How do bees prevent hive infections? The antimicrobial properties of propolis

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Propolis is a wax-like resin produced by honeybees from substances collected from plants, which are mixed with beeswax and other compounds of bee metabolism. Its chemical composition depends on the specific local flora at the site of collection and also of climatic characteristics, resulting in a striking diversity of constituents. Propolis is a mixture of balsams and resins, waxes, essential oils, pollen, and other substances which is used by bees in the construction, repair and protection of their hives, mainly due to its mechanical properties and antimicrobial activity. Because of the broad spectrum of biological activities and medicinal properties, propolis has been used by man since ancient times. Nowadays it is still used in traditional and alternative medicine, but also in the modern biocosmetic industry and in health foods. The renewed interest in this natural product is due to its antimicrobial, anticancer, antioxidant, antiviral, and other properties. Here we review the current knowledge about propolis diversity (geographic, compositional), its biological activities with emphasis to antimicrobial activity, and its potential therapeutic applications. Propolis ecological functions are also discussed.

Keywords propolis, bioactivities, antimicrobial

1. Introduction

Propolis or bee glue, as it is commonly named, is a natural resinous mixture produced by honeybees (*Apis mellifera*) from substances collected from parts of plants, buds and exudates [1]. This resin is masticated, salivary enzymes are added, and then it is mixed with beeswax and probably with other compounds of bee metabolism [2]. Etymologically the word *propolis* derives from the Greek *pro* (for 'in front of', 'at the entrance to') and *polis* (for 'community' or 'city'), meaning that this natural product contributes to hive defence. Due to its waxy nature and mechanical properties, bees use propolis in the construction and repair of their hives - for sealing openings and cracks and smooth out the internal walls [2, 3] - and as a protective barrier against external invaders or against weathering threats like wind and rain. They also use bee glue to embalm the carcasses of dead intruders, thus avoiding their decomposition and eliminating a potential source of microbial infections.

Propolis is a complex mixture composed of beeswax, resins and plant balsams, essential oils, pollen and some organic and mineral compounds [1, 2]. It has been extensively employed by man since ancient times, especially in folk medicine to treat or alleviate several maladies. Egyptians knew very well its anti-putrefactive properties and used bee glue to embalm their cadavers. Incas employed propolis as an anti-pyretic agent. Greek and Roman physicians used it as mouth disinfectant and as an antiseptic and healing product in wound treatment, prescribed for topical therapy of cutaneous and mucosal wounds [2]. These therapeutic applications were perpetuated in the Middle Age and among Arab physicians. Listed as an official drug in the London pharmacopoeias of the 17th century, propolis became very popular in Europe between the 17th and 20th centuries due to its antibacterial activity. In Italy, Stradivari used bee glue as a violin varnish [4]. In the end of 19th century, propolis was widely used due to its healing properties [5] and in the Second Global War it was employed in several Soviet clinics for tuberculosis treatment, due to the observed decline of lung problems and appetite recovery. In the Balkan states it was one of the most frequently used remedies, applied to treat wounds and burns, sore throat and stomach ulcer [6]. The first scientific work with propolis, reporting its chemical properties and composition, was published in 1908, and indexed to *Chemical Abstracts* (reference nº 192) [7]. Later, in 1968, the first patent [8] was obtained to produce bath lotions in Romania. The following years saw an increase in international patents, predominantly from the former USSR and satellite countries during the 1980s. In 2000 almost half of the commercial licenses were Japanese [9], but in the last decades there were patents registered worldwide.

Nowadays, propolis is a natural remedy found in many health-food stores in various forms for ingestion or topical use. It is still used in many regions of the world, including Japan and the European Union, either in cosmetics or as popular alternative medicine for self-treatment of various diseases. Current applications of propolis include preparations for cold syndrome (upper respiratory tract infections, common cold, flu-like infections), as well as dermatological preparations useful in wound healing, treatment of burns, acne, herpes simplex and genitalis, and neurodermatitis. Additionally, propolis is used in mouthwashes and toothpastes to prevent caries and treat gingivitis and stomatitis, and it is widely used in cosmetics and in health foods and beverages. It is commercially available in the form of capsules (either pure or combined with aloe gel, *Rosa canina* or pollen), extracts (hydroalcoholic or glycolic), mouthwash

solutions (combined with lemon balm, sage, mallow and/or rosemary), throat lozenges, creams, powder and also in more purified products from which the wax was removed.

