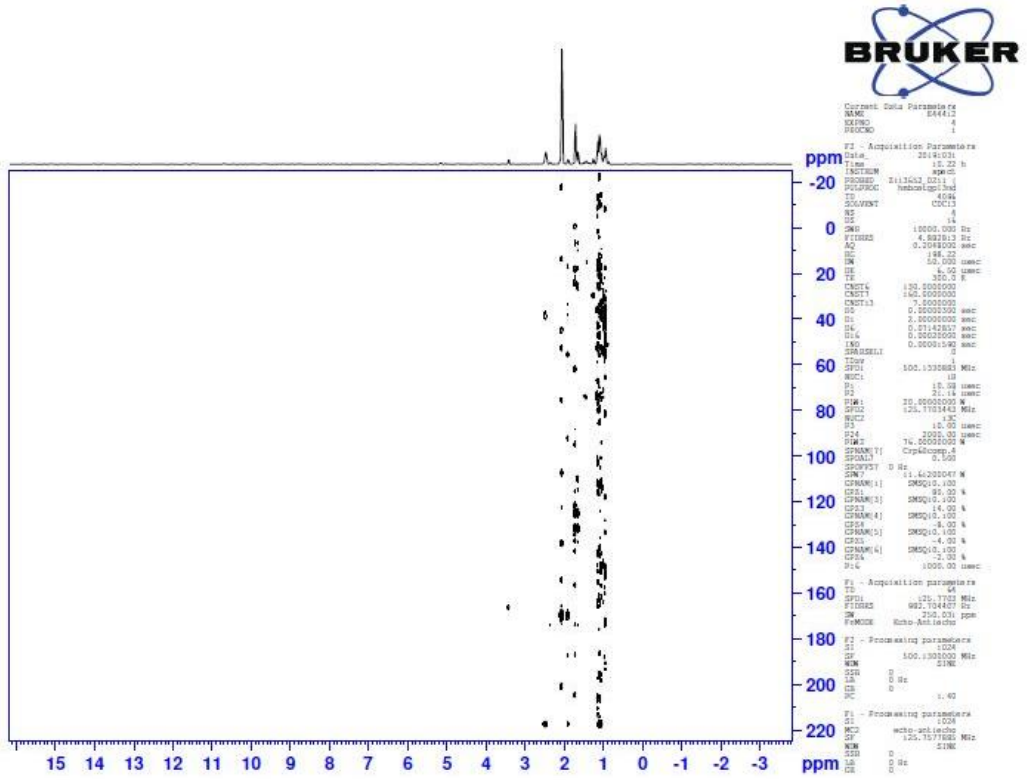


**Figure A1.12.** HMBC spectrum of **FC** in $CDCl_3$ (500 MHz).

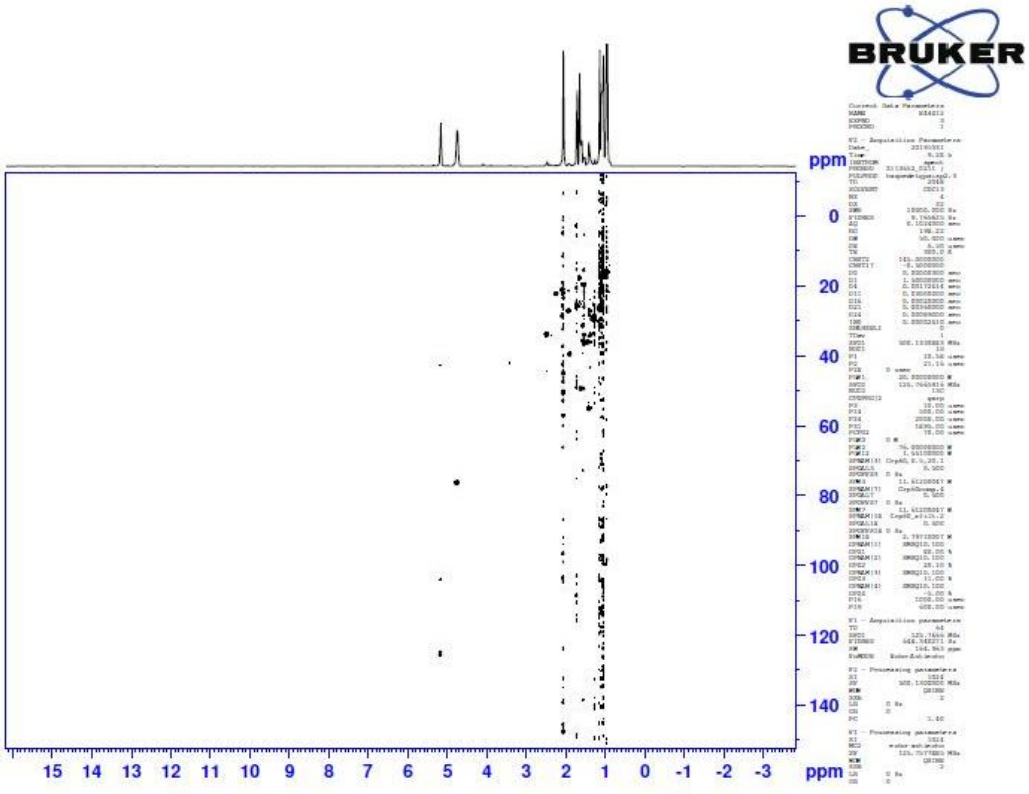


**Figure A1.13.** HSQC spectrum of **FC** in $CDCl_3$ (500 MHz).

