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Chemical characterization and bioactivity of poplar, green and red propolis: a screening study with a food preservation purpose

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Dedication

I fully dedicate this work to my parents, ***Dioukhamady Diallo*** *and* ***Nana Diawara****, who have supported me financially and spiritually, and I cannot forget their immeasurable advice, blessings, and love which have been and continue to be an unwavering contribution to climb the levels. In short, I can only thank them, because no word is powerful enough to describe my gratitude.*

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Table of contents

List of figures

List of tables

List of abbreviations

ATP: Adenosine triphosphate

CA: California

CAPE: Caffeic acid phenethyl ester

CID: Collision-induced dissociation

CIMO: Centro de Investigação de Montanha

DAD: Diode array detection

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNP: Dinitrophenylhydrazine

DPPH: Diphenylpicrylhydrazyl

DW: Dry weight

ESI: Electrospray ionization tandem

ESM: Standard error of the mean

HPLC: High-performance liquid chromatography

Inc: Incorporated

ITS: Internal transcribed spacer

LC: Liquid chromatography

LOX: Lipoxygenases

m/z: Mass to charge ratio

MG-Brazil : Minas Gerais-Brazil

MO: Missour

MPO: Myeloperoxidase

MRSA: Methicillin-resistant Staphylococcus aureus

MS: Mass spectrometry

NADPH: nicotinamide adenine dinucleotide phosphate

NF-κB: nuclear factor kappa-light-chain-enhancer

NI: Non identified

ORAC: Oxygen radical absorbance capacity

PDA: Potato dextrose agar

PP: Portuguese propolis

PT: Portugal

rpm: rotation per minute

TGI: Topway global incorporated

UPLC: Ultra Performance liquid chromatography

USA: United States of America

Abstract

Propolis is a resin made by honeybees from multiple plant sources surrounding the hive. Therefore, each type of propolis from different regions has intrinsic characteristics related to their chemical composition, such as antifungal, antioxidative properties, and extrinsic such as color, both specific to their botanical origin. Thus, propolis can be regarded as a functional food with preservative properties offering an alternative to the concern of the global and growing use of synthetic preservatives considerably harming human health. The aim of this work was to study three types of propolis, namely, Portuguese propolis (from *Populus* sp.), Brazilian red propolis (from *Dalbergia ecastophyllum),* and Brazilian green propolis (from *Baccharis dracunculifolia*) in order to evaluate the antifungal potential for its application as a preservative in food. For that, the chemical composition was characterized by LC/DAD/ESI-MS^n. Also, the total phenolic content, flavonoids and antioxidant activity was evaluated through UV-Vis spectrophotometry. Concerning the antifungal properties, the values differed in relation to the types of fungi, to the inoculating concentration and mode of action. With this in mind, an analysis of their antifungal activities at two levels of concentration (0.5-1.5 g/L and 5-15 g/L) was previously evaluated *in vitro*, with the aim of selecting the best concentration of inhibition of the rotting diameter of two types of grapes, namely, red and white. Subsequently, different fungi were used to test the activity of the aforementioned propolis, namely, *Alternaria* sp., *Botrytis cinerea*, *Cladosporium* sp., *Penicillium* sp. *2, Aspergillus carbonarius* MUM04.46, and *Aspergillus carbonarius* MUM04.52. This last experiment made it possible to measure the rotting diameter of each grape and to evaluate the antifungal capacity of each type of propolis, with the final aim of using it to design an effective product that can extend the shelf life of food.

Keywords: propolis, antifungal activity, food preservative, grapes, LC/DAD/ESI-MS^n, antioxidant activity, UV-Vis.

Resumo

A própolis é uma resina produzida pelas abelhas a partir de múltiplas fontes vegetais ao redor da colmeia. Portanto, cada tipo de própolis de diferentes regiões possui características intrínsecas relacionadas à sua composição química, como antifúngica, propriedades antioxidantes, e extrínsecas como a cor, ambas específicas à sua origem botânica. Assim, a própolis pode ser considerada um alimento funcional com propriedades conservantes, oferecendo uma alternativa à preocupação mundial e crescente do uso de conservantes sintéticos que prejudicam consideravelmente a saúde humana. O objetivo deste trabalho foi estudar três tipos de própolis, a saber, própolis portuguesa (de *Populus* sp.), própolis vermelha brasileira (de *Dalbergia ecastophyllum*) e própolis verde brasileira (de *Baccharis dracunculifolia*), a fim de avaliar o potencial antifúngico para sua aplicação como um conservante em alimentos. Para isso, a composição química foi caracterizada por LC/DAD/ESI-MS^n. Também os compostos fenólicos totais, flavonoide totais e atividade antioxidante foi avaliada por espectrofotometria de UV-Vis. Em relação às propriedades antifúngicas da própolis, os valores vão depender dos tipos de fungos, da concentração de inoculação e do modo de ação. Tendo isto em mente, foi efetuada uma análise *in vitro* da atividade antifúngica em dois níveis de concentração (0,5-1,5 g/L e 5-15 g/L), com o objetivo de selecionar a melhor concentração de inibição do diâmetro de apodrecimento de dois tipos de uvas, tintas e brancas. Posteriormente, diferentes fungos foram utilizados para testar a atividade da própolis, nomeadamente, *Alternaria* sp., *Botrytis cinerea*, *Cladosporium* sp., *Penicillium* sp. 2, *Aspergillus carbonarius MUM04.46* e *Aspergillus carbonarius MUM04.52*. Esta última experiência possibilitou medir o diâmetro de apodrecimento de cada uva e avaliar a capacidade antifúngica de cada tipo de própolis, com o objetivo final de usar essa informação para desenvolver um produto eficaz que possa prolongar a vida útil dos alimentos.

Palavras-chave: própolis, antifúngico, conservante de alimentos, uva, própolis, LC/DAD/ESI-MS^n, atividade antioxidante, UV-Vis.

1 General introduction

An interest in nutraceuticals, therapeutic or medicinal foods, has been perceived in recent years. This interest is due to the idea that these products can contribute to well-being through their competitive advantage and thus reduce the risk of developing certain diseases. Likewise, bee products are one of them and have stood out with their galloping success.

By their adaptability to the many changes that the world has undergone, bees have been able to remain and evolve in the animal kingdom for more than 125 million years. Moreover, for their chemistry and applicability, they have long been exploited by man for various purposes, such as remedies, or consumption. Among the bee products are honey, beeswax, venom, propolis, pollen and royal jelly. Since antiquity, man has been able to distinguish and use as a remedy one of the products of bees, which is none other than propolis. Indeed, it is seen as being the most important "chemical weapon" of the bees which use it to counter pathogenic microorganisms in addition to these various biological activities. That said, by its fame and its success in the business market, propolis has attracted the interest of several fields of study such as pharmacology and chemistry around the world.

The many natural benefits of propolis, which is a mixture of natural substances, are linked to climatic and geographical factors around the beehive where it is produced, in fact, the many varieties of plants that bees exploit make its phenolic composition unique which is produced from the secondary metabolism of plants correlating with its many biological properties. Indeed, phenolic compounds provide propolis with antioxidant characteristics, thus, a characterization of its chemical compounds is necessary for an accentuated production and at the same time its commercial value for the promotion of its application (Falcão SI, 2013).

Consequently, the aim of this work will be the evaluation of the different bioactivity of phenolic propolis extracts obtained from the most common types: European propolis and Brazilian green/red propolis. The objective will focus on the antimicrobial potential for its application as a preservative in food.

For this we proceeded:

1- To characterize the physicochemical parameters of the different propolis samples. Moreover, to quantify, through spectrophotometric methods, the total phenolic and flavonoids content.

2- To evaluate the antioxidant activity.

3- To study the antifungal properties and finally, a possible application as a preservative in food.

1.1 Propolis

1.1.1 Definition

Propolis or bee glue is a substance that is part of bee products, such as honey, bee pollen, bee bread, royal jelly, beeswax and bee venom. The word propolis comes from the Greek and can be dissected in two words, that means, the "pro" signifies "in front" and "polis" insinuates "city"; In other words, it is defined as a “hive defensive substance” (Anjum SI *et al*., 2019). Propolis is presented in the form of resin and has a resemblance to wax. Besides, there are many functions that it performs within the beehive such as a building and sealing material, and by its morphology, it acts as a defensive agent covering the internal walls of the hive. On sunny days, a handful of worker bees venture out to produce resin, by mixing different vegetable resins with wax (Bankova *et al*., 2018). Thereby, the color of propolis is linked to the different botanical sources surrounding the hive (geographic region) generally harvested from species of *poplar*, *birch*, *elm*, *alder*, *beech*, *horse chestnut, clusia,* and *conifers*. Apart from the color of propolis, its chemical composition can also differ from other types. Naturally, it is affected by environmental conditions (illumination, altitude, and food availability), bee species, and plant sources (Rojczyk E *et al*., 2020).

1.1.2 The history of propolis

In history, propolis was first seen on Egyptian vases, where the representation of producing bees was made. Recognized by its many benefits, the Egyptians appreciated it to the point of naming the pharaoh by the title of "Bee King", and apart from the fact that they associated the bees with the Gods, they used it to embalm the corpses. On the other hand, polyanthus is a Greek fragrance, of which propolis was used as the main ingredient (Rojczyk E *et al*., 2020). The recognition of the medicinal power of propolis has been made by many scientists and doctors (Greek and Roman) such as Aristotle, Pliny the Elder, Galen, Cornelius Celsus, and Dioscorides (Anjum SI *et al*., 2019; Toreti VC *et al*., 2013). Plus, in the old days it was used as a remedy, in order to treat ulcers. In fact, Hippocrates (father of modern medicine) is one of the first doctors to use this substance as an ointment. Incidentally, bee

glue was called "tzori" (Hebrew) in the Old Testament and made use of a therapeutic balm and a special incense component by the Persians, Arabs, and Jews (Rojczyk E *et al*., 2020). The use of propolis was also widespread in Europe where the London pharmacopeias of the seventeenth century listed propolis as a healing gel in the treatment of inflammation and wounds, which originated from the buds of black poplar (Kuropatnicki AK *et al*., 2013). Moreover, the Incas used propolis as an antipyretic agent and the Persians made use of it as a medicine against eczemas, myalgia, and rheumatism (Rojczyk E *et al*., 2020).

1.1.3 Type of propolis

The physicochemical characteristics of propolis are highly dependent on the plant source from which it was produced (**Figure 1**), therefore, there is a difference in the melting point of these resins, in fact, some types melt between 60 ° C and 70 ° C and others at 100 ° C (Martinotti S & Ranzato E, 2015; Anjum SI *et al*., 2019; Wagh VD, 2013). In addition, by its complex and hard nature at low temperature, a number of solvents have been able to demonstrate their extracting power of phenolic compounds from propolis compounds for commercialization, namely ethanol, methanol, chloroform, ether and acetone. And more specifically, ethanol is more appreciated (Martinotti S & Ranzato E, 2015; Ramos AFN & Miranda JL, 2007; Trusheva *et al*., 2007). On the commercial level, propolis can be represented in different aspects, namely in the form of toothpastes, lozenges, mouthwashes, creams, gels, cough syrups, wine, cakes, powder, soap, chewing gum and tablets (Anjum SI *et al*., 2019).

Figure 1. Aspect and botanical source of different propolis (Bogdanov S, 2016; Biodiversity4all, 2021). (a): Portuguese Propolis: *Populus* sp., (b): Brazilian green propolis: *Baccharis dracunculifolia*, (c): Brazilian red propolis: *Dalbergia ecastophyllum*.

1.1.4 The different fields of application of propolis

The nutritional composition of foods helping to protect the human body is attracting the attention of many consumers around the world. However, the development and the enrichment of these food products are of need. That said, propolis by its multifunctional effects namely antioxidants, anti-inflammatory, antibacterial and antiviral, and its food preservative properties such as bactericidal and bacteriostatic make it a useful raw material. In particular, the antioxidant power of propolis, which is widely sought by food industries is linked to the many compounds it contains and which are correlated with the total content of polyphenols and flavonoids (Liaudanskas M *et al*., 2021).

Along with the fields of medicine and dentistry as well as in the pharmaceutical, cosmetic, and food industries, where a strategic selection of propolis (**Figure 2**) based on the richness of its phenolic composition has been established (Azemin *et al*., 2017; Freiresa *et al*., 2016). Besides, pests and pathogens pose a great threat to honeycombs. To counter these intrusions, some tropical bees and more specifically the species *Apis mellifera* have resorted to the benefits of propolis (Drescher N *et al*., 2019). Indeed, many studies have confirmed the ameliorative power of the immune system of bees (Simone-Finstrom M *et al*., 2009; Borba RS *et al*., 2015; Simone-Finstrom M, 2017) as well as the inhibitory power of the resin against the growth of microbial pathogens, such as *Paenibacillus* larvae. These aforementioned benefits make the propolis a crucial weapon and remedy for the health of the bee colony (**Figure 2**).

Figure 2. Collection and discharge of resin by bees (*Apis mellifera*) (Wilson MB *et al*., 2013; Tomazzoli MM *et al*., 2019).

1.1.5 Propolis: composition and plant origin

1.1.5.1 Resin sources

Several types of resin exist throughout the world namely, in North America, in Europe, in non-tropical regions of Asia, and also in New Zealand, the type of propolis found in these areas correspond to mostly poplar species, likewise, in Egypt scientists have discovered that in the composition of Egyptian propolis are those of poplar as well as other types of constituents namely esters of caffeic acid and long-chain fatty alcohols, in particular tetradecanol, hexadecanol, and dodecanol (Anjum SI *et al*., 2019). On the other hand, the composition (flavonols and flavones) of *birch* propolis found in Russia was different from those of poplar having *Betula verrucosa* as a plant source. Also, diterpenes, lignans, prenylated derivatives of *p*-coumaric acid as well as acetophenone and flavonoids (not similar to those of poplars) enter into the composition of Brazilian propolis. Subsequently, it has been seen that the *Baccharis dracunculifolia* contains a level of artepillin C much higher than CAPE (phenethyl ester of caffeic acid) (Chan GCF *et al*., 2013, Ferreira *et al*., 2017). Strangely, the propolis of the tropical zones showed over time a unique constitution of the resin namely germacren D, ledol, and spatulenol (Anjum SI *et al*., 2019). Further, *Clusia rosea* represents the plant source of propolis for Cuba, and in its composition is polyisoprenylated benzophenone, and the latter is representatively different from the composition of propolis found in Brazil and Europe (Anjum SI *et al*., 2019).

1.1.5.2 Chemical components of propolis

In the past, the general composition of propolis has been established approximately as following 50% resin, 30% wax, 10% essential oils, and 5% pollen, to finish the other organic components and impurities represent the 5% remaining. By the time at least 300 chemical compounds have been identified in its composition harvested both in temperate and tropical zones (Anjum SI *et al*., 2019). A standard has been established for different propolis represented in **Table 1**.

Table 1. Standard for poplar and green propolis (Bogdanov S & Bankova V, 2011; Popova MP *et al*., 2007).

Component	**Poplar propolis minimum value, (g/100 g)**	**Green propolis minimum value (g/100 g)**
Balsam	45	34
Total phenolics	21	7
Total flavones and flavonols	5	-
Total flavanones and dihydrofalvonols	4	-
Total flavonoids	9	1
Beeswax	Maximum: 25	Maximum: 25
Insoluble matter	Maximum: 5	Maximum: 5
Ash content	-	Maximum: 5

-: Non specified

The many chemical compounds (**Table 2**) of a similar nature identified in propolis are such as polyphenols; benzoic acids and derivatives; cinnamic alcohol and cinnamic acid and their derivatives; sesquiterpenes and triterpene hydrocarbons; benzaldehyde derivatives; alcohols, ketones, and heteroaromatics; terpenes and sesquiterpene alcohols and their derivatives; aliphatic hydrocarbons; minerals; sterols and steroidal hydrocarbons; sugars and amino acids (Wagh VD, 2013). Moreover, amino acid compounds are 32 in number, and among which 7 are known as essential, including vitamin B1 (thiamine), vitamin PP

(nicotinic acid), and provitamin A (Cauich-Kumul R & Campos MRS, 2019). Furthermore, phenolic compounds are at the center of scientific interest and, in total 50 have been identified, the example is taken on benzoic acids, cinnamic acids and their derivatives, as well as flavonoids (flavones), flavonols (mainly quercetin and galangin) and flavanones, such as pinocembrin. Subsequently, calcium, potassium, sodium, magnesium, iron, aluminum, phosphorus, silicon, vanadium, and strontium are microelements also present in propolis (Cauich-Kumul R & Campos MRS, 2019).

Table 2. General composition of propolis (Bogdanov S, 2016).

	Substances
Balsam 40 - 70 % Ethanol soluble Poplar origin	Phenolic acids, flavonoids, lignans, alcohols, aldehydes esters, aliphatic acids and aliphatic esters and ketones. Mono-and sesquiterpenes, aromatic compounds.
Essential oils 1-3 % ethanol soluble	Mono-, and sesquiterpenes, aromatic compounds
Non-balsam Ethanol insoluble Wax: 20-35 % Beeswax origin	Beeswax components
Others: 5 % partly ethanol soluble bee and pollen origin	Mainly minerals average ash content 2.1% Polysaccharides: 2% proteins, amino acids, amines and amides: 0.7% traces of carbohydrates, lactones, quinones, steroids, vitamins

In its composition sugars are also present, however, their origins still remain ambiguous, which leads to assumptions such that they were accidentally inserted during the making process of the resin or from an interaction of the bees with the resin or both (Wagh VD, 2013). Besides, the plant sources that produce the volatile compounds have a low amount

(Anjum SI *et al*., 2019). It should be noted that polyphenols, aromatic acids, and diterpene acids represent the essential compounds of the diversity of propolis (Wagh VD, 2013).

1.1.6 Bioactive compounds

Flavonoids are widely present in propolis (**Figure 3**), in the literature 38 flavones, 12 derivatives of benzoic acid, 14 derivatives of cinnamyl alcohol and cinnamic acid, phenols and terpenes have been registered (Cauich-Kumul R & Campos MRS, 2019). The resin is known for its composition rich in polyphenols, and pinocembrin has been specifically identified by researchers from Chili as having significant biological activity within the main compounds (Cauich-Kumul R & Campos MRS, 2019). Besides, it has been noted that it also exhibits antimicrobial, anti-inflammatory, antioxidant and anticancer characteristics (Saad MA *et al*., 2015; Lan X *et al*., 2016). On the other hand, Li *et al*. (2016) from China revealed that in the composition of propolis are found 6 phenolic acids and 5 flavonoids (caffeic acid, ferulic acid, isoferulic acid, 3,4-dimethoxycinnamic acid, pinobanksin, benzyl ester of caffeic acid, ester caffeic acid phenylethyl, apigenin, pinocembrin, chrysin and galangin), **Table 3**.

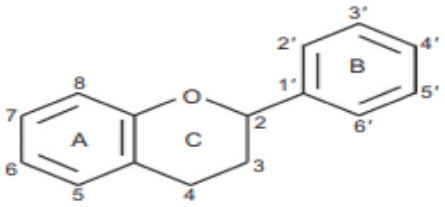

Figure 3. General structure of flavonoids (Cauich-Kumul R & Campos MRS, 2019).

Table 3. Propolis content in flavonoids (Li A *et al*., 2016).

Flavonoid	Quantity (%)
Pinocembrin	21.4
Galangin	5.0
Chrysin	4.8
Quercetin	2.2
Tectochrysin	1.1

Activities such as biological and pharmaceutical (anticancer, anti-inflammatory, immunomodulating, analgesic and antioxidant agents) are represented by polyphenols (**Figure 4**) (Gómez AL *et al*., 2006, 2016). The variation in the number of phenolic compounds (reported as main bioactive compounds) from different plant sources has influenced their biological properties (Cauich-Kumul R & Campos MRS, 2019). Naturally, it is essential to perform a study on the quantification of these compounds from different regions in order to define their quality parameters (Cauich-Kumul & Campos, 2019).