Due to its medicinal and biological properties, propolis became the focus of great scientific interest during the last 30 years mainly envisaging its application in human and veterinary medicine, pharmacology and cosmetics.

2. Propolis characteristics, origins and chemical composition

Propolis is a lipophilic, hard and brittle material when cold but becomes soft, pliable, gummy and very sticky when warm [10]. It possesses a characteristic and pleasant aromatic smell and varies in colour from yellow-green, to red and to dark brown depending on its source and age [3, 11].

As referred above, propolis is a complex mixture made from plant-derived and bee-released compounds. The proportion of the various substances is variable and depends upon the place and time of collection [1] but, in general, raw propolis is composed of around 50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% of various organic compounds [2, 12, 13]. More than 300 constituents were identified in different samples [11, 14], and new ones are still being recognized during chemical characterization of new types of propolis [3, 15-19].

Many analytical methods have been used for separation and identification of propolis constituents and the substances identified belong to the following groups of chemically similar compounds: flavonoids; benzoic acids and derivatives; benzaldehyde derivatives; cinnamyl alcohol, and cinnamic acid and its derivatives; other acids and respective derivatives; alcohols, ketones, phenols and heteroaromatic compounds; terpene and sesquiterpene alcohols and their derivatives; sesquiterpene and triterpene hydrocarbons; aliphatic hydrocarbons; minerals; sterols and steroid hydrocarbons; sugars and amino acids [20]. As it may be expected, volatile compounds (produced by the source plants) are present in low amounts [14]. Sugars are thought to be introduced accidentally during the elaboration of propolis and/or passage of bees over the resin. Some compounds are probably present in all propolis samples and contribute to its characteristic properties. Others are represented in many samples of different origins, but a few others only occur in propolis from particular plant species [1].

Many studies on the properties and composition of propolis have been made without knowing the plant(s) from which the sample was obtained, or the sites where bees collected the material. However, the large number of studies reporting to propolis chemistry allowed researchers to realize that its chemical composition is not only complex but also highly variable, depending on the season and local flora at the site of collection as well as on the type of bees foraging [3, 11, 17, 21]. The main visited plant species are poplars (*Populus* spp.), beech (*Fagus sylvatica*), horsechestnut (*Aesculus hippocastanum*), birch (*Betula alba*), alder (*Alnus glutinosa*) and various conifer trees [1, 20]. The source species can vary with geographical regions and determines propolis chemical composition [21]. Indeed, there are differences between propolis of temperate and tropical zones: while the materials used in temperate regions are essentially bud exudates from different poplar trees [14], these are absent in tropical zones and bees use exudates of other plants – mainly the leaf resin of *Baccharis dracunculifolia* - giving the resin a different composition. European propolis contains the typical "poplar bud" phenolics: flavonoid aglycones (flavones and flavanones), phenolic acids and their esters, whereas the major constituents of Brazilian propolis are terpenoids and prenylated derivatives of *p*-coumaric acids [3, 22]. HPLC analysis of the phenolic compounds present in *Populus nigra* bud exudates clearly support that this is the main origin for propolis in continental Europe, North America, West Asia, and New Zealand [1, 10, 23]. It is also reported that in areas where poplars are not native plants, such as in Australia and equatorial regions in South America, bees gather exudates from *Ambrosia deltoidea* and *Encelia farinose* to make propolis. Phenolic compounds of propolis from Venezuela have its origin in resin exudates of *Clusia minor* and *Clusia major* [12]. The most important biologically active constituents of propolis from different geographic locations and the corresponding source species are represented in Table 1. Apart from plant exudates collected by bees, the compounds identified in propolis are originated from other two sources: secreted substances from bee metabolism and also from materials introduced during resin elaboration [1, 11]. In the absence of natural materials, bees may use some man-made products like asphalt and mineral oils as substitutes [14, 24].