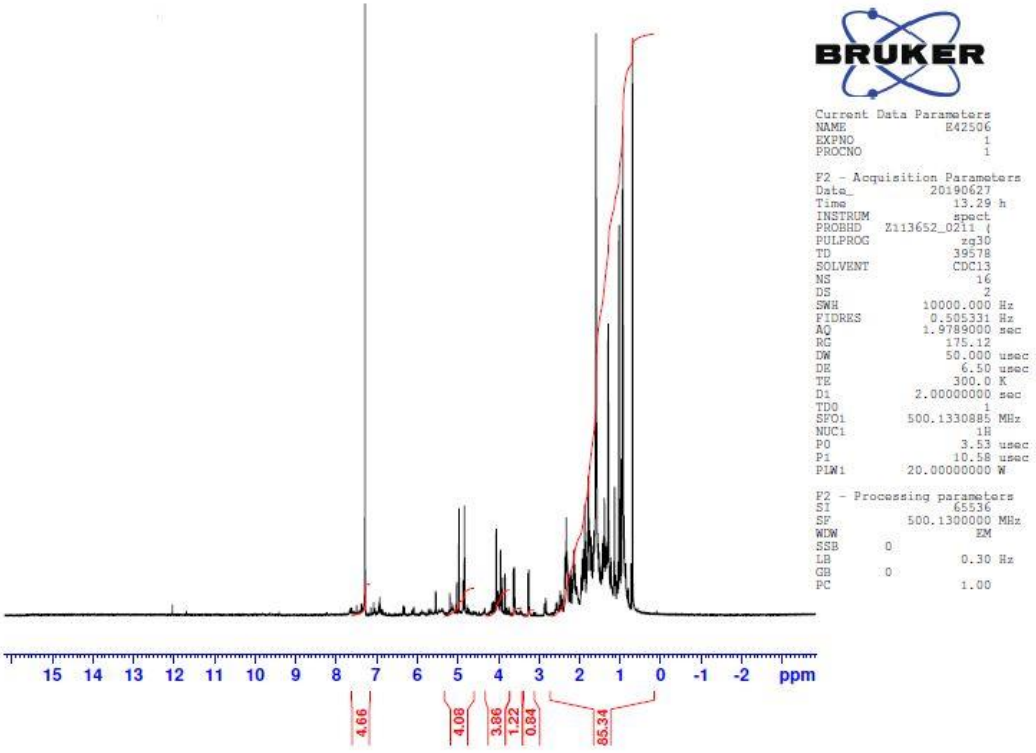


**Figure A1.14.** $^1$H NMR spectrum of **FD** in $CDCl_3$ (500 MHz).

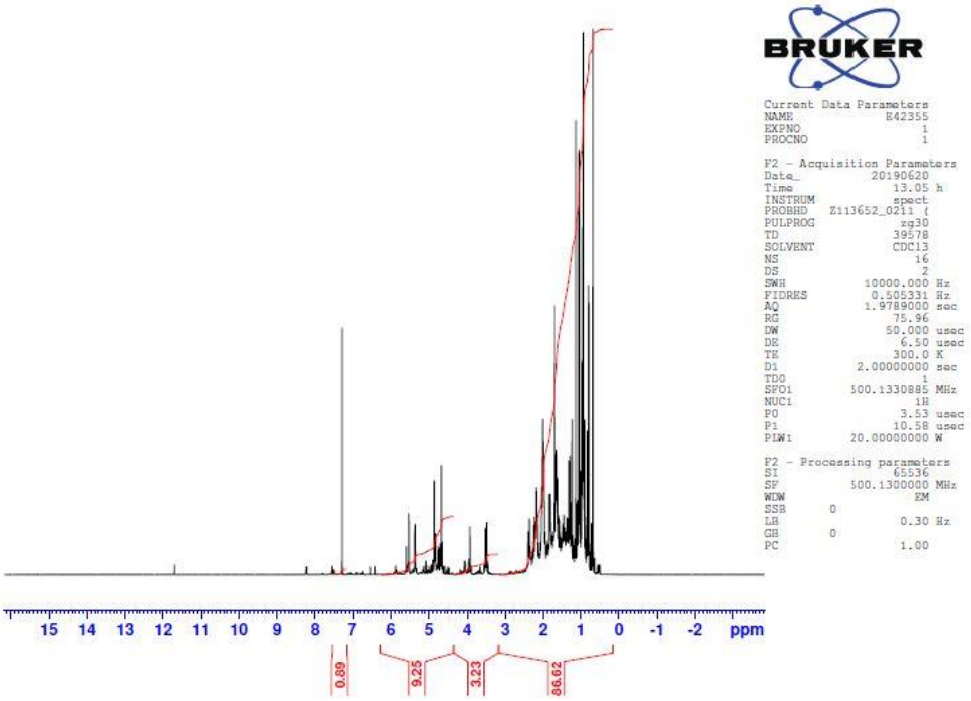