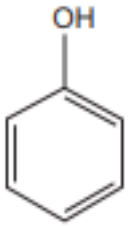

Figure 4. Structural representation of phenols (Cauich-Kumul R & Campos MRS, 2019).

1.1.7 Biological properties

Biological properties of propolis, such as, antibacterial, anti-inflammatory, antiviral, antioxidant, antiprotozoal, anesthetic, antitumoral, anticancer, antifungal, antiseptic, antimutagenic, antihepatotoxic, cytotoxic activity (Anjum SI *et al*., 2019) are summarized in the **Table 4** and **Figure 5**. These biological properties are associated with the powerful antioxidant and anti-free radical activity of the phenolic compounds it abounds. These said characteristics proved for years its broad spectrum of biological and pharmaceutical properties (Bogdanov S, 2011). Therefore, its medical application such as in stomatology and odontology, otorhinolaryngologic and respiratory diseases, gastroenterology, against cancer, and in the treatment of skin lesions, wounds, burns, and ulcers (Bogdanov S, 2011).

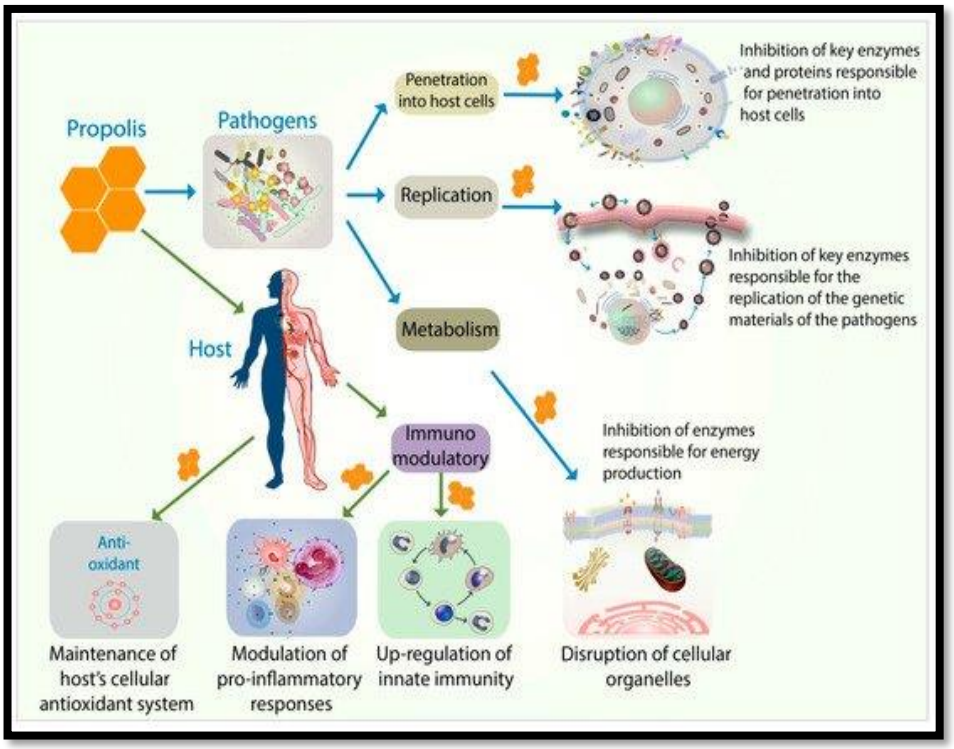

Figure 5. Various mechanisms of action of propolis (Zulhendri F *et al*., 2021).

Table 4. Summarizes the different biological properties of the propolis compounds.

Propolis type tested	**Component**	**Biological activity**	**References**
All propolis types	Polyphenols, flavonoids	Antibacterial, anti-inflammatory, antiviral, antioxidant, antiprotozoal, anesthetic, antitumoral, anticancer, antifungal, antiseptic, antimutagenic, antihepatotoxic and cytotoxic activity	(Sforcin JM *et al*., 2017; Rajpara S *et al*., 2009, Sforcin JM, 2016; Toreti VC *et al*., 2013)
Poplar type	CAPE and other caffeates	Antioxidant, anti-inflammatory, antitumor, antibacterial, antiviral, fungicide, immunomodulatory, cardioprotective, hepatoprotective and antiosteoporosis	(Bankova V, 2009), (Farooqui T & Farooqui A, 2010).

Poplar and green propolis	Caffeic acid	Antiviral, antioxidant, antitumor	(Bankova V, 2009)
Green propolis	Artepillin C	Antioxidant, antimicrobial, anti-inflammatory, antidiabetic, neuroprotective, gastroprotective, immunomodulatory and anticancer effects	(Shahinozzaman MD *et al*., 2020)
Pacific propolis	Prenylated flavanones	Antioxidant, anticancer and apoptosis inducing	(Bankova V, 2009)
Greece, Crete and Malta	Diterpenes	Antibacterial and antifungal	(Popova MP *et al*., 2012)
Brazil, Poland	Essential oils	Antibacterial	(De Alburquerque IL *et al*., 2008)
Red propolis, Brazil	Methyl eugenol	Anticancer, anti-inflammatory and antioxidant	(Balasundram N *et al*., 2006 ; Cheynier V, 2012)

1.1.7.1 Antioxidant activity

The mechanism of action of many toxins is related to free radicals and other oxidizing agents. That said, the protection of the defense system has become of great interest. More specifically, free radicals and other oxidizing agents act by impacting the oxidative systems inside biomolecules (carbohydrates, proteins, lipids, and nucleic acids) leading to cell destabilization (death), they are also responsible for the development of cardiovascular disease, rheumatoid arthritis, cancer, inflammation, and aging processes. Besides, as endogenous defense mechanisms against oxidative damage, the tissues of organisms resort to enzymatic antioxidants, such as superoxide dismutase, catalase, peroxidase, tocopherol, ascorbic acid, and polyphenols (Cauich-Kumul R & Campos MRS, 2019). In the case of propolis, several researchers have demonstrated its ability to scavenge free radicals (Yang H *et al*., 2011), but also its ability to absorb lipid peroxidation, accompanied by a chelating power of ferric (Fe^{+3}), cupric (Cu^{+2}), and ferrous (Fe^{+2}) ions, trap DPPH (2,2-diphenyl-1-picrylhydrazyl) (Gülçin *et al*., 2010). Further, its power is also exerted on the leukocyte by

the bias of its action on the significant inhibition of the xanthine oxidase activity of enzymes, such as myeloperoxidase (MPO), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and lipoxygenases (LOX) (Cauich-Kumul R & Campos MRS, 2019).

1.1.7.2 Antimicrobial Properties of Propolis

Propolis is a substance that has this extraordinary power to interact by stimulating the immune system leading to the activation of the body's natural defenses, but also to act directly on the microorganism. The different actions that it exerts on a microorganism are such as: to infer its effect on the permeability of the cell membrane of the microorganism, the disruption of the membrane potential and the production of adenosine triphosphate (ATP) as well as the reduction of bacterial mobility (**Figure 6**) (Sforcin JM & Bankova V, 2011). It has been seen that it acts more on Gram-positive than Gram-negative bacteria, and this reduced reaction is related to the species-specific structure of the outer membrane of bacteria, and the production of hydrolytic enzymes that break down the active ingredients of propolis (Sforcin JM, 2016; Kedzia B & Hołderna-Kedzia E, 2013). Veiga *et al*. (2017) reported a high concentration of artepellin C (3,5-diprenyl-*p*-coumaric acid) with ethanolic extraction rather than hexane. Indeed, artepellin C (from ethanolic extraction) is one of the many phenolic compounds (prenylic derivative of *p*-coumaric acid) of propolis, that showed higher bacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) (Veiga RS *et al*., 2017). On the other hand, against the anaerobic bacterium *Porphyromonas gingivalis*. From a particular point of view the activity of artepillin C is shown by bacteriostatic activity with membrane bubbles (Yoshimasu Y *et al*., 2018). In addition, this same phenolic extract demonstrated anti-inflammatory capacity, mediated by the modulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the inhibition of dinoprostone and nitric oxide. Besides, 3-prenyl-cinnamic acid allylic ester and 2-dimethyl-8-prenylchromene which represent derivatives from prenyl within propolis similarly had these antibacterial activities. Subsequently, against *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Listeria monocytogenes* and *Enterococcus faecalis*, the application of a high concentration resulting from the ethanolic extraction of kaempferide, artepillin C, drupanine and *p*-coumaric acid

showed antioxidant and antibacterial activity (Seibert JB, 2019). Additionally, through their main target for binding to topoisomerase II, flavonoids succeed in inhibiting the growth of *Escherichia coli* by inducing DNA cleavage (**Figure 6**) (Olegário L *et al*., 2019).

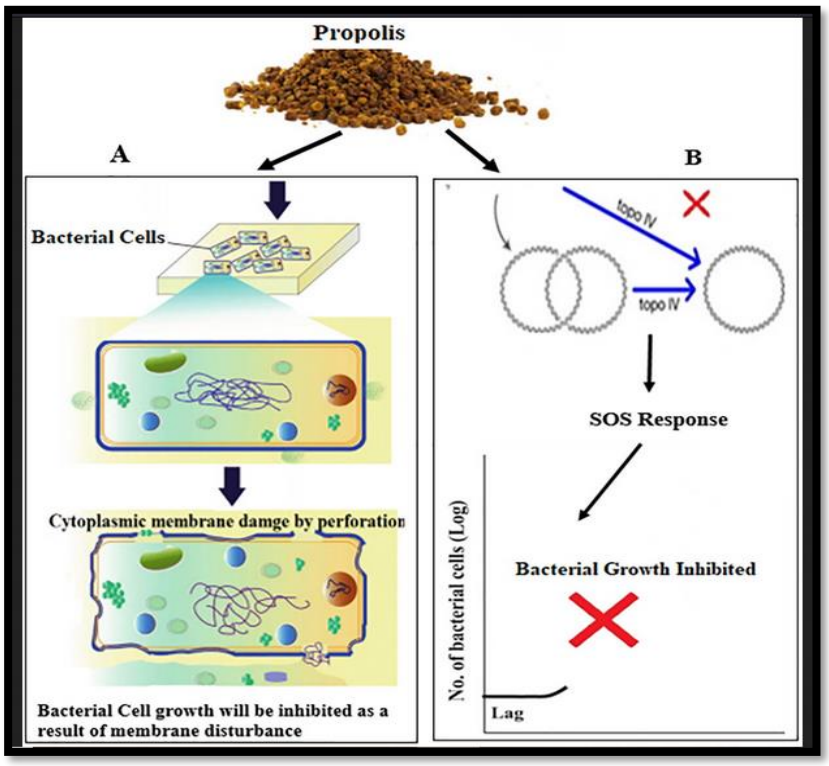

Figure 6. Antimicrobial actions of propolis (Almuhayawi MS, 2020). Mode of action of propolis against bacteria (A). Deterioration of structural integrity caused by components of propolis fixed on the cytoplasmic membrane. This leads to cell death by expelling cytoplasmic contents due to the perforation of the membrane (B). Inhibition of bacterial growth by the action of flavonoids on the activity of topoisomerase IV. SOS: associated cellular response and is known as SOS 'save our ship'.

1.1.7.3 Antifungal activity of propolis

The antifungal activity of propolis has been reported by Anjum *et al*. (2019). Indeed, propolis exerts inhibitory activity against aflatoxigenic fungi and also controls the growth of conidia (*Aspergillus flavus*). A frenetic action of the development of *Candida guilliermondii*,

C. krusei, C. *albicans* has also been studied in different plants from different regions. This inhibitory action of propolis effectively extends to human fungal pathogens *C. albicans, C. glabrata, Aspergillus fumigates*. In addition, pinocembrin showed activities against *Penicillium italicum*, by acting on mycelial growth, respiration, energy homeostasis of pathogens leading to rupture of the cell membrane, and disorders of the cell metabolism (Sforcin JM, 2016). Propolis also inhibits the growth of yeast (Anjum SI *et al*., 2019). In addition, *C. pelliculosa, C. parapsilosis*, and *Pichia ohmeri*, *C. famata*, *C. glabrata* are also affected by the flavonoid agents of the resin (Wagh VD, 2013). Subsequently, there are many other components of propolis that exhibit antifungal activities namely, Pinobanksin 3-*O*-acetate, pinobanksin-3-acetate, pinocembrin, *p*-coumaric acid, and caffeic acid on 26 or more. Singularly, caffeic acid also exerts an inhibition on the growth of fungi affecting the skin such as *mycobacteria*, *Candida*, *Trichophyton*, *Fusarium*, by applying an antimycotic activity (Anjum SI *et al*., 2019).

1.1.7.4 Antitumoral activity of propolis

Caffeic acid phenethyl ester (CAPE) (Anjum SI *et al*., 2019) and artepillin C, are components of propolis that have been shown to have antitumor properties (Chan GCF *et al*., 2013). Their mode of action is such as, cell cycle arrest, inhibition of matrix metalloproteinases, anti-angiogenesis effect, and also inhibit the transfer of the disease from one part of the body to another (Sforcin JM, 2016). As for the mode of action of propolis, it has been seen that it induces the aging of tumor cells (apoptosis) and has the ability to activate white blood cells, to generate agents capable of regulating the function of B, T cells, and natural killer cells (Salomão K *et al*., 2011, Wagh VD, 2013). Further, the prevention of the rapid division of tumor cells (Sforcin JM, 2016) is carried out by galangin, cardanol, nemorosone, and chrysin (Sforcin JM, 2016). Besides, the combination of the phenolic compounds of propolis makes it active against tumors, for example, Caffeic acid and esters as well as diterpenoids and phenolic compounds have a better destructive capacity (Anjum SI *et al*., 2019). Finally, the inhibitory power of propolis (Turkish) extends to the limitation

of DNA synthesis and indirectly it acts on a delay of leucine, thymidine, and uridine and thereby prevents the formation of carcinogenic cells (Watanabe *et al*., 2011).

Ethanolic extract of Indian propolis from stingless bees showed results of apoptosis and cytotoxic cancer cells. These anticancer actions are given to the antioxidant activity of propolis (Król *et al*., 2013). Besides, cancers of the breast, lungs, mouth as well as cancers of the esophagus, stomach, colorectal, prostate, and skin are inhibited by the action of the flavonoids contained in the resin (Martinotti S & Ranzato E, 2015). Likewise, Brazilian propolis resulting from ethanolic extraction has been shown to be anticancer activity, examined on 1,2-dimethylhydrazine which caused colon carcinogenesis in mice (Watanabe MAE *et al*., 2011). Against the colon carcinoma cell line SW-620, an aqueous extraction of Thai propolis produced more significant results than compared to methanolic extraction of propolis (Watanabe MAE *et al*., 2011). Subsequently, cytotoxicity of the ethanolic extract of the propolis against the adenocarcinoma cells of the colon HT-29 as well as against the human fibrosarcoma HT-1080 was noted. Finally, its action remains neutral with respect to the cutaneous fibroblast typical humans (Watanabe MAE *et al*., 2011).

1.1.7.5 Antiprotozoal activity of propolis

Protozoa are responsible for certain diseases in humans and animals, such as giardiasis (Freitas S *et al*., 2006), Chagas disease, leishmaniasis (Duran G *et al*., 2008). The antiprotozoal activity of propolis has been studied against Leishmania donovani, Trypanosoma cruzi, Giardia lamblia, Trichomonas vaginalis, Toxoplasma gondii and Giardia duodenalis (Fokt H *et al*., 2010; Wagh VD, 2013; Aminimoghadamfarouj N & Nematollahi A, 2017). More specifically, the components that makeup propolis have been shown to have antimicrobial activity, such as caffeic acid, chrysin, moronic acid, protocatechuic acid, *p*-coumaric acid, apigenin, and other constituents such as terpenoids, esters, and phenols (Sforcin JM, 2016). However, the type of solvent extraction is also involved in the effectiveness of the antiprotozoal activity of the resin, additionally, the example is taken by the extract of dimethyl sulfoxide and ethanolic extracts against *Trypanosoma cruzi*, and *Trichomonas vaginalis* where a tremendous effect of propolis has been observed (Lotfy M,

2006). 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, 3,5-diprenyl-4-hydroxycinnamic acid, 3-(2,2-dimethyl-8-prenylbenzopyran-6-yl) propenoic, and 3,5-diprenyl-4-hydroxycinnamic acid, are phenolic compounds of propolis and, further, a separation of these compounds to study their effect against this disease was carried out by Marcucci *et al*. (2001) and it results an antimicrobial activity against *Trypanosoma cruzi* (Anjum SI *et al*., 2019).

1.1.7.6 Anti-inflammatory activity of propolis

By results from scientific research done by Anjum *et al*. (2019), it has been studied that flavonoid are responsible for the anti-inflammatory capacity of propolis. Flavonoids are involved in several regulations within cells such as the control of NADPH-oxidase ornithine decarboxylase, myeloperoxidase activity, hyaluronidase of guinea pig mast cells, and tirosine-protein kinase (Lotfy M, 2006). The functions of these compounds mentioned above are to restrict the production of leukotrienes and prostaglandins by white blood cells (Machado *et al*., 2016) and to delay the activity of myeloperoxidase, ornithine decarboxylase, tyrosine-protein-kinase, and NADPH-oxidase (Ramos A and Miranda JD, 2007). In this case, an inhibition of edema with carrageenan, and arthritic inflammations useful in rats, have been studied through the use of the type of propolis called poplar, this inhibiting power is provided by the components of propolis, namely CAPE and galangin (Wagh VD, 2013). Two other types of propolis have been shown to be active against the pathogenesis of arthritis induced by collagen in mice, namely Chinese and Brazilian propolis (Sforcin JM, 2016).

1.1.7.7 Hepatoprotective activity of propolis

By its power to increase the level of glutathione while stopping the peroxidation of lipids and the level of glutathione oxide, propolis has been seen as a hepatoprotective agent (Anjum SI *et al*., 2019). These aforementioned actions of propolis induce an increase in the antioxidant activity against the toxicity induced by mercury. Likewise, it acts against hepatorenal oxidative stress which attracts CCL_4 and the resulting injuries (**Figure 7**) (Wagh VD, 2013). In addition, the damage created by paracetamol as well as CCL_4 allyl alcohol in the liver of rats was attenuated by the means of propolis (Anjum SI *et al*., 2019).

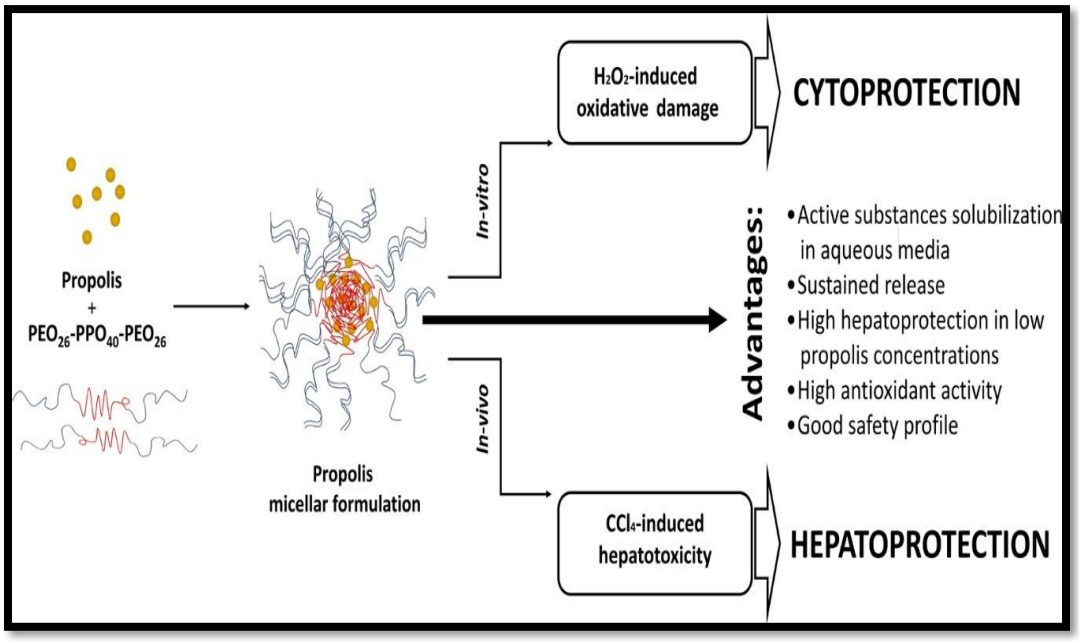

Figure 7. Cytoprotection and hepatoprotection of propolis (Tzankova V*et al*., 2019). Micellar formulations based on poly (ethylene oxide)–β-poly (propylene oxide)–β-poly (ethylene oxide) (PEO–PPO–PEO).