Poplar-type propolis is undoubtedly the most studied one but there are many other propolis types, as that found in some Mediterranean regions (Sicily, the Adriatic coast) which has diterpenic acids as main components [25, 26]. More recently, a new type of Brazilian propolis, popularly known as "red propolis", was collected in northeast Brazil [19]. Its intense red colour and chemical composition make it different from the 12 types formerly classified by Park *et al.* [12]. In fact, Brazilian propolis is quite diverse in chemical composition, due to Brazil's biological and climate diversity. Brazilian red propolis, like Cuban red propolis, contains isoflavonoids which have been associated with a variety of health benefits, including the relief of some symptoms in menopause, osteoporosis, hormonal cancer and prostate cancer [26]. In tropical countries of South America there are indigenous stingless bees which mix collected resinous material with bee wax and soil, forming geopropolis [3, 14, 28].

Table 1 Propolis types, geographic origin, main plant sources and chemical compounds [adapted from 11].

Propolis type	Geographic origin	Plant source	Main bioactive compounds	References
Poplar propolis	Europe, North America, non-tropic regions of Asia	*Populus* spp., most often *P. nigra* L.	Flavones, flavanones, phenolic acids and their esters	[3]
Birch propolis	Russia	*Betula verrucosa* Ehrh.	Flavones and flavonols (different from poplar propolis)	[3]
Green (rosemary) propolis	Brazil	*Baccharis* spp., predominantly *B. dracunculifolia* DC.	Prenylated *p*-coumaric acids, diterpenic acids	[11]
Red (Clusia) propolis	Cuba, Venezuela	*Clusia* spp.	Polyprenylated benzophenones	[25]
"Pacific" propolis	Pacific region (Okinawa, Taiwan)	Unknown	C-prenylflavanones	[22]
"Canarian" propolis	Canary Islands	Unknown	Furofuran lignans	[26]

Biological activities are always present in propolis but they can be associated with completely different chemical profiles in samples from different geographic and climatic zones [26]. The principal compounds responsible for propolis biological activities are flavonoids, aromatic acids, diterpenic acids and phenolic compounds, but very often different propolis types have distinct main bioactive compounds (Table 1). Hegazi and co-workers [16] tested propolis from Austria, Germany and France for antimicrobial activity and observed different activity spectra but some similarities in qualitative composition. In propolis from Northern Argentina the highest antimicrobial and antioxidant activity correlated with highest concentrations of flavonoids (pinocembrin) and phenolics [29]. Many studies confirm these results showing these compounds to be responsible for the antimicrobial and antioxidant properties of many propolis types [22, 26]. Table 2 points up some of the compounds that have been correlated with specific bioactivities in different propolis types [21], making evident that it is not possible to ascribe a certain property exclusively to one individual component [26].

Table 2 Compounds responsible for four biological activities of different propolis types [21].

Propolis type	Antibacterial	Anti-inflammatory	Antitumor	Antioxidant	References
Brazilian (*Baccharis* type)	Prenylated *p*-coumaric acids, labdane diterpenes	Unidentified	Prenylated *p*-coumaric acids, clerodane diterpenes, benzofuranes	Prenylated *p*-coumaric acids, flavonoids	[11]
Cuban	Prenylated benzophenones	Not tested	Prenylated benzophenones	Prenylated benzophenones	[11]
European (Poplar type)	Flavanones, flavones, phenolic acids and their esters	Flavanones, flavones, phenolic acids and their esters	Caffeic acid phenetyl ester	Flavonoids, phenolic acids and their esters	[6]
Taiwanese	Not tested	Not tested	Prenylated flavanones	Prenylated flavanones	[24]