**Figure A1.15.** $^{1}$H NMR spectrum of **FE** in $CDCl_3$ (500 MHz).

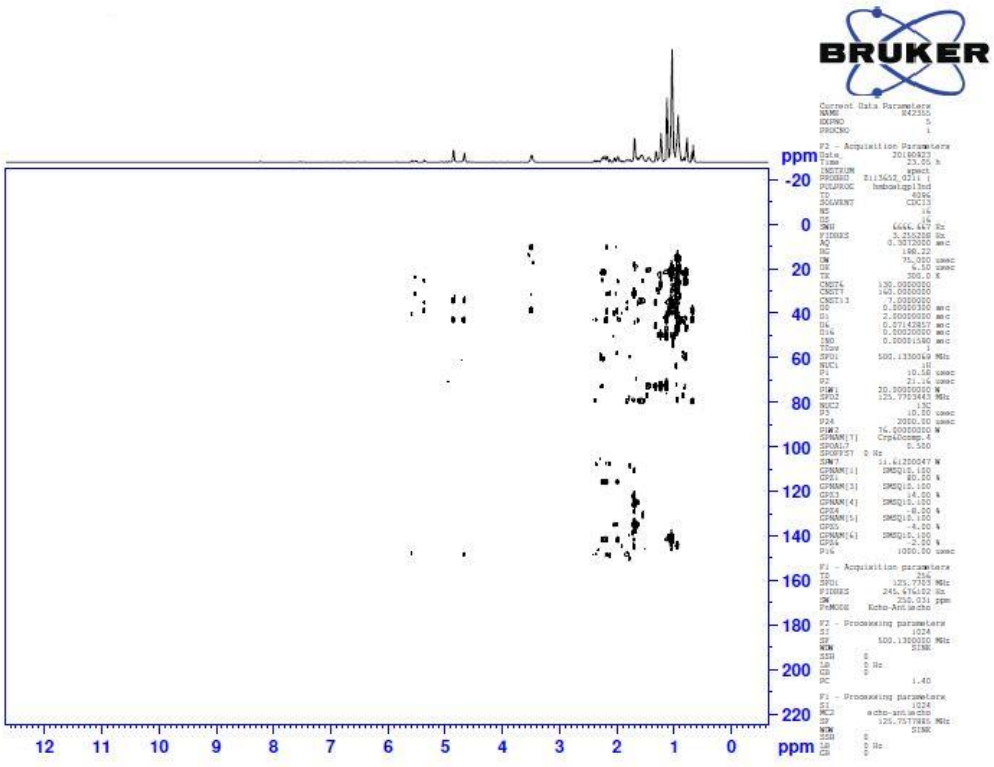


**Figure A1.16.** HMBC spectrum of **FE** in $CDCl_3$ (500 MHz).

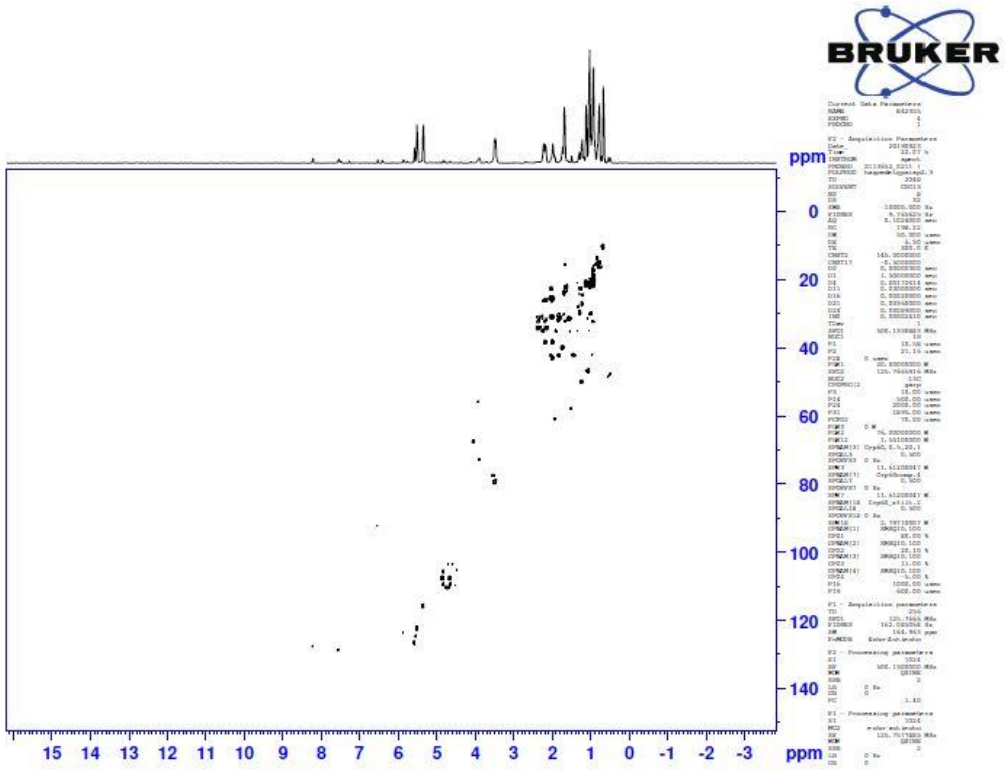