1.1.7.8 Dental action of propolis

Against different anaerobic bacterial strains such as *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Veillonella parvula*, *Lactobacillus acidophilus*, *Peptostreptococcus anaerobius*, *Peptostreptococcus micros*, *Prevotella oralis*, and *Prevotella melaninella*. An ethanolic extract of propolis gathered from four different regions of Brazil and Turkey was carried out. However, it was resolved from a weak inhibitory activity and also a weak bactericidal activity by the agar dilution method. On the other hand, galangin, chrysin, pinobanksin, quercetin, naringenin, which are compounds of flavonoids and aromatic acids have been shown to give more significant results against oral abnormalities (especially dental diseases) (Anjum SI *et al*., 2019; Sforcin JM, 2016). The prevention of microbial infections, as well as the treatment of inflammation of the gums, has become more effective by combining ethanolic extract of propolis with mouthwash and toothpaste (**Figure 8**) (Anjum SI *et al*., 2019).

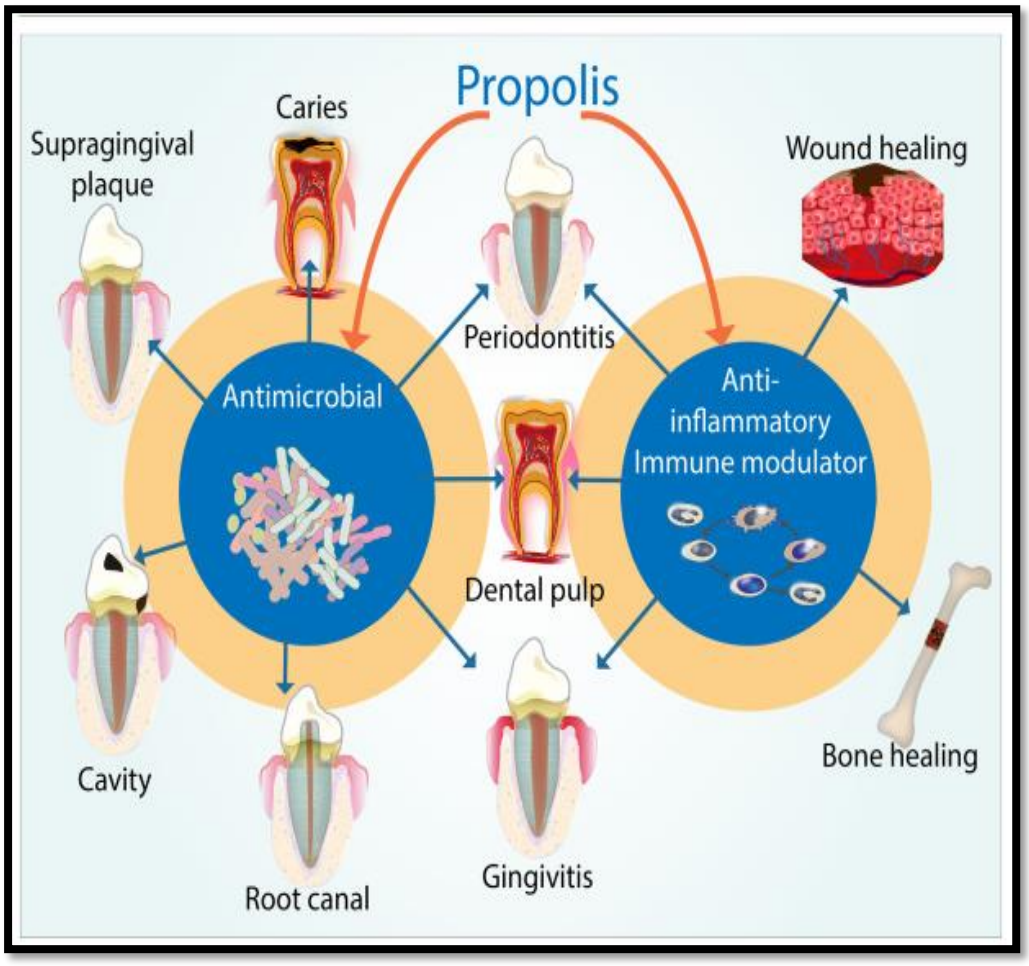

Figure 8. Potential uses of propolis in dentistry (Zulhendria F *et al*., 2021).

1.1.7.9 Wound healing activity of propolis

Injury regeneration is achieved by the components of propolis which act through their therapeutic capacity and on tissue repair (Anjum SI *et al*., 2019). As well as by their immunomodulatory, anti-inflammatory and antimicrobial characteristics (Martinotti S & Ranzato E, 2015; Sforcin JM, 2016) (**Figure 9**). It has also been studied that propolis increases the development of collagen and its constituents and also decreases the level of free radicals in inflammatory lesions (Król W *et al*., 2013; Martinotti S & Ranzato E, 2015). By other numerous compounds such as bioflavonoids, arginine, vitamin C, provitamin A as well as certain minerals within the propolis, the induction of an acceleration of various enzymatic

reactions, of cell metabolism, blood circulation, and also the formation of collagen fibers has been perceived (Anjum SI *et al*., 2019).

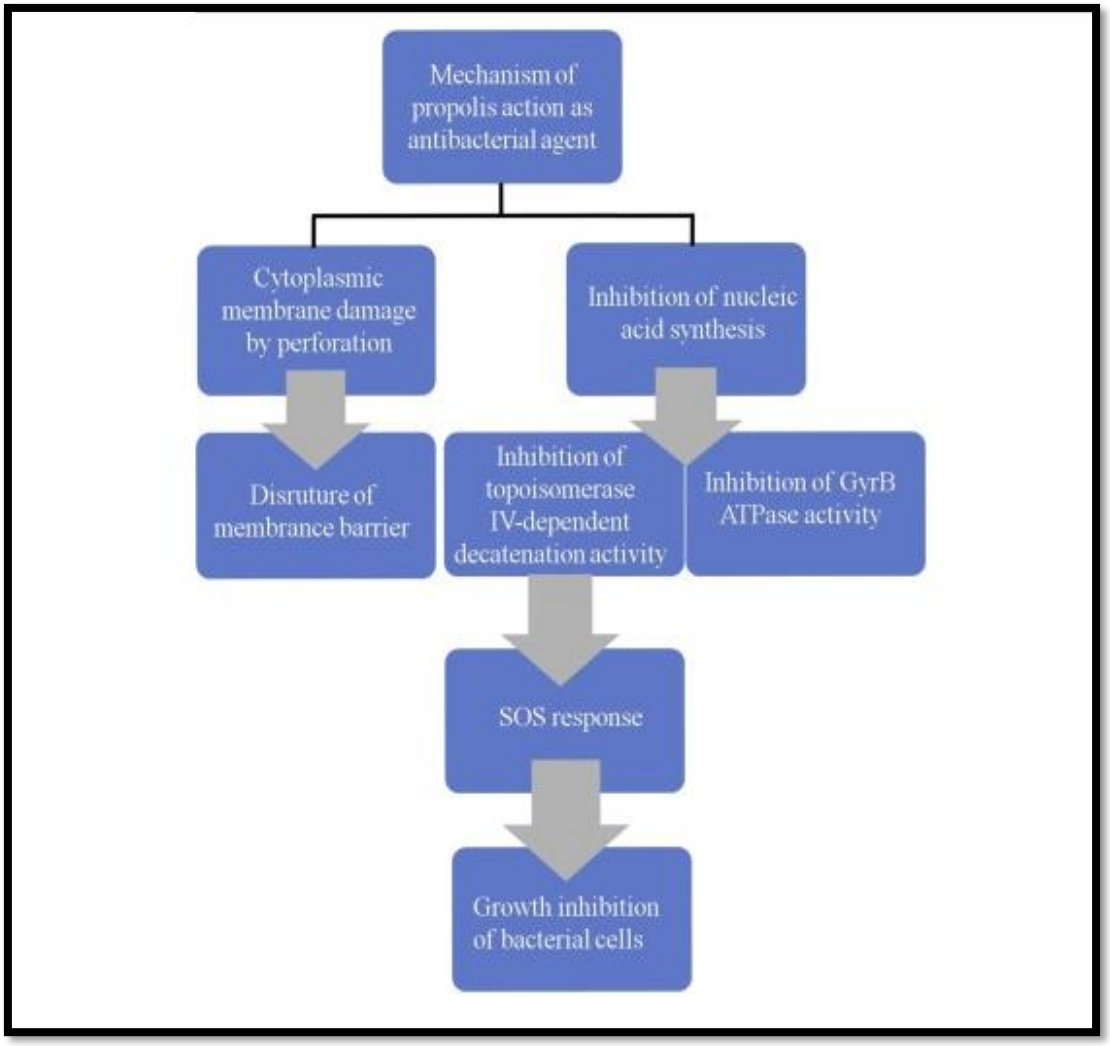

Figure 9. Antibacterial activity of propolis (Oryan A *et al*., 2018).

1.1.7.10 Propolis as natural preservative in foods

The benefits represented by propolis are those sought by the food industries, in fact, its characteristics such as: its richness of bioactive compounds, microbial stability, and the quality of food during storage (Silici S & Karaman K, 2014; Luis-Villaroya A *et al*., 2015). In addition, the use of flavonoids contained in propolis is an alternative for replacing certain

food preservatives. Besides, it is also shared that the specificity of flavonoids is that it is capable to carry out the capture of alkoxy, and superoxide radicals depending on the dose (Pobiega K *et al*., 2019; Silici S & Karaman K, 2014; Viera VB *et al*., 2016). From the consumer point of view, the reported detrimental effects of synthetic preservatives such as carcinogens and teratogens, as well as, residual toxicity have been of great concern, thus leading to a strong preference for natural preservatives which are seen and reported to be more reliable and better (Yang W *et al*., 2017). Many are the type of use of propolis, namely, immersing food directly in extracts of propolis, or as coatings made of polymers extracted from propolis, in short, the effect of both methods is to reduce or eliminate completely pathogens (by contamination of food) including saprophytic microbiota (Pobiega K *et al*., 2019).

2 Material and methods

2.1 Standards and Reagents

The phenolic compound, chrysin has been purchased from Sigma Chemical Co (St Louis, MO, USA). Analytical grade reagents as sodium carbonate, Folin—Ciocalteu reagent, acetic acid, and sulfuric acid, and ethanol, were acquired from Panreac (Barcelona, Spain). Aluminium chloride, potassium ferricyanide, ferric chloride, trichloroacetic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co (St Louis, MO, USA) and 2,4-dinitrophenylhydrazine (DNP) from Fluka (Buchs, Switzerland). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2 Propolis Origin

The raw propolis samples had different origins, the Portuguese propolis sample was collected in 2020 and supplied by Iberiensis, Lda (Portugal), while green and red propolis from Brazil were collected in 2019 and supplied by Bee Propolis Brasil (Bambui, Minas Gerais, Brazil). The samples were kept at -20ºC until further analysis.

2.2.1 Phenolic compounds and bioactivities

2.2.1.1 Procedure for obtaining the extract

The propolis extract was obtained following a previous methodology (Falcão SI *et al*., 2010) (**Figure 10**), 10 g of each type of propolis was weighed and grounded to obtain a fragmented form, then 100 mL of 80% ethanol was added as an extraction solvent. After this procedure, the samples were placed in the hot bath set at a temperature of 70 ºC and for a duration of 1 hour at a rate of 60 rpm. The mixture was then filtered through the Whatman No 4-filter paper and the residue was re-extracted under the same conditions.

After extracts were mixed and the solvent was evaporated in a rotavapor (Rotary Evaporator model Hei-VAP from Heidolph, Schwabach, Germany) at 40 °C. In the last step, the propolis extracts were freeze dried using a lyophilizer (FreeZone 4.5 model 7750031 from Labconco, Kansas City, KS, USA) and stored at room temperature until further analysis.

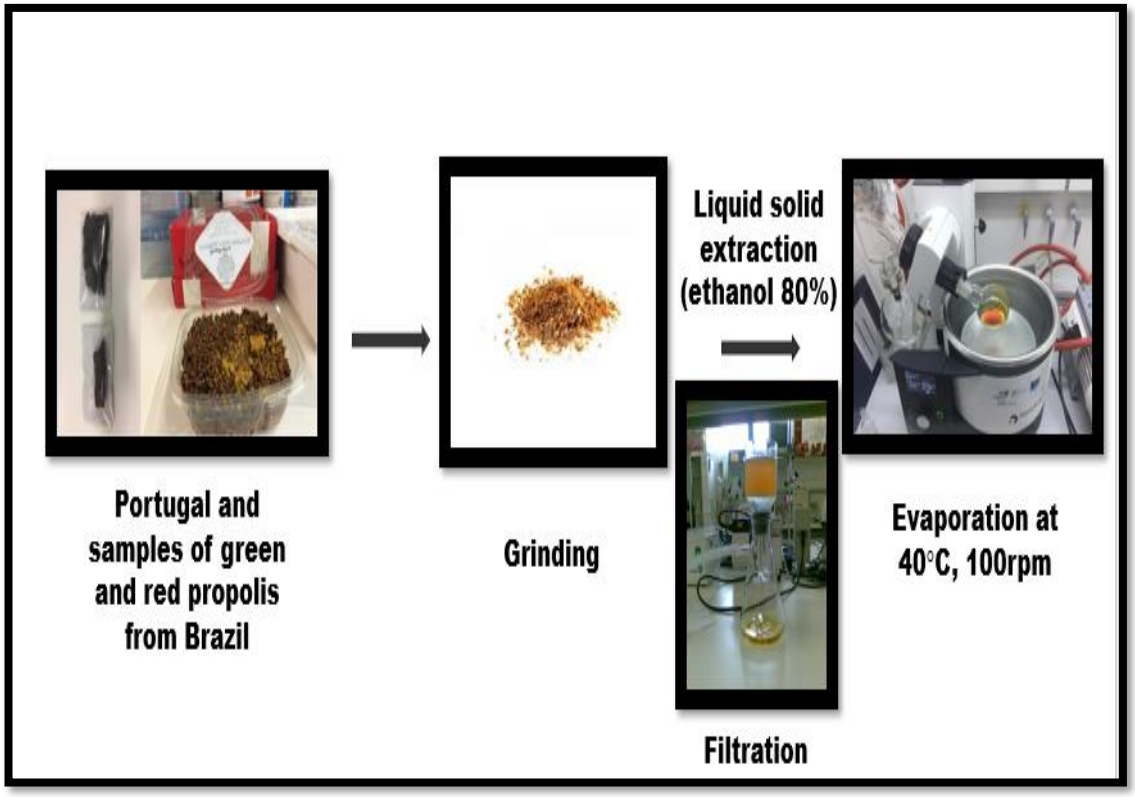

Figure 10. Process of obtaining the extract of propolis.

2.2.2 Spectrophotometric analysis of the phenolic compounds

2.2.2.1 Total phenolic content

The estimation of the phenolic concentration was made according to the method of Singleton and Rossi (1965) adapted by Falcão *et al*. (2013). Total phenolic content was determined by the Folin-Ciocateu method with some modifications (Falcão SI *et al*., 2019). According to the procedure, 1.5 mL of ethanolic extract was pipetted to a 10 mL volumetric flask and diluted with 80 % ethanol/water. An aliquot of the working solution, 0.2 mL, was mixed with 1.5 mL of water and 0.4 mL of the Folin-Ciocalteu's reagent. Then, 0.6 mL of a sodium carbonate solution (20%) was added to the mixture, and the final volume of 5 mL was adjusted by adding 2.3 mL of deionized water. The mixture was left in the dark for 2 hours at room temperature and the absorbance was read at 760 nm using a spectrophotometer

(Analytikijena 200–2004 Spectrophotometer, Analytik Jena, Germany). The blank (0.2 mL) was prepared in the same conditions as the samples, utilising 80 % of ethanol/water. For the quantification, a calibration curve of gallic acid was prepared applying the same procedure as for the samples (5 points at the following concentrations: 0.025; 0.050; 0.100; 0.200; 0.300 mg/mL). If the sample absorbance did not followed within the calibration curve, the concentration of the working solution were adapted. The total phenolic content value of the propolis samples was expressed as milligram of gallic acid equivalent per g of sample (mg GAE/g).

2.2.2.2 Total flavonoid content

Total flavonoid content was recorded spectrophotometrically according to Falcão *et al*. (2019). Briefly, a solution of 1.5 mL of propolis ethanolic extract was pipetted to a 10 mL volumetric flask and diluted with 80 % ethanol/water. Then, in a 25 mL volumetric flask, a working solution of 1 mL was mixed with 10 mL of methanol and 0.5 mL of 5% $AlCl_3$ solution (5g in 100 mL of methanol), adjusting the final volume with methanol. The mixture was left in the dark for 30 min at room temperature and after the reaction, the absorbance was measure at 425 nm. A blank solution was prepared in the same conditions, using instead 1 mL of 80 % ethanol/water. For the quantification, a calibration curve of quercetin was prepared using the same procedure as for the samples (5 points at the following concentrations: 0.005; 0.020; 0.050; 0.100; 0.250 mg/mL). The total flavonoid content value of the propolis samples was expressed as milligram of quercetin equivalent per g of sample (mg QE/g).

2.2.3 Chemical Characterization of the Samples by LC/DAD/ESI-MS^n

The LC/DAD/ESI-MS^n analyzes were performed on a Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, San Jose, CA, USA) equipped with a diode-array detector and coupled to a mass detector. The column used for high-performance liquid chromatography (HPLC) was a Macherey-Nagel Nucleosil C18 (250 mm × 4 mm id; 5 mm particle diameter, end-capped) and its temperature was maintained at 30 ºC. The LC conditions used followed previous work (Falcão SI *et al*., 2013). A flow rate of 1 mL/min

and an injection volume of 10 µL were applied. Spectral data for all peaks were accumulated in the range of 190–600 nm. The mass spectrometer was operated in the negative ion mode using Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, CA, USA) equipped with an electrospray ionization (ESI) source. ESI source parameters were as follows: Source voltage, 5 kV; capillary voltage, −20 V; tube lens voltage, −65 V; capillary temperature, 325 ºC; and sheath and auxiliary gas flow (N_2) set as 50 and 10 (arbitrary units), respectively. Mass spectra were acquired on full range acquisition covering 100–1000 *m/z*. For the fragmentation study, a data dependent scan was performed by deploying collision-induced dissociation (CID). The normalized collision energy of CID cell was set at 35 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (Thermo Scientific, San Jose, CA, USA). The elucidation of the phenolic compounds was achieved by comparison of their chromatographic behavior, UV spectra, and MS information, to those of reference compounds. When standards were not available, the structural information was confirmed with UV data combined with MS fragmentation patterns previously reported in the literature. Quantification was achieved using calibration curves for caffeic acid (0.0187-0.4 mg/mL; y = 6 x10^7x-26360; R^2 = 0.996), *p*-coumaric acid (0.0187-0.4 mg/mL; y = 9x10^6x - 35105; R^2 = 0.999), genistein (0.0375-0.8 mg / mL; y = 1x10^6x + 48333; R^2 = 0.999), kaempferol (0.075-1.6 mg/mL; y = 1x10^6x – 5867; R^2 = 0.997), pinocembrin (0.0375-0.8 mg / mL; y = 2x10^6x + 5250; R^2 = 0.997) and chrysin (0.0375-0.8 mg / mL; y=4x10^6x - 18959; R^2 = 0.999). When the standard was not available, the compound quantification was expressed in equivalent terms of the structurally closest compound. The assays were performed in duplicate and the results expressed as mg/g of sample.