**Figure A1.17.** HSQC spectrum of **FE** in $CDCl_3$ (500 MHz).

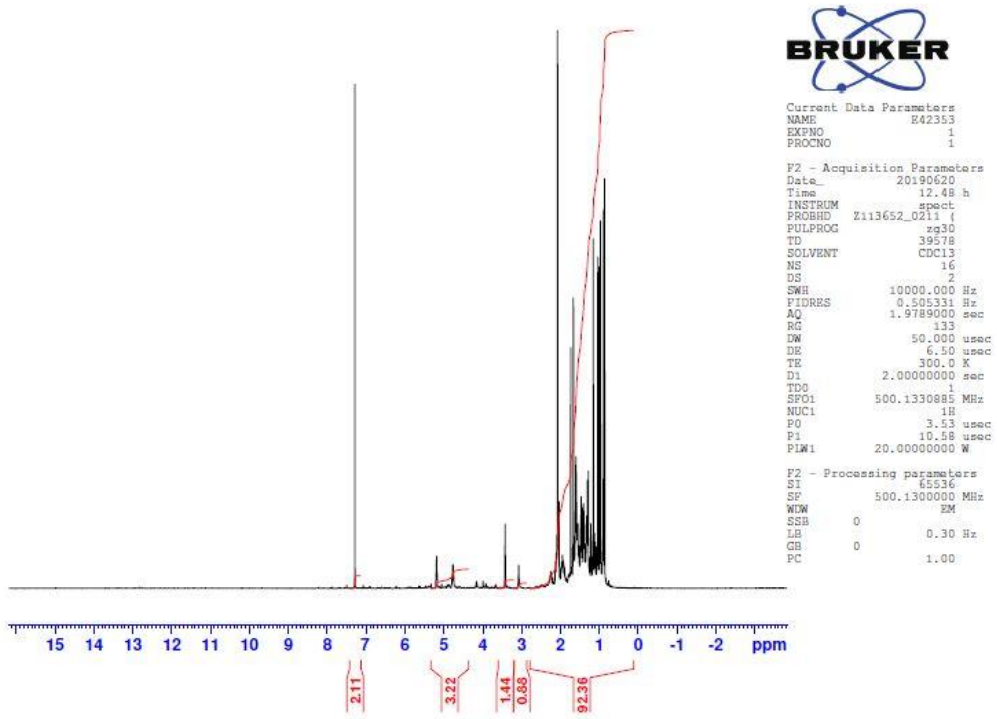


**Figure A1.18.** $^1H$ NMR spectrum of **FF** in $CDCl_3$ (500 MHz).

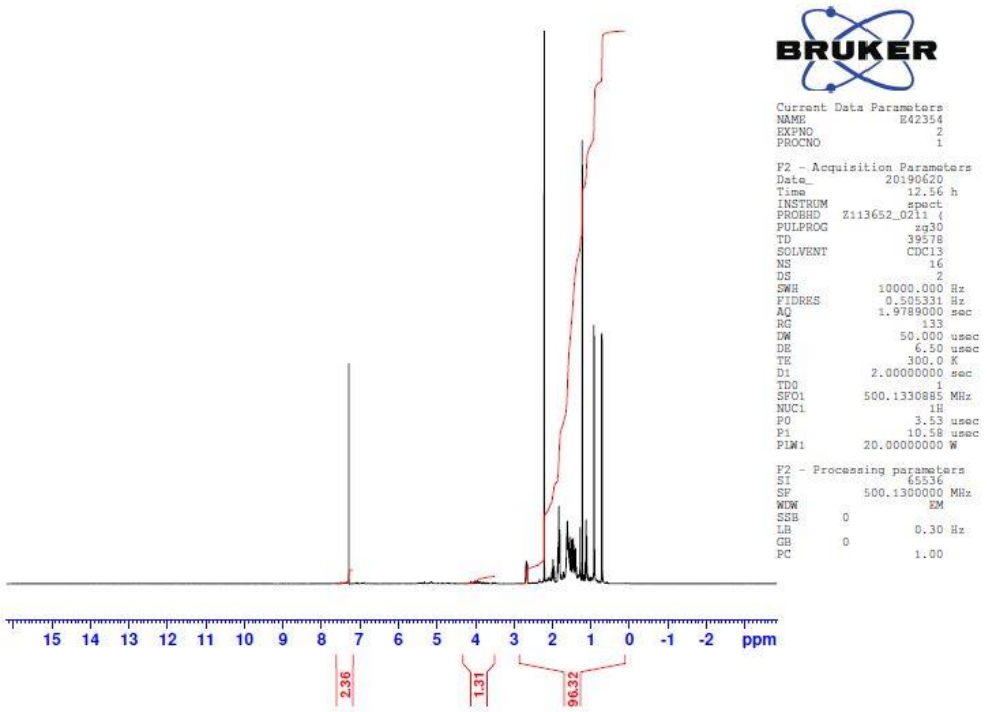


**Figure A1.19.** $^{1}$H NMR spectrum of **FG** in $CDCl_3$ (500 MHz).

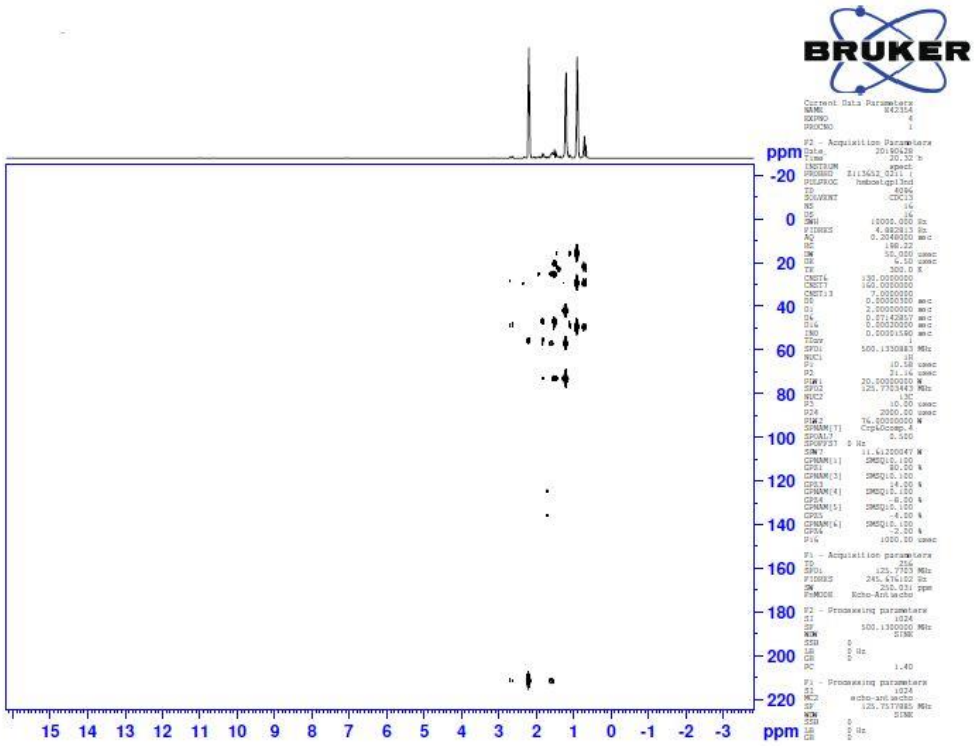