2.3 Antioxidant activity

2.3.1 DPPH Free Radical-Scavenging Activity

DPPH free radical scavenging activity of samples was performed according to the method of Brand-Williams *et al.* (1995), with some modifications. The principle of the DPPH method is to induce a reduction in DPPH, with the presence of a hydrogen donor antioxidant.

In fact, it belongs to the compounds possessing a proton-free radical with an absorption characteristic of exposure to proton radical scavengers, the latter decreasing considerably.

A 96-well microplate was selected to perform the experiment. Different quantities of propolis extracts (10, 20, 40, 80 and, 160 µL) were mixed with 80% ethanol (190, 180, 160, 120, and 40 µL) and 150 µL of DPPH (0.025 g/L). Finally, the mixture was placed in the dark for 45 minutes. The absorbance was measured at λ = 515 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.). Gallic acid was used as standard. The percentage of radical inhibition was obtained from the following formula:

$$\%Inhibition=[(A_{DPPH}-A_{sample}/A_{DPPH}] * 100$$

In order to obtain the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50% (EC_{50}), the percentage was plotted against the extract concentration. The assay was performed in triplicate.

2.3.2 Reducing power

The reducing power of propolis extract was obtained by following the procedure described by Oyaizu (1986). This method is based on the principle that substances that have a reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), the latter in turn reacting with ferric chloride to form a complex ferro-ferrous which has an absorption maximum at 700 nm.

A volume of 1250µl of the propolis ethanolic extract (0.01-2 mg/mL) was mixed with 1250µl phosphate buffer (0.2 mol/L, pH 6.6) and 1250µl of 1% potassium ferricyanide. After mixing in the vortex, the mixture was incubated at 50ºC for a period of 20 minutes. Then 1250µl of trichloroacetic acid (10%) was added, and then centrifugated (Centurion K2R series) at 3000 rpm for 10 min. The upper layer of the solution (1250µl) was mixed with distilled water (1250µl) and $FeCl_3$ (250µl, 0.1%), and the absorbance was measured at 700 nm. Gallic acid was used calculate the standard curve (0.06-1 mg/mL; y = 0.3778x-0.0296; R^2 = 0.998) and the results were expressed as mg of gallic acid equivalent per g of extract

(mg GAE/g). Increased absorbance of the reaction mixture indicates increased reducing power.

2.4 Antifungal activity of propolis

2.4.1 Preparation of the propolis extracts and fungicides

The propolis solutions were prepared as follows: 1.5 g of propolis were weighed and mixed with 1 mL of pure dimethyl sulfoxide (DMSO). DMSO is known as an organic polar aprotic molecule with an amphipathic nature, dissolving poorly soluble polar and non-polar molecules (Novak, 2002). Subsequently, the mixture was dissolved in sterilized distilled water to a volume of 100 mL, to prepare a stock solution at 15 g/L. This solution was used to prepare the working solutions 1.5 g/L and 0.5 g/L; 5 and 15 g/L by diluting in sterilized distilled water. For the preparation of the commercial fungicide (Teldor, Bayer, based on fenhexamid, used for the control of grape bunch rot caused by *Botrytis cinerea*) and *in vitro* fungicide (Tebuconazol, used as a standard fungicide in *in vitro* studies) at 1 mg/mL, a volume of 10 mL of distilled water was used for a mass of 10 mg of the fungicide powder.

2.4.2 Fungal strains, culture conditions and inoculum preparation

To evaluate the activity of propolis films on microorganisms, several microorganisms were used. **Table 5** lists the strains used in the present study. The strains G1 to G6 were isolated from grapes, and were provided by Professor Paula Rodrigues, from the Mountain Research Center (CIMO-IPB). The strains *Aspergillus carbonarius* MUM 04.46 and MUM 04.52 were also isolated from grapes, and were provided by the culture collection Micoteca da Universidade do Minho (MUM). These microorganisms are common in grapes and other similar fruits, and are often blamed for rotting and general post-harvest deterioration of stored fruits.

Table 5. List of fungi used in the study

Working code	Identification
G4	*Alternaria* sp.
G3	*Botrytis cinerea*
G1	*Cladosporium* sp.
G6	*Penicillium* sp.
Ac MUM04.46	*Aspergillus carbonarius* MUM04.46
Ac MUM04.52	*Aspergillus carbonarius* MUM04.52

All strains were grown on potato dextrose agar (PDA, BioLife, Italy) and incubated at 25 ºC for 7 days whenever necessary for experimental assays.

For the inoculation procedures, spore suspensions were prepared from these cultures by flooding the Petri dishes with 1 mL of an aqueous solution of 0.9% NaCl containing 0.1% Tween-80 and transferring the spore suspension into a 1.5 mL *eppendorf*. Spore concentrations were determined by microscopy using a Neubauer counting chamber and the suspensions were diluted up to a final concentration of 10^6 spores/mL.

2.4.3 Preliminary *in vitro* antifungal assays

To determine the effectiveness of the propolis extracts, two different preliminary *in vitro* assays were implemented, based on Balouiri *et al*., (2016): Agar well diffusion and agar disk diffusion.

2.4.3.1 Agar well diffusion

Two square dishes (120 x 120 mm) for each fungus were prepared with 50 mL of PDA and 9 wells (6 mm diameter) were punched in each dish with the back of a sterile glass Pasteur pipette, following the scheme presented in **Figure 11**. A sterile swab was soaked in each spore suspension and was uniformly spread on the medium, and then 100 µL of each test solution were deposited in the corresponding wells. The plates were incubated at 25 ± 2 °C, for 3 to 5 days, until fungal growth was evident.

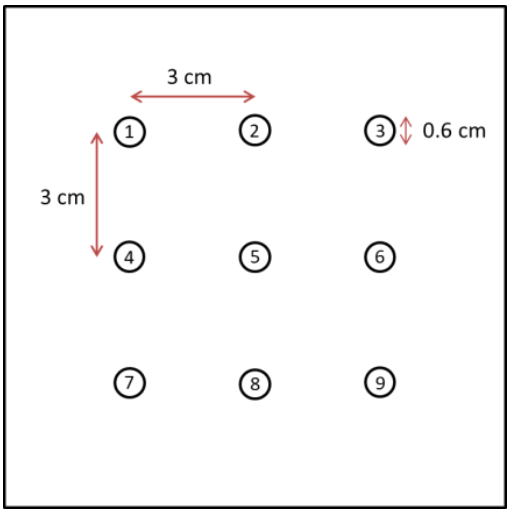

Figure 11. Schematic diagram of the plate preparation for each fungus. 1 – Positive control (Teldor, 1 mg/mL), 2 – Negative control (base solution, no extract, Blank); 3 – Positive control (Tebuconazol, 1mg/mL); 4 – Portuguese propolis, 0.5 g/L; 5 – Brazilian red propolis, 0.5 g/L; 6 – Brazilian green propolis, 0.5 g/L; 7 – Portuguese propolis, 1.5 g/L; 8 – Brazilian red propolis, 1.5 g/L; 9 – Brazilian green propolis, 1.5 g/L.

2.4.3.2 Agar disk diffusion

Plates were prepared in a similar way as described previously, but wells were not punched. Instead, 9 mm diameter filter disks (PRAT DUMAS, France) were put on the top of the agar, using the same positions as described in **Figure 11**. Each disk was flooded with 100 µL of each solution (0.5 g/L and 1.5 g/L for low concentration and 5 g/L and 15 g/L for high concentration). The plates were incubated at 25 ± 2 °C, for 3 to 5 days, until fungal growth was evident.

2.4.4 Antifungal assays with application on table grapes

By observing the results of the preliminary assay, it was concluded that the optimal concentration of propolis for best antifungal activity was 15 g/L. Indeed, Portuguese propolis and Brazilian red propolis were seen as the best types of propolis with evident inhibitory activity. The antifungal assay on grapes was based on these preliminary results.

Red and white table grapes were obtained from a local market (Bragança, Portugal). Healthy grapes were visually selected and submitted to superficial disinfection. For that, 232 red grapes and 232 white grapes were washed in tap water, dipped in 10 % commercial bleach (0.5% hypochlorite) for 2 minutes, and rinsed twice with sterile water. From these, grapes were divided into four sets of eight grapes, and each one was submitted to a different treatment, as follows: Blank, Portuguese propolis (15 g/L), Brazilian red propolis (15 g/L), and commercial fungicide Teldor (1 g/L). Grapes were dipped in the solutions for 2 minutes, were put in trays (eight grapes by treatment; one tray for each test fungus), and were left to dry in a sterile flow hood.

Eight grapes submitted to each treatment were then inoculated with 10 µL of the spore suspensions prepared as previously described. The fungal spores were inoculated by perforating the grapes with a pipette tip. Eight grapes of each type (red and white) not submitted to any treatment were conserved (negative control) and 64 grapes submitted to each treatment were not inoculated. A total of 464 grapes of each type was used in this assay. Finally, the samples were stored under refrigerated conditions at 5 ºC for 15 days, periodic reviews were realized to check the fungal contamination of grapes.

2.5 Statistical analysis

Statistical analysis was performed using Microsoft Excel 2013 Analysis Utility (Washington, USA). All measurements were performed in duplicate and the representation of results was performed as mean ± standard error of the mean (ESM). A comparison of all means of the experimental groups was made by one-way *ANOVA*, accompanied by Tukey's Honestly Significant Difference test. The *p* probability value less than 0.05 was considered statistically significant, and less than 0.001 as highly significant. Different letters mean significant (a-d; A-D).

3 Results

3.1 Total phenolic and flavonoid content

Total phenolic content of the various propolis extracted with 80% ethanol are shown in the **Table 6**; **Figure 12A**. A non-significant result between Brazilian red propolis and Brazilian green propolis is seen ($p > 0.05$). On the other hand, between the Brazilian green propolis and the Portuguese propolis a significant result is noticed ($p < 0.05$) as for Brazilian red propolis and Portuguese propolis. Concerning the total flavonoid content (**Figure 12B**), a non-significant quantity is shown between Brazilian red propolis *versus* Brazilian green propolis with $p > 0.05$, unlike Brazilian red propolis and Portuguese propolis where the difference is significant $p < 0.05$. The comparison between Portuguese propolis and Brazilian green propolis also showed a significant difference ($p < 0.05$).

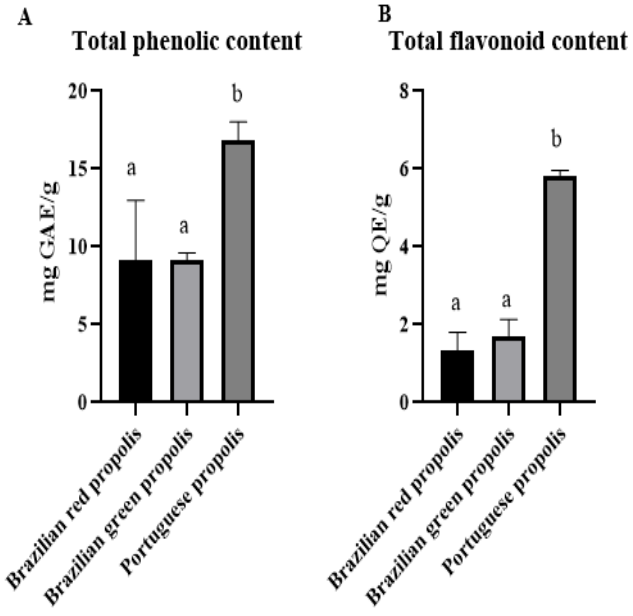

Figure 12. Total phenolic and flavonoids content of Brazilian red propolis, Brazilian green propolis, and Portuguese propolis, different letters (a–c) mean significantly different ($p < 0.05$).

3.2 Phenolic profile by LC/DAD/ESI-MS^n

The analysis of the propolis phenolic extract by LC/DAD/ESI-MS^n allowed the detection of the different phenolic compounds present in the different types of propolis (**Tables 7**, **8** and **9**). In Brazilian red propolis, 26 phenolic compounds were (**Figure 13**, **Table 7**) tentatively identified, among them 4 phenolic acids, 17 flavonoids (1 dihydroflavonol 1 flavone, 4 flavanones, 8 isoflavonoids, 1 chalcone, 2 flavonoid pigments), 1 pterocarpan, 1 triterpene, 3 benzophenones. The main compounds were (**Figure 14**), liquiritigenin (*m/z* 255), vestitol (*m/z* 271), neovestitol (*m/z* 271), guttiferone E (*m/z* 601). Specifically, Guttiferone E/Xanthochymol (27.95 ± 0.30 mg/g) had the highest concentration.

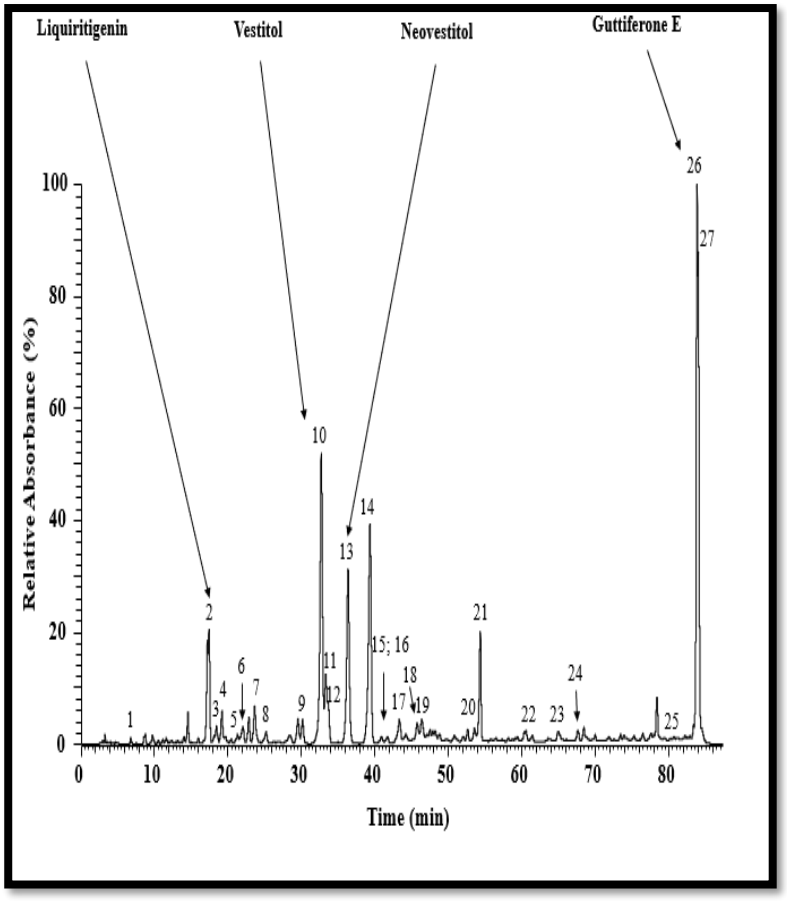

Figure 13. Chromatographic profile of Brazilian red propolis phenolic extract at 280 nm.

Table 6. Characterization of the phenolic compounds from Brazilian red propolis.

Nr	t_R (min)	λ_{max} (nm)	$[M-H]^-$ m/z	MS^2 (% base peak)	Proposed compound	mg/g
1	6.8	292, 323	179	135	Caffeic acid[a,b]	0.11 ± 0.00
2	17.4	276, 312	255	135 (100), 119 (10)	Liquiritigenin[b,c]	5.20 ± 0.06
3	18.4	279, 310	285	270	Vestitone[b,d]	0.86 ± 0.03
4	19.1	289	283	268	Calycosin[b,c]	1.83 ± 0.02
5	21.3	276, 309	315	300	Violanone[b,e]	0.41 ± 0.02
6	22.0	280, 342	285	270 (100), 267 (17), 179 (4)	3,4-Dihydroxy-9-methoxypterocarpan[b, e]	1.29 ± 0.02
7	23.6	291	271	151	Naringenin[a,b]	2.64 ± 0.00
8	25.1	280	283	268	Biochanin A[b,d]	0.98 ± 0.00
9	30.1	281	299	284	Sativanone[b,f]	1.81 ± 0.02
10	32.7	282	271	227 (100), 109 (86), 135 (83)	Vestitol[b,d]	26.16 ± 0.02
11	33.3	280, 320	267	252	Formononetin[b,d]	5.67 ± 0.04
12	33.6	240, 370	255	135 (100), 119 (25)	Isoliquiritigenin[b,d]	2.42 ± 0.00
13	36.3	282	271	135 (100), 227 (74), 109 (62)	Neovestitol[b,d]	17.01 ± 0.00
14	39.3	298, 325	247	179 (100), 135 (16)	Caffeic acid isoprenyl ester[a,b]	20.95 ± 0.05
15	40.9	298, 325	247	179 (100), 135 (16)	Caffeic acid isoprenyl ester (isomer)[a,b]	0.37 ± 0.01
16	41.7	298, 325	269	178 (100), 135 (96)	Caffeic acid benzyl ester[b,g]	0.47 ± 0.00
17	43.3	289	255	213 (100), 211 (55), 151 (36)	Pinocembrin[a,b]	2.14 ± 0.01
18	45.7	268, 313	253	209	Chrysin[a,b]	1.52 ± 0.01
19	46.3	294	313	253 (100), 271 (20)	Pinobanksin-3-*O*-acetate[b, g]	1.97 ± 0.01
20	53.5	324	239	197 (100), 135 (36), 148 (19)	7-Hydroxyflavanone[b,d]	1.02 ± 0.02
21	54.3	283	397	123 (100), 167 (97), 351 (40)	NI	
22	60.5	285, 481	521	397 (100), 491 (45)	Retusapurpurin B[b,h]	0.47 ± 0.01
23	64.9	284, 481	521	397 (100), 491 (60)	Retusapurpurin A[b,h]	0.94 ± 0.01
24	67.5	264, 327	425	410 (100), 367 (43), 355 (41)	Cycloartenol/α-amyrin/β-amyrin[b,h]	
25	81.2	244, 351	617	465 (100), 481 (40), 521 (15)	16-Hydroxyguttiferone[b, h]	0.02 ± 0.00
26	83.8	244, 351	601	465	Guttiferone E/Xanthochymol[b,d]	27.95 ± 0.30
27	84.0	244, 351	601	327 (100), 273 (26), 271 (15)	Oblongifolin B[b,d]	22.13 ± 0.21

[a]Confirmed with standard, [b]Confirmed with MS^n fragmentation , [c](Omar RMK *et al*., 2016), [d](Morais DV *et al*., 2021), [e](Righi AA *et al*., 2011), [f](Falcão SI *et al*., 2019), [g](Falcão SI *et al*., 2013), [h](Silva VC *et al*., 2020), NI: non identified.