**Figure A1.20.** HMBC spectrum of **FG** in $CDCl_3$ (500 MHz).

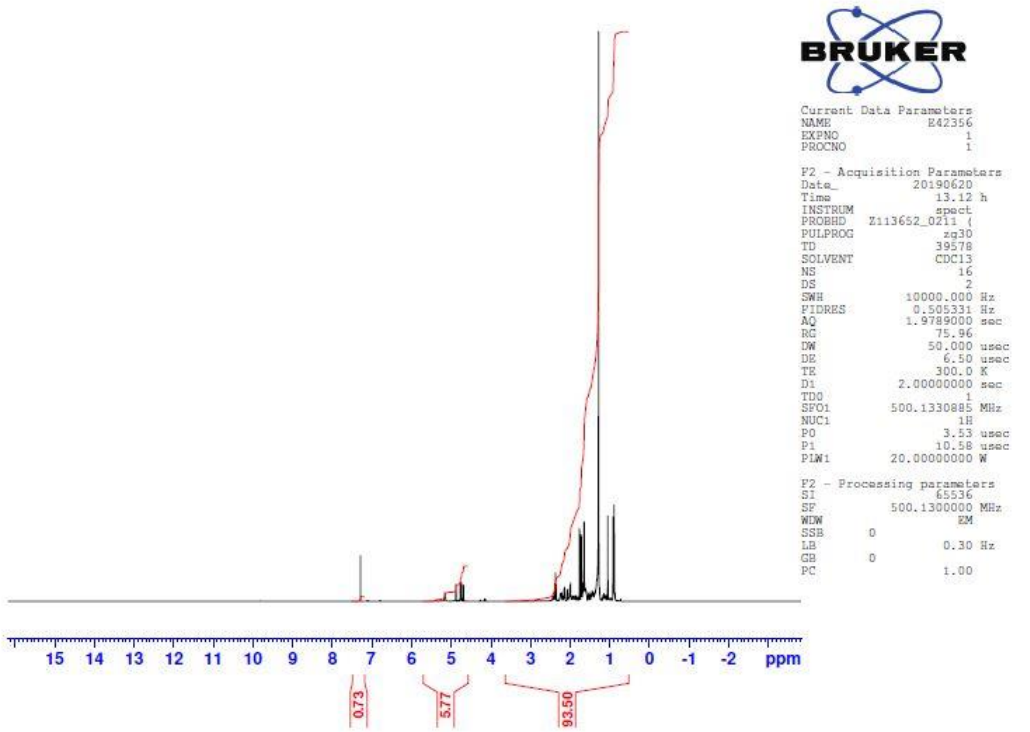


**Figure A1.21.** $^{1}$H NMR spectrum of **FH** in $CDCl_3$ (500 MHz).

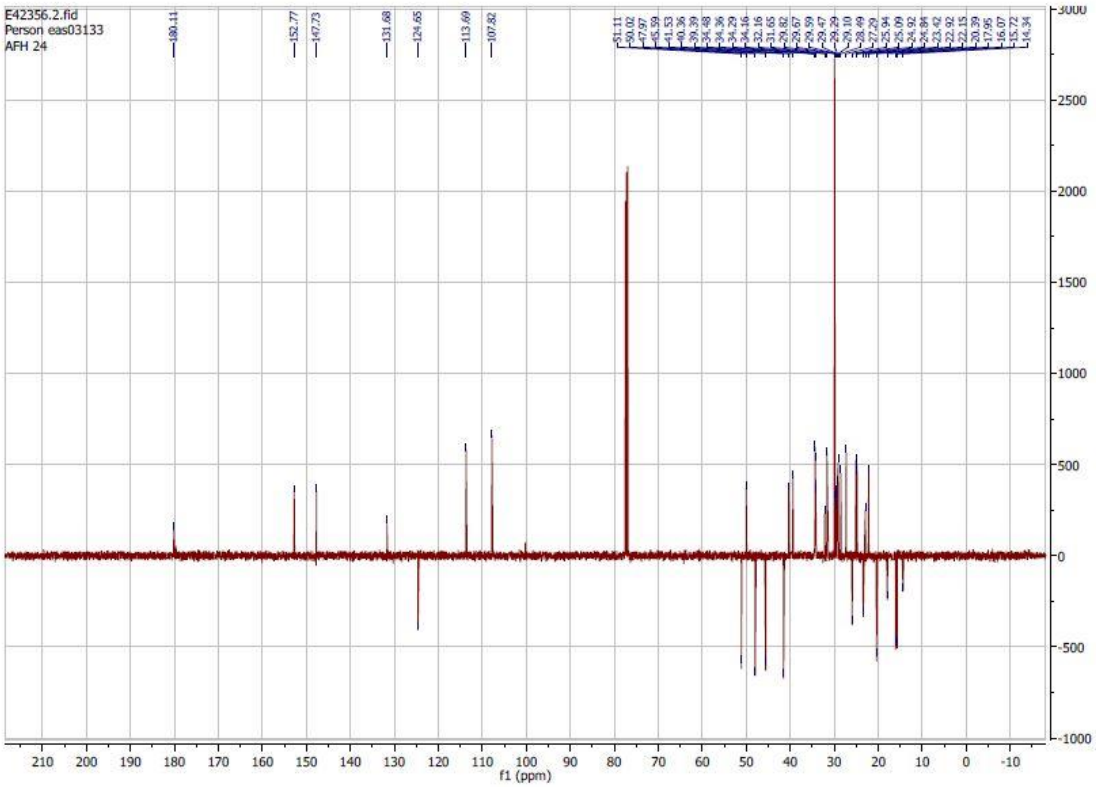


**Figure A1.22.** $^{13}$C NMR spectrum of **FH** in $CDCl_3$ (100 MHz).

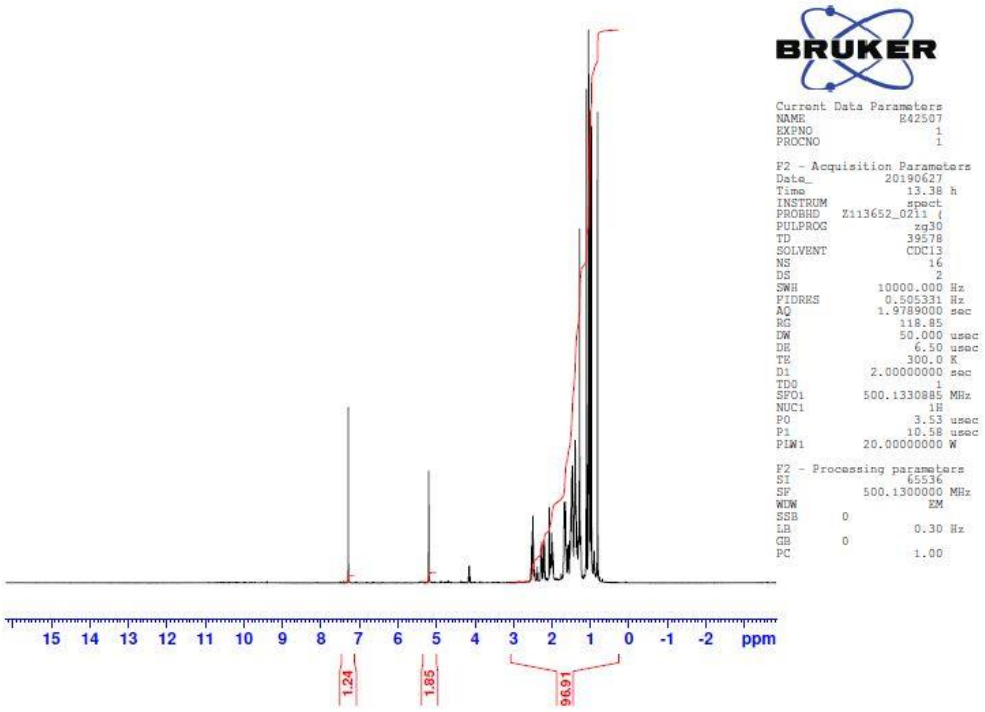


**Figure A1.23.** $^{1}$H NMR spectrum of **FI** in $CDCl_3$ (500 MHz).