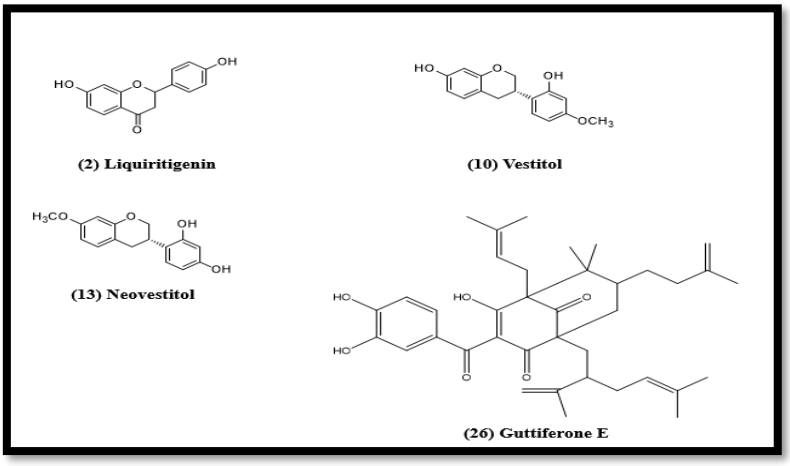

Figure 14. Most abundant compounds in Brazilian red propolis.

In Brazilian green propolis, 21 phenolic compounds have been reported (**Figure 15**, **Table 8**) in which 6 phenolic acids, 11 terpenic phenolic compounds, 4 flavonoids (1 dihydroflavonols, 3 flavonols). Subsequently, p-Coumaric acid (*m/z* 163), 5-*O*-caffeoylquinic acid (*m/z* 353), dihydrokaempferide (*m/z* 301), drupanin (*m/z* 231), kaempferide (*m/z* 285), and artepillin C (*m/z* 299), were the majority compounds (**Figure 16**), with kaempferide (35.66 ± 0.13 mg/g) representing the highest concentration.

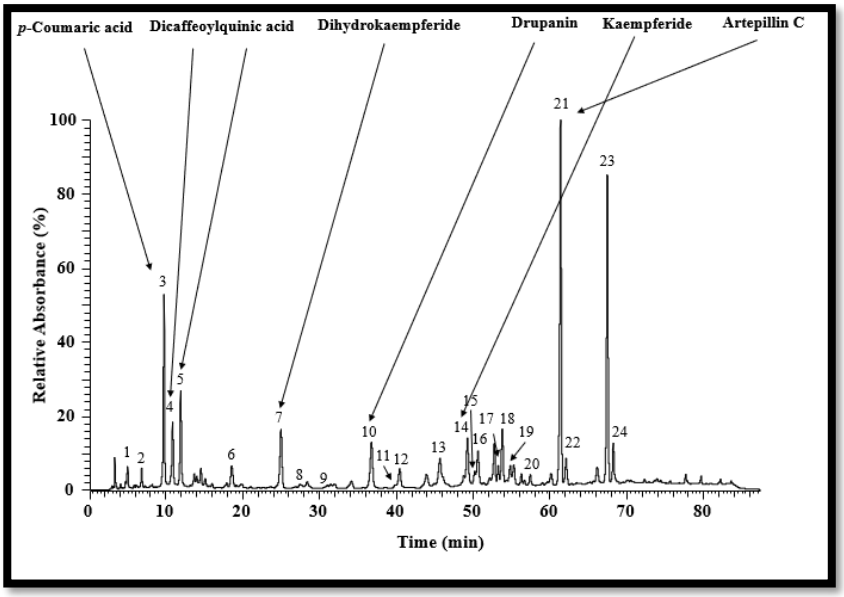

Figure 15. Chromatographic profile of Brazilian green propolis phenolic extract at 280 nm.

Table 7. Characterization of the phenolic compounds from Brazilian green propolis.

Nr	t_R (min)	λ_{max} (nm)	$[M-H]^-$ m/z	MS^2 (% base peak)	Proposed compound	mg/g
1	4.9	294sh, 325	353	191 (100), 179 (8), 135 (1)	5-O-Caffeoylquinic acid[a,b]	1.35 ± 0.04
2	6.8	292, 323	179	135	Caffeic acid[a,b]	1.18 ± 0.04
3	9.7	310	163	119	*p*-Coumaric acid[a,b]	9.92 ± 0.01
4	10.8	294sh, 325	515	353	Dicaffeoylquinic acid[b,c]	6.04 ± 0.40
5	11.8	294sh, 325	515	353	Dicaffeoylquinic acid (isomer)[b,c]	8.81 ± 0.04
6	18.5	294sh, 325	677	515	Tricaffeoylquinic acid[b,c]	3.34 ± 0.01
7	24.9	292	301	283 (100), 151 (29)	Dihydrokaempferide[b,c]	24.53 ± 0.06
8	27.7	267, 365	285	285 (100), 257 (13), 151 (20)	Kaempferol[a,b]	1.47 ± 0.01
9	30.9	321	247	203	5-Isoprenyl caffeic acid[b,d]	0.36 ± 0.02
10	36.7	315	231	187	Drupanin[b,c]	4.90 ± 0.02
11	39.8	310	327	283	Dihydroconiferyl *p*-coumarate[b,c]	0.44 ± 0.01
12	40.3	315	315	271 (100), 241 (70), 285 (59)	Cappilartimisin A[b,c,d]	2.03 ± 0.01
13	45.6	315	315	271 (100), 241 (72), 285 (55)	Cappilartimisin A (isomer)[b,d]	3.72 ± 0.02
14	49.2	266, 365	299	284	Kaempferide[b]	35.66 ± 0.13
15	50.1	266, 365	299	284	Kaempferide (isomer)[b]	14.95 ± 0.02
16	50.5	269, 363	329	314	NI	
17	53.2	316	393	349 (100), 163 (91), 145 (53)	5-Isoprenyl caffeic acid-p-coumaric acid ester[b,d]	4.05 ± 0.02
18	53.7	319	315	245 (100), 201 (41), 271 (11), 257 (11)	Cappilartimisin A (isomer)[b,d]	1.57 ± 0.01
19	56.2	315	379	231	Drupanin derivative[b]	0.68 ± 0.01

20	57.4	284	377	245 (100), 319 (95), 349 (66)	*E*-Baccharin 5''-aldehyde[b,e]	1.23 ± 0.01
21	61.3	314	299	255	Artepillin C[b,c]	24.03 ± 0.04
22	62.1	284	363	187	Baccharin[b,e]	2.10 ± 0.01
23	67.4	282	447	297 (100), 149 (10)	NI	
24	68.2	277, 320	613	511	NI	

[a]Confirmed with standard, [b]Confirmed with MS^n fragmentation , [c] (Coelho J *et al*., 2017), [d](Xu X *et al*., 2020), [e](Rodrigues DM *et al*., 2020) NI: non identified.

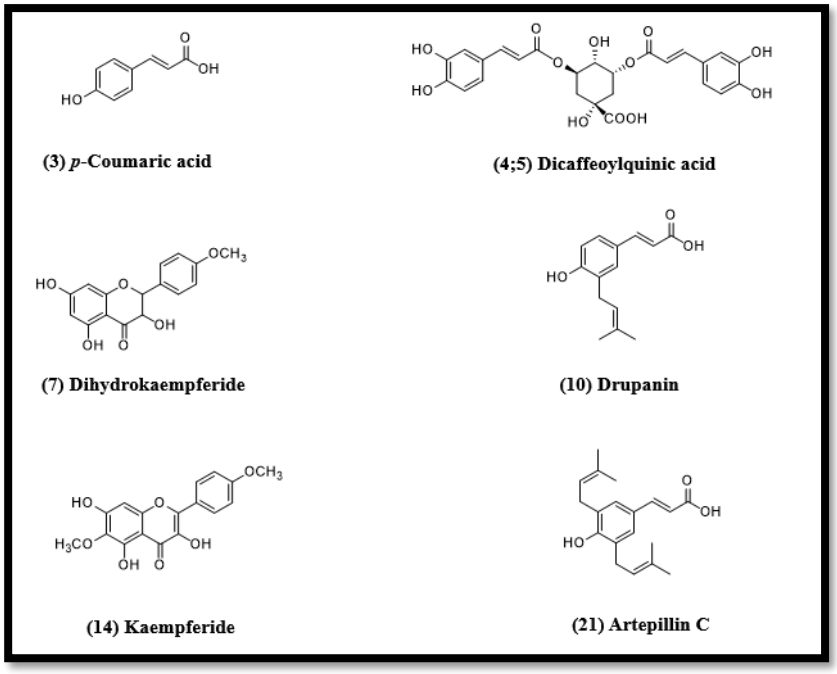

Figure 16. Most abundant compounds in Brazilian green propolis.

The results obtained in Portuguese propolis (**Figure 17**, **Table 9**) indicate that 39 phenolic compounds were tentatively identified including 14 phenolic acids and 25 flavonoids (11 dihydroflavonols, 7 flavonols, 5 flavones, 2 flavanones). The main compounds (**Figure 18**) found were respectively, cinnamylidenacetic acid (*m/z* 173), pinocembrin (*m/z* 255), chrysin (*m/z* 253), pinobanksin-3-*O*-acetate (*m/z* 313), galangin (*m/z* 269) and having the pinocembrin the highest concentration (140.57 ± 0.16 mg/g).

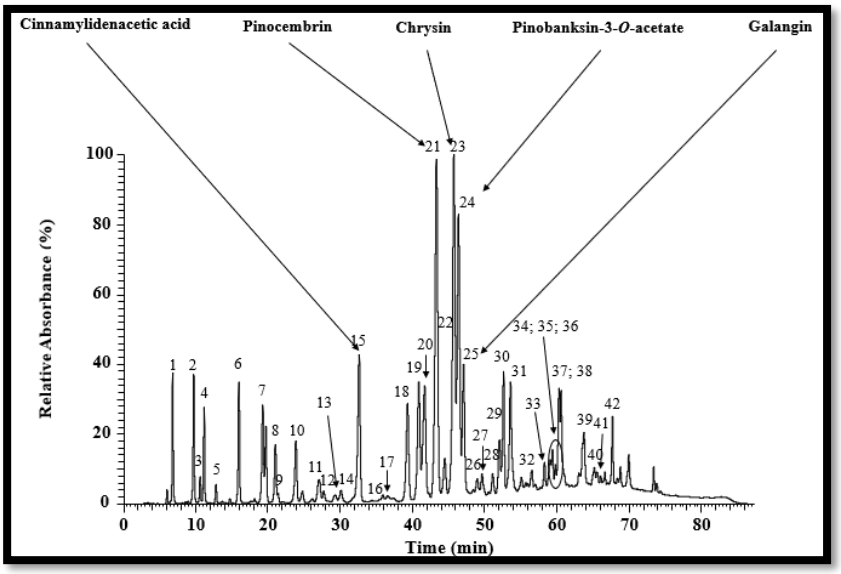

Figure 17. Chromatographic profile of Portuguese propolis phenolic extract at 280 nm.

Table 8. Characterization of the phenolic compounds from Portuguese propolis.

Nr	t_R (min)	λ_{max} (nm)	$[M-H]^-$ m/z	MS^2 (% base peak)	Proposed compound	mg/g
1	6.8	292, 323	179	135	Caffeic acid[a,b]	6.27 ± 0.09
2	9.7	310	163	119	*p*-Coumaric acid[a,b]	4.84 ± 0.03
3	10.6	295, 322	193	133 (100), 149 (49), 177 (15)	Ferulic acid[a,b]	1.40 ± 0.01
4	11.1	298, 319	193	133 (100), 149 (49), 177 (15)	Isoferulic acid[a,b]	5.25 ± 0.09
5	12.8	228	121		Benzoic acid[a,b]	1,07 ± 0.01
6	15.9	295sh, 322	207	192 (100), 163 (62)	3,4-Dimethyl-caffeic acid[a,b]	8.25 ± 0.04
7	19.2	287	285	267 (100), 239 (25), 252 (16)	Pinobanksin-5-methyl ether[b,c]	23.95 ± 0.09
8	21.0	309	177	163 (100), 119 (16)	*p*-Coumaric acid methyl ester[a,b]	3.22 ± 0.02
9	21.3	256, 355	315	300	Quercetin-3-methyl ether[b,c]	3.95 ± 0.11
10	23.8	292	271	253 (100), 225 (22), 151 (8)	Pinobanksin[b,c]	19.79 ± 0.12
11	27.0	269, 337	269	225 (100), 151 (20)	Apigenin[a,b]	5.06 ± 0.01

12	27.7	267, 365	285	285 (100), 257 (13), 151 (20)	Kaempferol[a,b]	6.94 ± 0.04
13	29.3	253, 370	315	300	Isorhamnetin[a,b]	6.63 ± 0.09
14	30.1	267, 352	299	284	Kaempferol-methyl ether[b,c]	10.05 ± 0.07
15	32.6	311	173	129	Cinnamylidenacetic acid[b,c]	18.14 ± 0.12
16	35.9	256, 367	315	165	Rhamnetin[b,c]	2.76 ± 0.10
17	36.5	265, 300sh, 352	283	268 (100), 239 (76)	Galangin-5-methyl ether[b,c]	3.66 ± 0.02
18	39.3	298, 325	247	179 (100), 135 (16)	Caffeic acid isoprenyl ester[a,b]	12.49 ± 0.04
19	40.9	298, 325	247	179 (100), 135 (16)	Caffeic acid isoprenyl ester (isomer)[a,b]	15.45 ± 0.20
20	41.7	298, 325	269	178 (100), 135 (96)	Caffeic acid benzyl ester[b,c]	16.78 ± 0.03
21	43.3	289	255	213 (100), 211 (55), 151 (36)	Pinocembrin[a,b]	140.57 ± 0.16
22	44.5	290	285	139 (100), 145 (42)	NI	
23	45.7	268, 313	253	209	Chrysin[a,b]	66.93 ± 0.21
24	46.4	294	313	253 (100), 271 (20)	Pinobanksin-3-*O*-acetate[b, c]	105.51 ± 0.05
25	47.1	266, 300sh, 359	269	269 (100), 241 (61)	Galangin[a,b]	95.17 ± 0.19
26	48.9	268, 331	283	269	Acacetin[a,b]	4.72 ± 0.01
27	49.6	265, 300sh, 350sh	283	269	6-Methoxychrysin[b, c]	4.88 ± 0.02
28	51.1	250, 268sh, 343	313	298	Chrysoeriol-methyl ether[b,c]	5.48 ± 0.01
29	52.0	294, 310	231	163 (100), 119 (12)	*p*-Coumaric isoprenyl ester[b,c]	4.72 ± 0.04
30	52.6	295, 324	295	178 (100), 135 (60)	Caffeic acid cinnamyl ester[b,c]	11.74 ± 0.04
31	53.6	289	327	253 (100), 271 (10)	Pinobanksin-3-*O*-propionate[b, c]	48.64 ± 0.11

32	56.5	289	269	254 (100), 251 (54), 165 (22)	3-Hydroxy-5-methoxyflavanone[b, c]	11.90 ± 0.02
33	58.2	292	417	297 (100), 402 (85), 267 (67)	Pinobanksin-methyl-ether-3-*O*-phenylpropionate[b, d]	13.29 ± 0.01
34	59.1	292	475	415	Pinobansin-3-*O*-acetate-5-*O*-hydroxyphenylpropionate[b,c]	16.84 ± 0.10
35	59.4	308	431	281	NI	
36	59.8	292	417	267 (100), 281 (100)	Pinobanksin-methyl-ether-3-*O*-phenylpropionate (isomer)[b,d]	8.93 ± 0.04
37	60.3	292	475	415	Pinobansin-3-*O*-acetate-7-*O*-hydroxyphenylpropionate (isomer)[b,c]	30.23 ± 0.15
38	60.6	294, 320	413	161	NI	
39	63.7	292	355	253	Pinobanksin-3-*O*-pentanoate or 2-methylbutyrate[b, c]	15.87 ± 0.10
40	65.1	292, 322	315	179 (100), 135 (31)	Caffeic acid derivative	3.00 ± 0.01
41	65.5	292	403	253 (100), 271 (21)	Pinobanksin-3-*O*-phenylpropionate[b, c]	10.69 ± 0.01
42	67.0	292	369	253 (100), 271 (14)	Pinobanksin-3-*O*-hexanoate[b, c]	23.90 ± 0.06

[a]Confirmed with standard, [b]Confirmed with MS[n] fragmentation, [c] (Falcão SI *et al*., 2013), [d] (Falcão SI *et al*., 2010), NI: non identified.

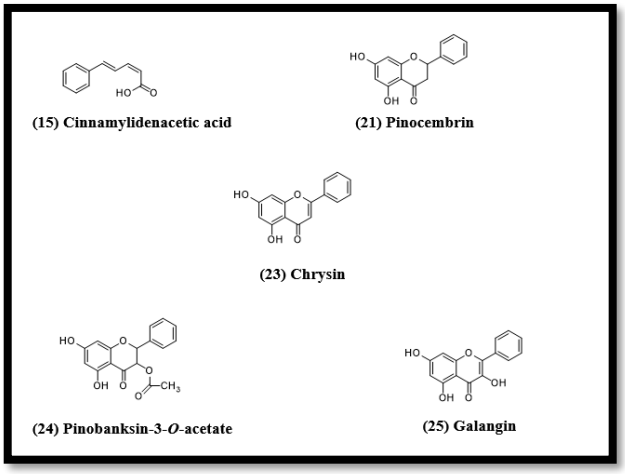

Figure 18. Most abundant compounds in Portuguese propolis.

3.3 Antioxidant activity

3.3.1 Scavenging of DPPH radicals

Regarding the **Figure 19**, a significant and non-significant difference is observed. Indeed, the comparison of the antioxidant activity between Brazilian red propolis and Brazilian green propolis did not show any significant results. The Brazilian red propolis had a radical scavenging activity of 77.19% compared to Brazilian green propolis showing a result of 71.92% ($p > 0.05$). In contrast, the Portuguese propolis showed a radical scavenging activity of 93.10% hence the significant difference compared to the other two types of propolis with $p < 0.05$.

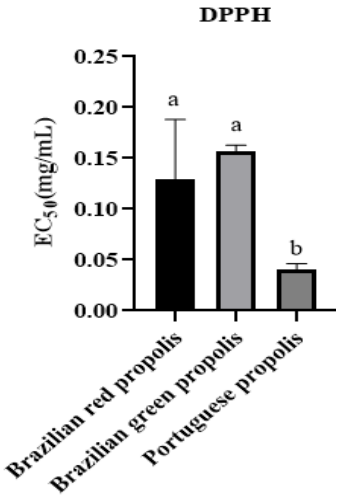

Figure 19. Total antioxidant activities of Brazilian red propolis, Brazilian green propolis, and Portuguese propolis determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, different letters (a–c) mean significantly different ($p < 0.05$).

3.3.2 Reducing power

Figure 22 expresses the results as gallic acid equivalents. The results vary from 0.48 to 0.98 mg GAE/g. Indeed, Portuguese propolis showed the highest activity rate which is 0.98 mg GAE/g, therefore, it showed a highly significant result compared to Brazilian red propolis and Brazilian green propolis ($p > 0.001$). Regarding Brazilian red propolis (0.52 ± 0.01 mg

GAE/g), it displayed a higher value than that of Brazilian green propolis (0.48 ± 0.03 mg GAE/g), however, the result is in no way significant between these two types of propolis ($p > 0.05$).

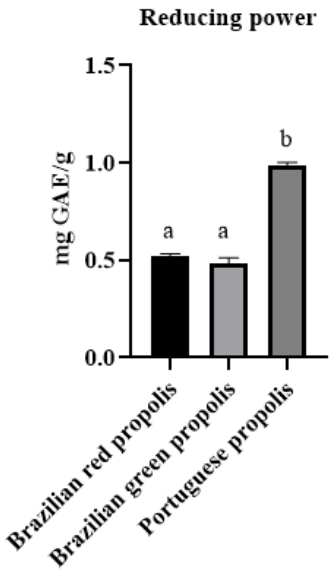

Figure 20 . Total antioxidant activities of Brazilian red propolis, Brazilian green propolis, and Portuguese propolis, different letters (a–c) mean significantly different ($p < 0.05$).