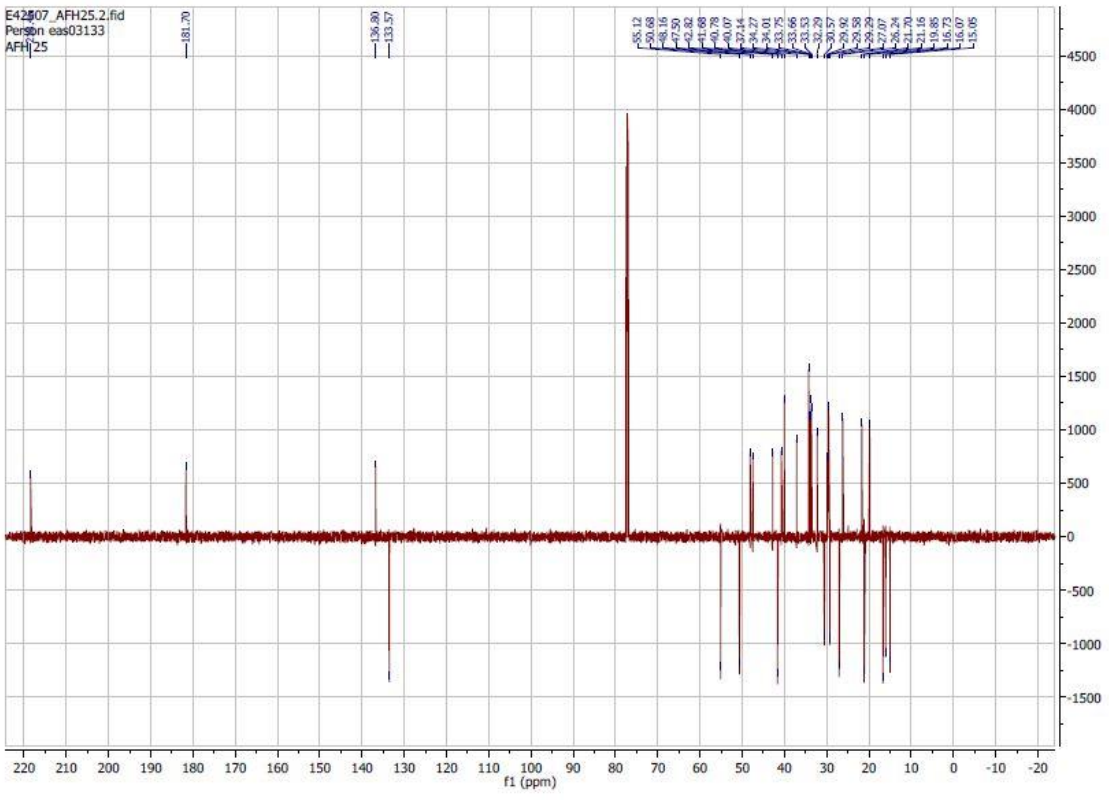


**Figure A1.24.** $^{13}$C NMR spectrum of **FI** in $CDCl_3$ (100 MHz).

## A1.4. References

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