3.4 Antifungal activity of propolis

3.4.1 *In vitro* test

An experiment to determine the best concentration (0.5, 1.5, 5 and 15 g/L) at which the different treatments (Portuguese propolis, Brazilian red propolis, and Brazilian green propolis) react better to the growth of different fungi (*Alternaria* sp. G4, *Botrytis cinerea* G3, *Penicillium* sp. G6, *Aspergillus carbonarius* MUM04.52 Ac MUM04.52, *Aspergillus carbonarius* MUM04.46 Ac MUM04.46, *Cladosporium* sp G1) by the appearance or not of a halo took place in different plates with the disk diffusion and well diffusion methods (**Figure 21**). The different candidates were placed in the Petri dishes as described in **Figure 11**.

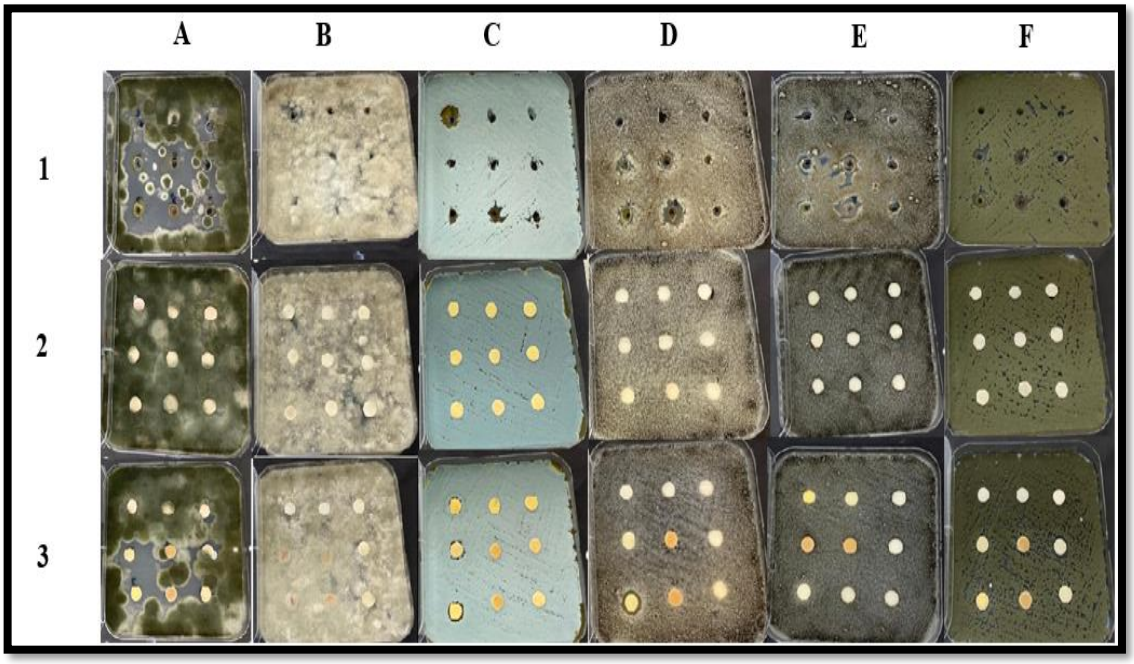

Figure 21. Inhibitory effect of the commercial fungicide (Teldor), Standard fungicide (Tebuconazol), Portuguese propolis, Brazilian red propolis, and Brazilian green propolis. A: *Alternaria* sp. G4, B: *Botrytis cinerea* G3, C: *Penicillium* sp. G6, D: *Aspergillus carbonarius* MUM04.52 (Ac MUM04.52), E: *Aspergillus carbonarius* MUM04.46 (Ac MUM04.46) F: *Cladosporium* sp G1. 1: Concentration at 0.5 and 1.5 g/L (wells), 2: Concentration at 0.5 and 1.5 g/L (disks), 3: Concentration at 5 and 15 g/L (disks).

First, with a series of concentrations of 0.5 and 1.5 g/L within the plates with wells, it is noted at position A1 in **Figure 21**, that facing *Alternaria* sp. G4, the different treatments which effectively inhibited its growth by the appearance of a clear halo are respectively the Portuguese propolis and Brazilian red propolis. Unlike them, the activity of Brazilian green propolis could not be expressed effectively, indeed the growth of the fungus within its well is observable. At a concentration of 1.5 g/L, a better inhibition on the part of the Portuguese propolis against the fungus is seen followed by Brazilian red propolis and finally Brazilian green propolis. Then at position B1, where *Botrytis cinerea* G3 is located, none of the treatments (Portuguese propolis, Brazilian red propolis, and Brazilian green propolis) were

able to induce the formation of a halo for any concentration. Concerning *Aspergillus carbonarius* MUM04.52 Ac MUM04.52, the formation of halo is observed at 0.5 and 1.5 g/L of the plate positioned at level D1, in fact, the treatment with Brazilian red propolis at 1.5 g/L gave a better inhibitory effect on the growth of Ac MUM04.52, however, the halos formed by these last two, faced the resistance of the fungus which evolved gradually towards the wells. On the other hand, in the same position but at 1.5 g/L, Brazilian red propolis denoted more efficiency against the fungus development than Portuguese propolis, indeed, the formation of the halo is much more visible than that of the Portuguese propolis. Regarding treatment with Brazilian green propolis, no activity was observed by its application at the same types of concentration. Subsequently, at 0.5 g/L of concentration at the E1 position where the *Aspergillus carbonarius* MUM04.46 Ac MUM04.46 is located, the application of Portuguese propolis and Brazilian red propolis have largely given better results than that of Brazilian green propolis, in fact, the latter did not reveal any inhibitory activity (growth of the fungus to the well) while the other two presented a halo. At 1.5 g/L, the Brazilian red propolis was able to stand out from the Portuguese propolis, indeed its application exposed a clear halo than that of the Portuguese propolis which tends to be invaded by the fungus. To finish the Brazilian green propolis presented a better activity but did not exhibit a satisfactory activity vis-à-vis the evolution of Ac MUM04.46 which progressed despite the formation of a weak halo (resistance). The last well test (position F1) corresponds to *Cladosporium* sp. G1, the different propolis treatments all showed inhibitory activity but at different levels, in fact, Brazilian green propolis reacted better at 0.5 g/L (with halo formation) than at 1.5 g/L where its activity was totally absent. On the other hand, the appearance of a clearer and wider halo is observed at 0.5 g/L than at 1.5 g/L with Portuguese propolis. Finally, Brazilian red propolis, unlike the two previous propolis, demonstrated its activity more at 1.5 g/L than at 0.5 g/L by the formation of a more apparent halo.

Second, the application of the different propolis treatments, at low concentration in the disks, namely, at 0.5 and 1.5 g/L, could not give a formation of a halo (locations: A2, B2, C2, D2, E2, F2).

Third, for a high concentration of application of the treatments (5 g/L and 15 g/L), at the A3 position, only the Portuguese propolis was able to effectively inhibit the growth of *Alternaria* sp. G4, observable by the formation of a halo at 5 and 15 g/L. For these same concentration levels Brazilian red propolis reacted better where a greater reduction in the fungal growth is observable, mentioning a better result than that of the treatment with Brazilian green propolis attesting no inhibition. Subsequently, at the B3 position corresponding to *Botrytis cinerea* G3, no activity of the different treatments is seen. Against the *Penicillium* sp. 2 G6 (position C3) only the treatment with the Portuguese propolis evinced its effectiveness by the creation of a halo with the two levels of concentration, however, the inhibition is better at 15 g/L than at 5 g/L where fungicidal resistance is perceived. As for the other two types of propolis (Brazilian red propolis and Brazilian green propolis), no inhibition was performed. Also, with the *Aspergillus carbonarius* MUM04.52 Ac MUM04.52, the Portuguese propolis was able to stand out, but only at 15 g/L with a clear presence of a halo, which the Brazilian red propolis and Brazilian green propolis did not manifest. Next, faced with *Cladosporium* sp. G1 (position D3) only treatment with Portuguese propolis reacted at 15 g/L (presence of a halo), treatment with Brazilian red propolis and Brazilian green propolis did not give an inhibitory effect. Finally, the plate at position F3, indicated that the growth force of *Cladosporium* sp. G1 is much greater than that of the activity of different propolis. Indeed, no inhibition is observed.

By this test, it was revealed that the activity of propolis depends not only on the type of fungus but also on the concentration, and that Portuguese propolis and Brazilian red propolis have more fields of antifungal activity than Brazilian green propolis. Based on these results, the Portuguese propolis and the Brazilian red propolis at the highest concentration (15 g/L) were selected to evaluate the ability of propolis to inhibit fungal growth and rot on table grapes.

3.4.2 Antifungal activity of propolis on grapes

3.4.2.1 Effect of treatments on the growth of *Alternaria* sp.

The results of the rotting diameter on the red and white grapes according to the different experimental treatments carried out are exposed in **Figure 22A**. The treatment with Portuguese propolis (3.52 ± 0.50 mm rotting diameter) compared to the treatment with the blank did not reveal significant results within 10 days ($p > 0.05$). While the observation of the results at 15 days proved an increase in the rotting diameter *via* the action of Portuguese propolis (12.54 ± 0.02 mm) compared to the blank ($p > 0.05$). On the other hand, Portuguese propolis displayed a significant regression of the rotting diameter compared to the action of the fungicide ($p < 0.05$) at 10 days. Conversely, the fungicide exhibited a lower median (non-significant) at 15 days of treatment. Besides, Brazilian red propolis (3.15 ± 0.55mm) induced a considerable regression of the rotting diameter after 10 days of treatment, compared to the experiment made with the blank and the fungicide ($p < 0.05$). However, at 15 days of treatment a regression of its activity is observed by the evolution of the rotting diameter (8.68 ± 2.69 mm), displaying a higher median (non-significant) compared to the two controls. For white grapes (**Figure 22B**), after 10 days of treatment, a smaller rotting diameter is observed with the Portuguese propolis treatment attesting a diameter of 3.18 ± 0.66 mm compared to the blank ($p > 0.05$). In contrast, Brazilian red propolis experiment marked a greater rotting progression with a diameter of 4.04 ± 1.79 mm ($p > 0.05$), however the two propolis exhibited a considerable regression against the fungicide ($p < 0.05$). This same case is also observed at 15 days of treatment, where the Portuguese propolis (9.55 ± 1.22 mm) and the Brazilian red propolis (8.08 ± 0.17 mm) compared to the blank did not indicate significant results, unlike with the fungicide where the regression is significant ($p < 0.05$). The information indicates that the rotting of white and red grapes at 10 days and 15 days are close.

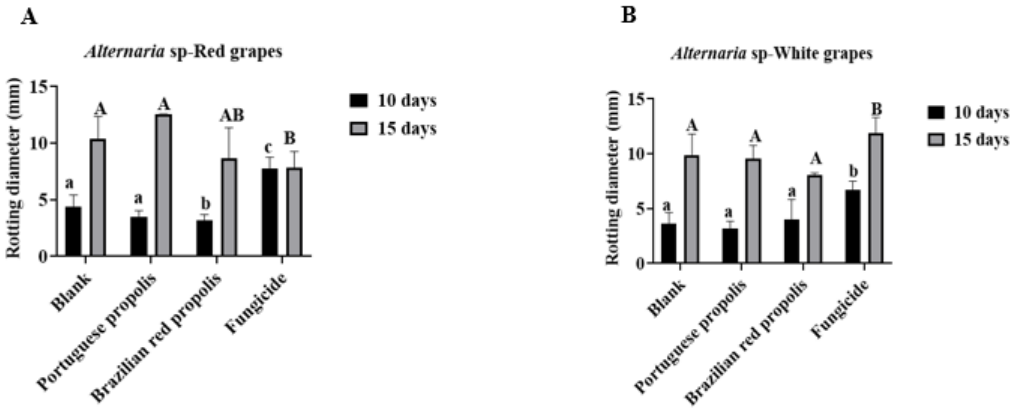

Figure 22. Effect of *Alternaria*. sp on the rotting of red grapes (A) and white grapes (B) treated with: blank, Portuguese propolis, Brazilian red propolis, and fungicide. Different letters (a–d; A-D) mean significantly different ($p < 0.05$) averages (n=8).

3.4.2.2 Effect of treatments on the growth of *Aspergillus carbonarius* 04.46

The effects of the evaluation of the treatment of red grapes against *Aspergillus carbonarius* Ac MUM04.46 are expressed in **Figure 23A**. After 10 days of incubation of the red grapes, evaluation with the Portuguese propolis presented the largest diameter which is 9.08 ± 0.90 mm compared to the control groups (blank $p > 0.05$, fungicide $p < 0.05$). Contrary, the treatment with the Brazilian red propolis (6.87 ± 1.05 mm) showed the lowest value of non-significant ($p < 0.05$) nature compared to the controls. On the other hand, after 15 days of treatment, the Brazilian red propolis treatment (15.17 ±0.92 mm) presented the greatest value by confrontation with the blank ($p > 0.05$) and fungicide ($p < 0.05$). The process with the Portuguese propolis (14.80 ±1.07 mm) communicated a higher decay value than blank and fungicide. With regard to the white grapes **Figure 23B**, after 10 days of monitoring, the intervention with Brazilian red propolis (5.23 ± 1.18 mm) and Portuguese propolis (5.68 ±1.28 mm) did not give any significant result compared to controls. An extension of the incubation to 15 days, did not provide any better result either (Brazilian red propolis: 10.96 ± 1.45mm; Portuguese propolis: 11.81 ± 2.82 mm). From the analysis of the

result, the two types of propolis are more effective against *Aspergillus carbonarius* Ac MUM04.46 with white grapes at 10 and 15 days of treatment.

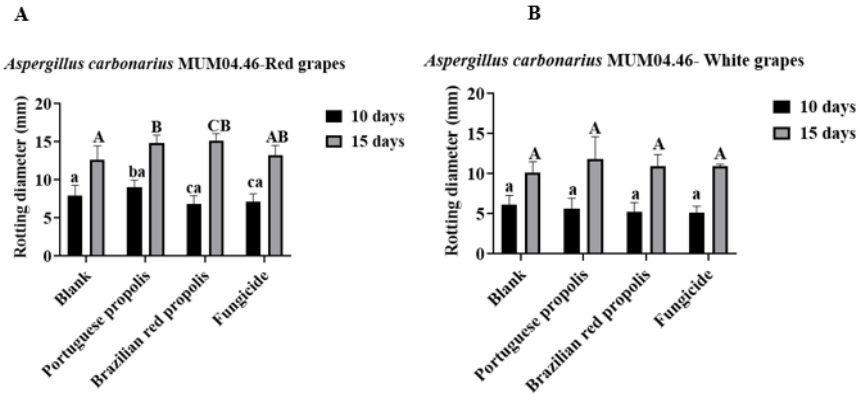

Figure 23. Effect of *Aspergillus carbonarius* MUM04.46 on the rotting of red grapes (A) and white grapes (B) treated with: blank, Portuguese propolis, Brazilian red propolis, and fungicide. Different letters (a–d; A-D) mean significantly different ($p < 0.05$) averages (n=8).

3.4.2.3 Effect of treatments on the growth of *Aspergillus carbonarius* 04.52

The inhibition diameter of the rotting caused by *Aspergillus carbonarius* Ac MUM04.52 on the red grapes is presented in **Figure 24A**. The different values found for Brazilian red propolis (7 ± 1.50 mm) and Portuguese propolis (8.11 ± 1.28 mm) for red grapes did not demonstrate significant results by comparison with those of the controls (blank and fungicide) and this at 10 days of incubation. Similarly at 15 days of incubation, the different propolis (Brazilian red propolis: 10, 96 ± 1.26 mm; Portuguese propolis: 11.34 ± 0.80 mm) did not display any significant difference as opposed to the controls. Concerning the white grapes in the **Figure 24B**, the Brazilian red propolis significantly induced regression of the rotting diameter to 4.83 ± 0.56 mm compared to the blank with a value of 6.26 ± 1.25 mm, but not significantly higher as opposed to fungicide ($p > 0.05$). A greater median of 5.52 ± 1 mm is observed with the Portuguese propolis, in fact, this value is not significantly higher than that of the fungicide and lower than that of the blank. On day 15, the activity of the Brazilian red propolis lowered allowing the evolution of the rotting diameter to 12.04 ± 0.93 mm and that of the Portuguese propolis to 11.17 ± 1.29 mm as opposed to the blank and

fungicide insignificantly. It is observed that both types of propolis have been more effective against the progression of the rotting diameter with the white grapes than the red grapes at 10 days of evaluation. On the other hand, they induced an effect more or less similar at 15 days

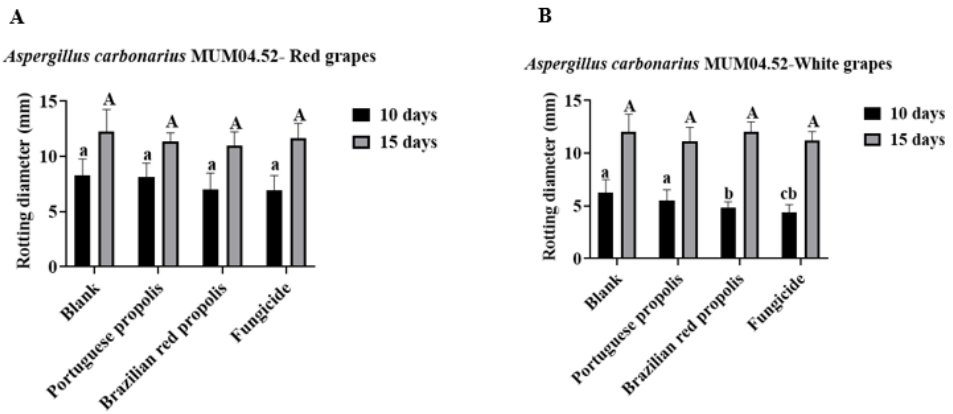

Figure 24. Effect of *Aspergillus carbonarius* MUM04.52 on the rotting of red grapes (A) and white grapes (B) treated with: blank, Portuguese propolis, Brazilian red propolis, and fungicide. Different letters (a–d; A-D) mean significantly different ($p < 0.05$) averages (n=8).

3.4.2.4 Effect of treatments on the growth of *Cladosporium* sp.

The diagram of the activity of the different candidates against *Cladosporium* sp. G1 on white grapes is represented in **Figure 25A**. First, at 10 days of growth at the red cluster level, it is observed that the Portuguese propolis with a value of 8.18 ± 1.31 mm is significantly greater than that of the blank. Always with a higher result, the rotting diameter with the experimentation of the Portuguese propolis is not significantly higher liken to the activity of the fungicide. As for the Brazilian red propolis, the Figure exhibited a result of 6.97 ± 0.93 mm which is not significantly higher than that of the blank and lower than that of the fungicide ($p > 0.05$). The observation at 15 days of growth, attested that the *Cladosporium* sp. G1 induced a rotting of the grape with a diameter of 11.57 ± 0.54 mm with the Portuguese propolis treatment, this value is much higher than that of the blank, as well as the fungicide ($p < 0.05$). On the other hand, a reduce value is observed, with the treatment with Brazilian red propolis (11.11 ± 0.78 mm) but in a manner non-significance by assimilation with controls. Compared to white grapes (**Figure 25B**), a minimize rotting is observed, the results project that the treatment with Portuguese propolis at 10 days of treatment reduced the rotting

of the grapes to a non-significant value of 5.52 ±0.63 mm compared to the controls (p > 0.05). Brazilian red propolis was able to inhibit grape rotting at a value of 5.17 ± 1.29 mm. This result, however, was not significant compared to the blank and significantly compared to the fungicide. For a 15-days incubation, the experimentation with Portuguese propolis induced an activity with a diameter of 7.80 ± 0.43 mm *versus* the rotting of the grape, a result which is lower than that of the blank (p > 0.05) and higher than that of the fungicide (p > 0.05). The Brazilian red propolis has meanwhile displayed a result of 8.69 ± 2.59 mm. This result turns out to be insignificant (p > 0.05) compared to those of the controls. Information from the graph inferred that treatment with white grapes gave lower rotting diameter at 10 and 15 days.

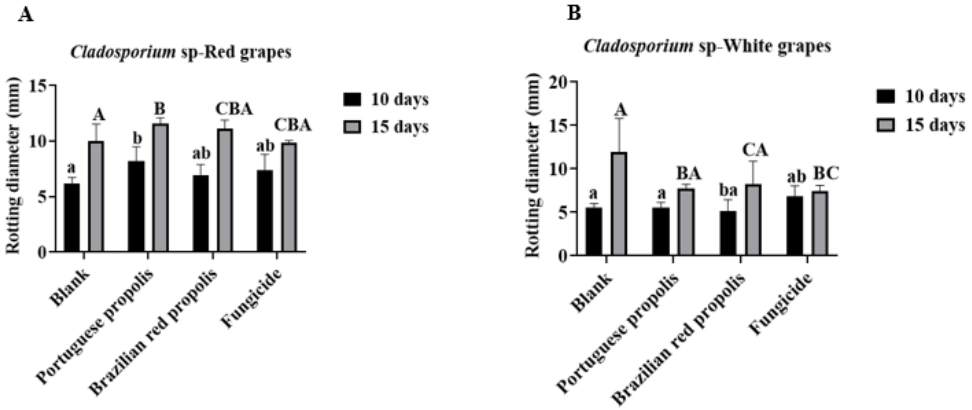

Figure 25: Effect of *Cladosporium* sp. on the rotting of red grapes (A) and white grapes (B) treated with: blank, Portuguese propolis, Brazilian red propolis, and fungicide. Different letters (a–d; A-D) mean significantly different (p < 0.05) averages (n=8).

3.4.2.5 Effect of treatments on the growth of *Botrytis cinerea*

The different results for the red grapes contaminated by *Botrytis cinerea* are imaged in **Figure 26A**. With the red grapes, it exhibited that at 10 days of treatment the Portuguese propolis (10.47 ± 0.82 mm) presented a significantly higher value than that of the blank (p < 0.05), and non-significant on the fungicide side (p > 0.05). In response, it is noted that its activity induced a greater regression of the rotting diameter of the fungus at 15 days (11.72 ± 1.81 mm) compared to the blank, and a value non-significantly higher set side by side to

the fungicide (11.54 ±0.57 mm). As for the Brazilian red propolis, it is seen that at 10 days of treatment, it presented a significantly higher value (8.37 ± 1.29 mm) compared to the blank, and in a non-significant manner compared to the fungicide. At 15 days, the growth of *Botrytis cinerea* G3 (against Brazilian red propolis) increased in a non-significant manner (12.31 ± 1.78 mm) vis-à-vis the treatment with fungicide and indicated lower growth (significant) opposed to the blank. Regarding the white grapes in the **Figure 26B**, after 10 days of observation, it is noted that none of the treatments were able to induce regression in the rotting diameter, namely the Brazilian red propolis (13.57 ± 2.37 mm) and the Portuguese propolis (14.80 ± 2.16 mm) compare to blank and fungicide. On the other hand, at 15 days of treatment, it was noticed at the level of Brazilian red propolis that a significant regression on the rotting was induced (14.12 ± 2.77 mm) compared with the blank, and significant result with the fungicide. In the case of Portuguese propolis (14.77 ± 1.48 mm), even at 15 days of treatment, no significant regression is observed by comparison to the controls. Unlike the previous fungal tests, the effect of the treatments was more expressed against *Botrytis cinerea* G3 into red grapes than in white grapes (10 and 15 days).

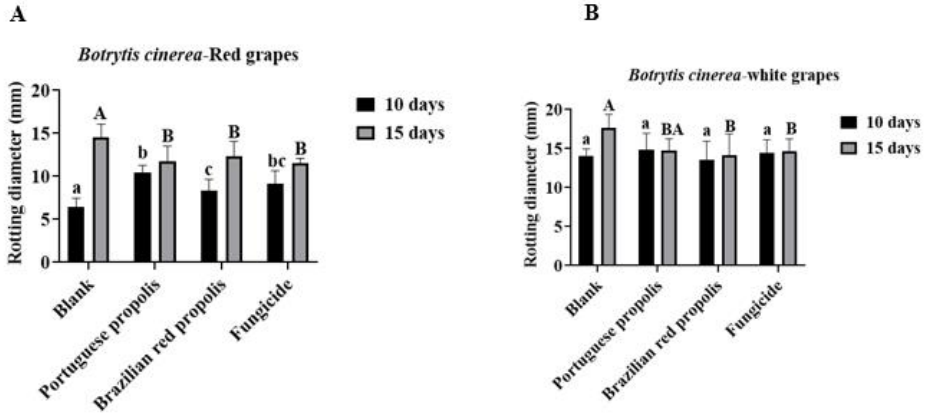

Figure 26. Effect of *Botrytis cinerea* on the rotting of red grapes (A) and white grapes (B) treated with: blank, Portuguese propolis, Brazilian red propolis, and fungicide. Different letters (a–d; A-D) mean significantly different ($p < 0.05$) averages (n=8).

3.4.2.6 Effect of treatments on the growth of *Penicillium* sp.

The upshots inferred from the activities of the different treatments on red grapes are revealed in the **Figure 27A**. The effect of *Penicillium* sp. G6 on the red grapes was significantly reduced by the Brazilian red propolis at 10 days of incubation, indeed the rotting of the grapes in the latter is 8.56 ± 1.19 mm which is lower compared to the blank and fungicide. The Portuguese propolis at 10 days of observation indicated a value of 11.80 ± 1.80 mm, this rotting diameter is smaller than those of the controls but is however not significant ($p > 0.05$). For a period of 15 days, it is observed with the Brazilian red propolis treatment, a diameter of 16.98 mm ± 0.12 mm, a value significantly greater than that of the blank and significantly lower than that of the fungicide. The treatment with the Portuguese propolis gave a result of 14.41 ± 1.07 mm, and compared to the blank it is slightly lower, and significantly lower than that of the fungicide. Concerning the white clusters in the **Figure 27B**, the two propolis did not mark any significant difference compared to the controls at 10 days of treatment, in fact, it is observed that the different treatments displayed close values (15.90 ± 1.35 and 15.59 ± 0.83 mm) respectively for the Portuguese propolis, Brazilian red propolis. On the other hand, after 15 days of observation, the treatment with the Brazilian red propolis presented a significant result on the regression of the rotting (9.11 ± 0.68 mm) compared with the treatment carried out with the blank and not significantly lower compared to the activity of the fungicide. The case of Portuguese propolis (14.77 ± 3.91 mm) revealed a different outcome, because a result not significantly lower is found compared to the treatment with blank, and significant compared to the fungicide. It can be concluded that the Brazilian red propolis has induced more regression of the rotting diameter at 15 days with the white grapes, and at 10 days with the red grapes. Besides, a better regression with red grapes from Portuguese propolis is observed, while at 15 days its effect has been almost similar.

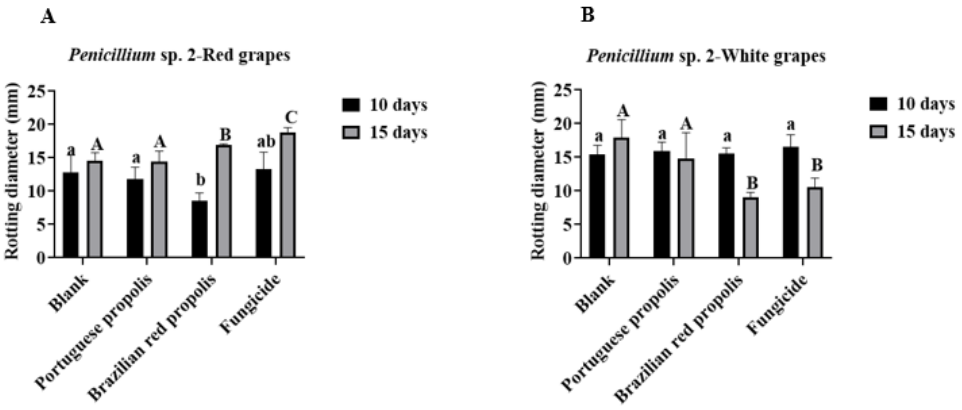

Figure 27. Effect of Penicillium sp. on the rotting of red grapes (A) and white grapes (B) treated with: blank, Portuguese propolis, Brazilian red propolis, and fungicide. Different letters (a–d; A-D) mean significantly different ($p < 0.05$) averages (n=8).

3.4.3 Effect on rotting pruning of white and red grapes

The assessment of the different treatments respectively on the red grapes and white grapes is respectively introduced in **Figure 28A**, **28B**, **29** and **30**. At 10 days of observation, no significant difference is observed between the red grapes and the white grapes respectively for the blank (red grapes: 46.04 ± 8.13 mm and white grapes: 51.06 ± 6.17) Portuguese propolis (red grapes: 51.18 ± 6.61 mm and white grapes: 50.61 ± 7.09 mm), Brazilian red propolis (red grapes: 40.93 ± 6.51 mm and white grapes: 48.44 ± 8.03 mm) and fungicide (red grapes: 51.62 ± 8.92 mm and white grapes: 54.03 ± 7.08 mm). On the other hand, at 15 days, the activity of Brazilian red propolis on the white grapes is significantly noticeable, with a value of 75.22 ± 7.55 mm for the red grapes against 62.26 ± 8.60 mm for the white grapes. Followed by the fungicide which also acted significantly on white grapes (white grapes: 66.70 ± 6.10 mm and red grapes: 72.97 ± 5.52 mm). Regarding the other treatments, no significant difference is observed, namely: Portuguese propolis: red grapes: 76.37 ± 5.84 mm and white grapes: 69.82 ± 11.15 mm; and the blank: red grapes: 74.43 ± 10.10 mm and the white grapes: 79.66 ± 13.13 mm.

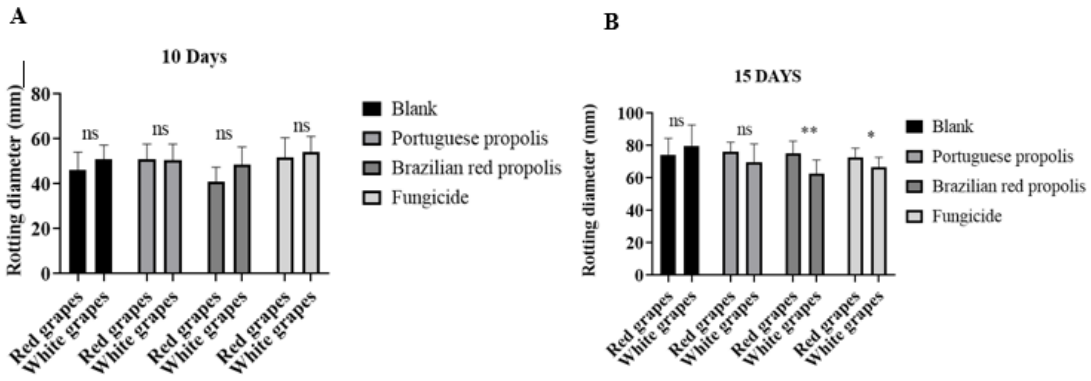

Figure 28. Rotting diameter of the different white grapes and red grapes at 10 days (A) and 15 days (B) by treatment with blank, Portuguese propolis, Brazilian red propolis, and fungicide. ns: non-significant, **: $p < 0.05$, *: $p < 0.001$.

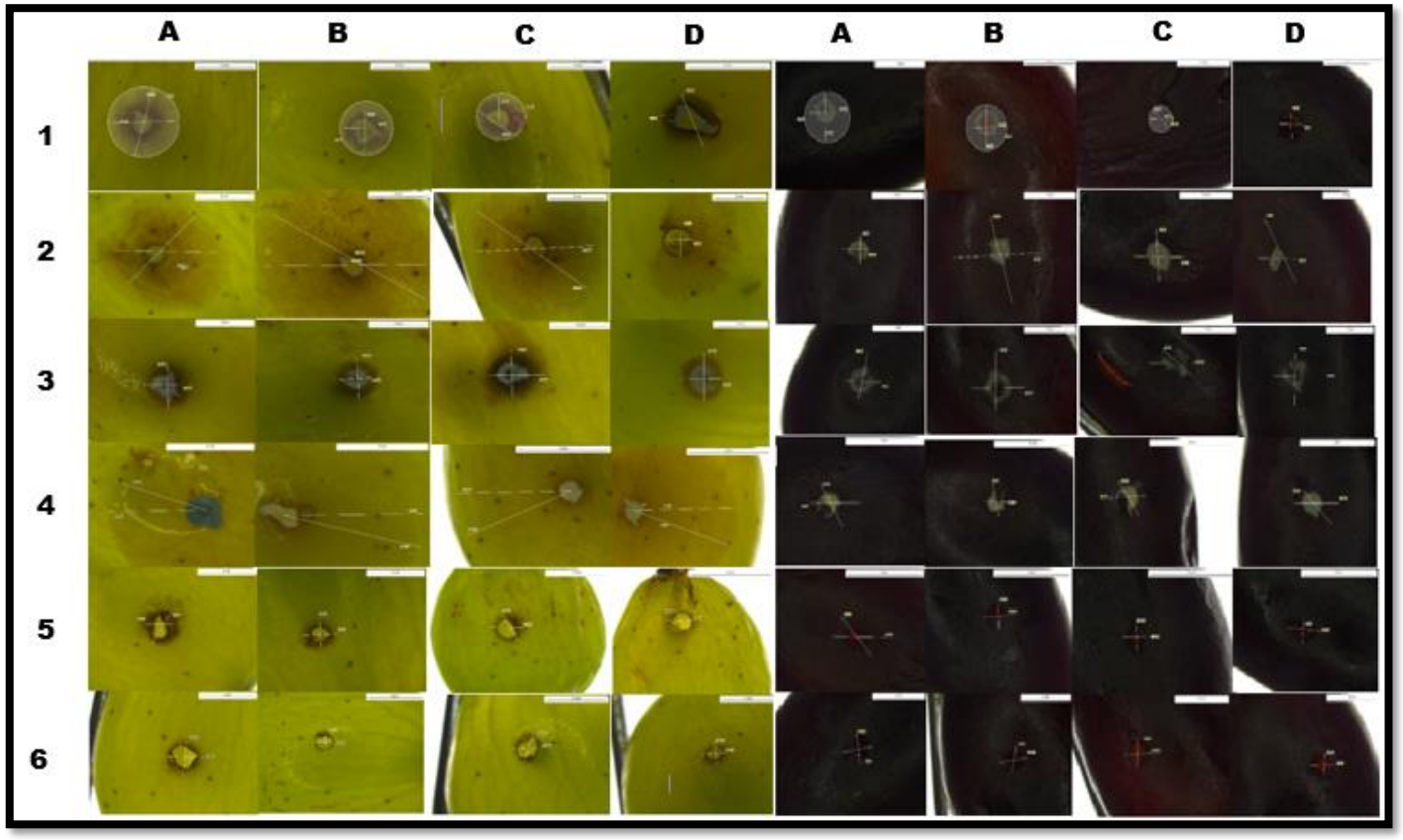

Figure 29. Fungal growth and rot development 10 days after the inoculation, with incubation at 4 ºC, on white (left) and red (right) grapes. 1: *Alternaria* sp. G4, 2: *Botrytis cinerea* G3, 3: *Cladosporium sp.* G1, 4: *Penicillium* sp. G6, 5: *Aspergillus carbonarius* MUM04.46 Ac MUM04.46, 6: *Aspergillus carbonarius* MUM04.52 Ac MUM04.52. A: Blank, B: Portuguese propolis, C: Brazilian red propolis, D: Commercial fungicide.

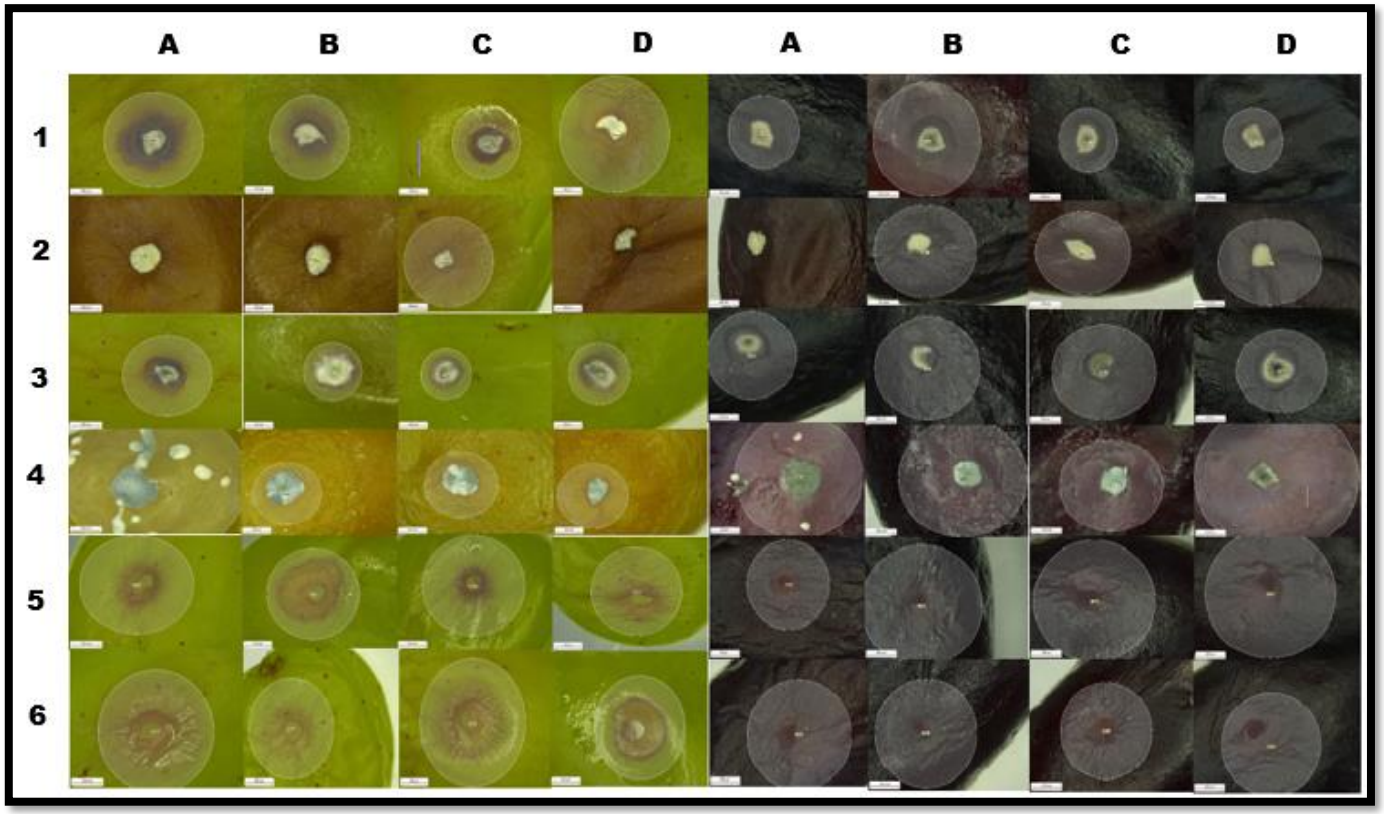

Figure 30. Fungal growth and rot development 15 days after the inoculation, with incubation at 4 ºC, on white (left) and red (right) grapes. 1: *Alternaria* sp. G4, 2: *Botrytis cinerea* G3, 3: *Cladosporium* sp. G1, 4: *Penicillium* sp. 2 G6, 5: *Aspergillus carbonarius* MUM04.46 Ac MUM04.46, 6: *Aspergillus carbonarius* MUM04.52 Ac MUM04.52. A: Blank, B: Portuguese propolis, C: Brazilian red propolis, D: Commercial fungicide.

4 Discussion

Initially the study was based on the chemical characterization of different types of propolis, namely Brazilian red propolis, Brazilian green propolis, Portuguese propolis different parameters and analysis techniques were used in order to not only identify the compounds but also to measure the level of polyphenols and flavonoids, DPPH, and the reducing power in each type of the aforementioned propolis. Finally, an evaluation of the antifungal activity of each type of propolis was carried out.

4.1 Phenolic extracts

Two major factors can mainly influence the quality of the propolis extract, namely the polarity of the solvent and the composition of the propolis which are also influenced by the setting of the time and the maintenance of the temperature in a constant manner (Sun C *et al*., 2015). In the literature, it is reported by a previous study made by Fikri *et al*. (2019) that the total phenolic content determination of propolis from Indonesian was in the range of 10.30 ± 0.16 to 28.65 ± 10.67 mg GAE/g. By comparison with the result found in this study, the values are more or less close, demonstrating that the results are in agreement (9.16 ± 3.81 mg GAE/g, Brazilian red propolis; 9.18 ± 0.42 mg GAE/g, Brazilian green propolis; 16.83 ± 1.19 mg GAE/g, Portuguese propolis). In the same study carried out by Fikri *et al*. (2019), the rate of flavonoids varied from a value of 0.76 ± 0.26 to 3.39 ± 1.08 mg QE/g. The levels of flavonoids from this experiment are slightly close to the interval, namely, 1.33 ± 0.48 mg QE/g (Brazilian red propolis), 1.71 ± 0.43 mg QE/g (Brazilian green propolis), and 5.84 ± 0.12 mg QE/g (Portuguese propolis). Even if the results are roughly in rank, the difference in total phenolic compound and total flavonoid content levels is explained by the diversity of the concentration of polyphenols in each type of propolis, as well as by the extraction method applied across the world (Silva VC *et al*., 2012). Furthermore, it has been reported by Fabris *et al*. (2013) that great variability exists between the phenolic compounds of European propolis and that of Brazilian. The Brazilian propolis by comparison to the European showed a lower rate of compounds (Fabris *et al*., 2013).

4.1.1 Chemical characterization by LC/DAD/ESI-MS^n

Liquid chromatography with detection by diode array coupled with tandem electrospray ionization mass spectrometry (LC/DAD/ESI-MS^n) was used for the phenolic identification and quantification, in fact, it is one of the best-suited techniques to the phenolic complexity of propolis (Falcão SI, 2013).

The results found for Brazilian red propolis concerning its major compounds in the literature correspond to isoflavones, isoflavans, pterocarpans and compounds such as isoliquiritigenin, liquiritigenin and naringenin, xanthochymol, oblongifolin A and guttiferone E (Piccinelli *et al*., 2011). These results are in line with those in this experience (**Table 9**) where the detection of 26 phenolic compounds has been noted and mainly consists of liquiritigenin (Nr 2), vestitol (Nr 10), neovestitol (Nr 13), guttiferone E (Nr 26). The Brazilian green propolis, showed a phenolic composition of 21 (Table 8), with *p*-coumaric acid (Nr 3), dicaffeoylquinic acid (Nr 4 5), dihydrokaempferide (Nr 7), drupanin (Nr 10), kaempferide (Nr 14), and artepillin C (Nr 21). These results are in agreement with those of previous studies (Machado JL *et al*., 2012; Saito Y *et al*., 2015; Lustosa SR *et al*., 2008) where the identified compound have been the artepillin C, baccharin, kaempferide, isosakuranetin, dihydrokaempferide, drupanin, *p*-coumaric acid, caffeic acid, aromadendrin, caffeoylquinic acid derivatives, and triterpene lupeol-3-(3′R-hydroxy)-hexadecanoate. Concerning the Portuguese propolis, according to its European brown poplar propolis origin, the raw propolis used to obtain the various preparations was found to possess flavonoids as the major species, with flavones (chrysin and apigenin), flavanones (pinocembrin), and flavonols (quercetin, pinobanksin, galangin, and their derivatives) being the most common components (Ristivojevic P *et al*., 2015). Likewise, in our study, the major compounds among 39 identified were cinnamylidenacetic acid (Nr 15), pinocembrin (Nr 21), chrysin (Nr 23), pinobanksin-3-*O*-acetate (Nr 31), galangin (Nr 25) (**Table 7**). On the other hand, the total number of phenolic compounds found in the different propolis varies slightly from those of the literature, this difference can be explained by the type of propolis but also in the climatic conditions around the beehive, these factors influence thus the composition of polyphenols

(Apostolou A *et al*., 2013). In fact, 30 phenolic compounds were found for Brazilian red propolis (Morais DV *et al*., 2021) against 26 in our study, 14 phenolic compounds for Brazilian green propolis (Coelho J *et al* 2017) against 21 studies and 38 (Falcão SI *et al*., 2013) for Portuguese propolis against 39 analyzed in this study. And among these examples of references cited different detection instruments have been applied, Coelho *et al*. (2017) used the HPLC technique, while Morais *et al*. (2021) used the LC-ESI-QTOF-MS/MS tool, and finally Falcão *et al*. (2013) used LC/DAD/ESI-MS^n. Hence the explanation for the approximation of the number of compounds detected for Portuguese propolis. Thus, providing confirmation that detection technique is crucial in establishing the content of resin of complex nature.

4.2 Antioxidant activity

4.2.1 Scavenging of DPPH radicals

The hydrogen donor capacity of the antioxidants present in the samples is related to the anti-free radical capacity. Thus, a low representation of the values of EC_{50} showed a stronger anti-free radical capacity. Indeed, the EC_{50} is the number of antioxidants necessary to reduce the initial concentration of DPPH by 50% (Coelho J *et al*., 2017). The analysis of the data showed that the antioxidant activity of different propolis is essentially linked to the number of phenolic compounds present (Mello & Hubinger, 2012). That said, The Brazilian red propolis stated a total of 26 phenolics compound with a concentration of 0.10 ± 0.00 mg/mL, while the Brazilian green propolis (21 identified compound) showed the highest concentration (with the lowest number of phenolic compounds) of 0.16 ± 0.01 mg/mL, this result explains the non-significant difference between Brazilian red propolis and Brazilian green propolis. Finally, the Portuguese propolis with a content of 39 phenolic compounds had a value of 0.04 ± 0.00 mg/mL of concentration, hence a significant difference compared to the other two propolis. Our different results are slightly in line with those of Mărghitaş *et al*. (2009) where a range of 0.3 to 5.6 mg/mL was found for different propolis collected in Transylvanian stationary apiaries. Additionally, a study carried out by Falcão *et al*. (2013)

reported the DPPH of several propolis from different regions of Portugal, the values were between 0.008 mg/mL to 0.093 mg/mL, which are in line with those found.

4.2.2 Reducing power

In fact, the reducing power indicates that the compounds can act as primary and secondary antioxidants through electron donation and the reduction of intermediate oxides (Chanda S & Dave R, 2009). With a value of 0.48 ± 0.03 mg GAE/g, the Brazilian green propolis has manifested the lower reducing power, followed by Brazilian red propolis which has displayed a value of 0.52 ± 0.01 mg GAE/g, and even if a non-significant difference is observed between the last two, Brazilian red propolis has demonstrated a greater antioxidant capacity than that of Brazilian green propolis. Finally, the experience has demonstrated that with a significant value of 0.98 ± 0.02 mg GAE/g, the Portuguese propolis showed the greatest reducing power. The reducing power of different propolis from Portugal studied by Moreira *et al*. (2008) gave results between 0.009 and 0.055 mg/g, these values are comparably low to those found in this study. In fact, the type of solvent extraction used in his study was methanol, hence the difference. Besides, a relationship is made between the structure of polyphenols as well as flavonoids and their ferric reduction capacity, moreover, many studies have in the past shown a strong reducing power of plant extracts (Mohtar LG *et al*., 2020).

4.3 Antifungal activity of the different propolis

4.3.1 In vitro antifungal activity

The antifungal efficacy of the different types of propolis and its contribution to reducing the rotting diameter of red and white grapes commonly found in public sales areas was tested. The latter which too often are found contaminated by different fungi such as *Alternaria* sp., *Botrytis cinerea*, *Cladosporium* sp., *Penicillium* sp. 2, *Aspergillus carbonarius* MUM04.46 and, *Aspergillus carbonarius* MUM04.52. To this end, an analysis of its impact on the activity of the various aforementioned fungi was done. A demonstration of their activity in inhibiting the growth of different fungi has been demonstrated. In this experiment it was seen

that the activity of the different propolis is based on the type of fungi, as well as on the chosen concentration (Petruzzi *et al*., 2020). All the tested fungal pathogens were sensitive. In the study carried out by Curifuta *et al*. (2012), the activity of propolis was tested against different types of fungi, namely, *Ulocladium* sp., *Trichoderma reesei*, *Penicillium expansum*, *Alternaria alternata*, *Botrytis cinerea* and *Fusarium* sp., and this at different concentration of propolis (0.5%, 1.0%, 2.5% and 5%) the best inhibition was found with the greatest percentage of concentration of propolis, namely, at 5%. This leads to the deduction that the fungal growth against the activity of propolis is highly dependent on the dose of concentration applied. Along with the study of Soylu *et al*. (2008) where the antifungal activity of propolis against postharvest disease agent *Penicillium digitatum* was tested *in vitro*. In the experiment, propolis was used at different concentrations namely 10, 50, and 100 µg/mL. It turned out that only propolis at 100 µg/mL was able to induce complete inhibition of the fungus.

4.3.1.1 Antifungal activity on grapes

The effectiveness of the antifungal activity of Brazilian red propolis has been proven against the growth of several fungi compared to the action of the fungicide and blank. The results obtained showed that Brazilian red propolis exerted a strong antifungal activity against *Alternaria* sp. (red grapes), *Penicillium* sp. 2 (red grapes and white grapes), and moderately with *Alternaria* sp. (white grapes), *Cladosporium* sp. (white grapes), *Cladosporium* sp. (red grapes), *Alternaria* sp. (red grapes), *Aspergillus carbonarius* MUM04.52 (red grapes/white grapes), *Botrytis cinerea* (red grapes/white grapes), *Aspergillus carbonarius* MUM04.46 (red grapes/white grapes). Indeed, several studies have demonstrated the antifungal activity of Brazilian red propolis due to their ability to inhibit the proliferation of several fungi namely, *Candida albicans* and *Candida glabrata (*Martorano-Fernandes L *et al*., 2020), *Candida parapsilosis, Candida tropicalis (*Francisco LD *et al*., 2018), *Paracoccidioides brasiliensis (*Santos LA *et al*., 2021). The phenolic compounds are linked to the antibacterial activity of propolis and some have been identified such as formononetin, biochanin A, and liquiritigenin, flavonoids (Hanski I *et al*., 2014; Kong W *et al*., 2015; Gaur R *et al*., 2016) which were also identified in this study.

Concerning the Portuguese propolis, it is observed that its antimicrobial activity was moderately expressed against the rot of grapes by different fungi compared to the controls also, namely against the *Alternaria* sp. (white grapes/red grapes), *Aspergillus carbonarius* MUM04.52 (white grapes), and *Aspergillus carbonarius* MUM04.46 (white grapes) and the Portuguese propolis had weak activity against *Botrytis cinerea* (white grapes/red grapes), *Cladosporium* sp. (white grapes/red grapes), *Penicillium* sp. 2 (white grapes/red grapes), and *Aspergillus carbonarius* MUM04.46 (white grapes/red grapes). These results thus demonstrate the antimicrobial activity of Portuguese propolis by the reduction (inhibition) of the growth of the aforementioned different fungi. The antimicrobial activity of Portuguese propolis was also demonstrated by the study made by Popova *et al*. (2007), where propolis from different regions were used in an antimicrobial test, it was resolved that propolis derives its activity from phenolic compounds, especially flavonoids, phenolic acids, and their esters. Besides, in the study conducted by Curifuta M *et al*. (2012) a demonstration on the antifungal activity of Chilean propolis against *Penicillium expansum*, *Alternaria alternata* and, *Botrytis cinerea*, it was resolved that propolis demonstrated inhibitory activity strongly against *Penicillium expansum*, moderately against *Alternaria alternata* and weakly against *Botrytis cinerea*. The same outcomes were observed in the results of this study with the Brazilian red propolis and Portuguese propolis.

The difference between the activity of Brazilian and Portuguese propolis is probably due to the botanical origin of the two resins, thus affecting their antimicrobial activity (Pobiega *et al*. 2019). In **Table 9**, it was shown that the Portuguese propolis had the greatest number of chemical compounds, but yet a strong activity was not observed on its part Falcão *et al*. (2013) reported that the synergy of the chemical compounds of propolis can induce a decrease in its activity, while singular isolation of its compounds can give greater results. In our case, although the Portuguese propolis demonstrated strong activity in the *in vitro* test, an interaction of these phenolic compounds and those of the grapes may have induced a loss of its activity. In addition, the different propolis have been shown to exert a delay in microbial growth thus acting as a protective film in accordance with those of Petruzzi *et al*. (2020).

Regarding the antifungal activity of Brazilian green propolis, it was seen that among the other two propolis (Brazilian red propolis and Portuguese propolis) it showed the weakest activity against the different fungi. The nature and composition of the different resins can be involved. Without forgetting the concentration of phenolic compounds which it abounds. From a special point of view, flavonoids are reputed to be the main antimicrobial components of propolis. As in the study made by Yang *et al*. (2011), where different propolis from different regions of China have demonstrated antifungal activities, and this activity has been given to pinocembrin, pinobanksin, chrysin, and galangin they contained. Likewise, another study by Campana *et al*. (2009) where a demonstration of the antifungal activity of Bulgarian propolis was tested, and as a result, galangin was seen as a main antimicrobial compound. In addition, pinocembrin, pinobanksin 3-acetate, chrysin, CAPE, acacetin, and galangin were present in Mexican propolis which has shown antimicrobial activity (Valencia D *et al*., 2012). In this study, on the one hand, due to the high concentration of pinocembrin (140.57 ± 0.16 mg/g), pinobanksin-3-*O*-acetate (105.51 ± 0.05 mg/g) in the Portuguese propolis, on the other hand, the presence of guttiferone E/xanthochymol (27.95 ± 0.30 mg/g), vestitol (26.16 ± 0.02 mg/g) in Brazilian red propolis, finally, the presence of kaempferide (35.66 ± 0.13 mg/g) in Brazilian green propolis can be taken as not only the main antifungal compounds but also the difference in activity between propolis. Additionally, these different compounds have in the past demonstrated several activities, such as, pinocembrin which inhibited the growth of *Penicillium italicum* and *Candida albicans* by interfering with energy homeostasis and cell membrane damage of the pathogen, in fact, pinocembrin is known for different characteristics, namely, neuroprotective activity, anticancer activity, antimicrobial activity, anti-inflammatory activity (Rasul A *et al*., 2013). Lyles *et al*. (2014) shared the effectiveness of guttiferone E against *Plasmodium falciparum* strain D6 and W2. Bueno-Silva *et al*. (2013) relayed that the vestitol was able to exert anti-inflammatory and antimicrobial activity (against *Streptococcus mutans*, *Streptococcus sobrinus*, *Staphylococcus aureus*, and *Actinomyces naeslundii*). Finally, Wang *et al*. (2018) have successfully experimented the antitumor, the antioxidant and the anti-inflammatory activity of kaempferol.

Regarding the two types of grapes, the analysis of the graphs showed that by using the different treatments on the red grapes and white grapes, that at 10 days of treatment no significant difference is seen between them, unlike at 15 days of storage where the viability of the white grapes is significantly seen with the treatment with the Brazilian red propolis and the fungicide. Red grapes are renowned for their higher antioxidant capacity, and contain a high level of resveratrol (trans-3,5,4′-trihydroxystilbene) (Singh CK *et al*., 2015; Tang GY *et al*., 2018). The study made by Petruzzi *et al*. (2020) showed that propolis at high concentration is not compatible for a direct application, due to the possible strong impact on the organoleptic characteristic of foods, moreover, the control (blank) proved that at 10 and 15 days that the red grapes decay less quickly (**Figure 28**).

Conclusion and perspectives

Through this work it was intended to evaluate the different bioactivity of phenolic propolis extracts obtained from the most common types: European propolis from poplar trees and Brazilian green and red propolis, with the final goal of the assessment of the antifungal potential for a potential application as a preservative in food.

The evaluation of phenolic compounds enabled a distinction between the propolis types, the Portuguese propolis type had the highest number of chemical compounds, followed by Brazilian red propolis and finally Brazilian green propolis. In addition, Portuguese propolis had a higher total phenolic compound and flavonoids than that of Brazilian red propolis which in turn is higher than that of Brazilian green propolis. About the DPPH, the Portuguese propolis has shown to have the lowest concentration (0.04 ± 0.00 mg/mL) followed by the Brazilian red propolis (0.10 ± 0.00 mg/mL) and then Brazilian green propolis (0.16 ± 0.01 mg/mL). Regarding the reducing power, Portuguese propolis comes out major with a value of 0.98 ± 0.02 mg GAE/g significantly compared to Brazilian red propolis and Brazilian green propolis (0.52 ± 0.01 and 0.48 ± 0.03 mg GAE/g, respectively). The detection of the components of the different propolis carried out by LC/DAD/ESI-MS^n showed that for the Portuguese propolis, cinnamylidenacetic acid, pinocembrin, chrysin, pinobanksin-3-*O*-acetate, galangin represent its major compounds. Subsequently, the major components of Brazilian red propolis were liquiritigenin, vestitol, neovestitol, guttiferone E. Regarding Brazilian green propolis, the following compounds were detected as major compounds namely: *p*-Coumaric acid, 5-*O*-caffeoylquinic acid, dihydrokaempferide, drupanin, kaempferide, and artepillin C.

The antifungal potential of the different propolis types is strongly linked to different factors such as type of grapes, the type of microorganisms, the concentration of inoculation, and that propolis is a product that acts by retarding fungicidal growth. A high concentration of propolis applied directly to the grapes induced browning of the grape-surface, this said by the result of the control (blank) it is seen that the red bunches have a longer tolerance to the contamination.

As a future perspective, a definition of the exact dose/response of propolis to be applied to grapes will prevent grape rotting, but also an identification and isolation of chemical compounds with antioxidant, antibacterial, and antifungal characteristics for which will be used precisely in the grape by food industry as preservative. The effectiveness of propolis also depends on the surrounding microenvironment, in other words, it is not only related to the sum of the phenolic compounds and their bioactivities. The combination of all these factors can induce an inhibitory or synergistic effect, and therefore, to facilitate of the use of propolis is more than necessary. Among these solutions, there is encapsulation which aims not only to protect the active compounds of propolis but also to mask the taste and odor which represent obstacles to its use by industrialists. In addition, several studies recommended the use of the propolis-based spray drying technique with a combination with other compounds such as maltodextrin, Arabic gum, or inulin in order to increase the activity spectrum of the product.

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