The distinct chemical compositions of propolis from different origins led to the expectation that their biological properties would be dissimilar, but this is amazingly untrue in many cases [21] and samples of different origins can display identical biological activity. Kujumgiev and co-workers [26] observed that in spite of the phytochemical differences found in propolis samples from different geographic locations, they all exhibited significant antibacterial and antifungal activities, revealing that different combinations can result in a same bee glue´s biological activity. This chemical redundancy, very common in nature, suggests that the antimicrobial activity is a vital property that bees must guarantee independently from the geographic area they inhabit. Popova and collaborators [31, 32] used European, Brazilian and Central American samples of propolis and showed that samples from Europe and Brazil had similar activities despite the drastic differences in chemical composition, and that they were more active than Central American propolis. It is clear that propolis research should include not only a chemical characterization but also combine different biological tests [33]. It also comes out from these observations that, probably, the differences in antimicrobial potency and specificity of propolis sampled from different geographic regions might be related with the risk of emerging (specific) microbial infections in those sites. Pushing forward this line of reasoning, it would be very interesting to study in what extent propolis antimicrobial activity against specific pathogenic species might constitute an indicator of the presence of those pathogens in the local or the risk of a certain disease.

3. Propolis analysis

Propolis cannot be used directly as raw material and a simple fractionation to obtain compounds is difficult due its complex composition. The usual procedure is the use of a solvent [13], which should remove the inert material and preserve the desired compound(s). As the composition of propolis primarily depends upon the vegetation from where it was collected but secondarily upon the methods used for extraction [11], the solvent should be carefully chosen [34]. The principal solvents used for extraction of bioactive compounds, and the correspondent classes of chemical compounds extracted are depicted in Table 3.

Table 3 Solvents used for active component extraction. Compounds in bold are commonly obtained only in one solvent [adapted from 34].

Water	Methanol	Ethanol	Chloroform	Dichloromethane	Ether	Acetone
Anthocyanins **Starches** Tannins Saponins Terpenoids **Polypeptides** **Lectins**	Anthocyanins, Terpenoids, Saponins, Tannins, **Xanthoxylline, Totarol,** **Quassinoids, Lactones,** Flavones**, Phenones,** Polyphenols**, Polypeptides,** **Lectins**	Tannins Polyphenols **Polyacetyle** **nes** Flavonols Terpenoids **Sterols** Alkaloids	Terpenoids Flavonoids	Terpenoids, Tannins, Polyphenols, **Polyacetylenes**, Flavonols, **Sterols**, Alkaloids	Alkaloids Terpenoids **Coumarins** **Fatty acids**	Flavonols

A routine and common procedure consists to extract the fraction soluble in alcohol, called "propolis balsam", discarding the insoluble fraction or wax fraction [1]. Ethanol is the most common solvent choice, but other solvents have also been used for separation and identification of many constituents in propolis [35-37]. Studies regarding the evaluation of propolis´ bioactivities have been performed using mainly ethanol extracts of propolis (EEP) or water extracts (WEP).

In the last decade the idea that propolis has a complex but more or less constant chemistry, has radically changed. A new paradigm arose, supported by the analysis of numerous propolis samples collected in different seasons and from different geographic regions, leading to the discovery that the chemical composition of bee glue is highly variable. This diversity in propolis chemistry is reflected on its pharmacological properties and demands for standardization [33]. In order to be accepted officially into the main stream of the healthcare system, chemical standardization is effectively an obligatory criterion. Such standardization could be achieved by formulating different propolis types according to plant source/chemical profile but for that it is essential to have detailed and reliable comparative information on every type of biological activity and chemical data. Propolis is a widely accepted product and has an established safety profile. However, it is also a known contact allergen and may seldom induce some adverse reactions as revealed by some reported cases of allergy and contact dermatitis [38-40]. Furthermore, when natural plant materials are absent for propolis elaboration, bees may use some man-made products like asphalt and mineral oils as substitutes [14]. This can introduce some unsafe substances such as lead (Pb) and other metals like copper (Cu), cadmium (Cd) and zinc (Zn) which can contribute to the increase of propolis toxicity [24]. International markets are also very demanding in terms of trace compounds, heavy metals and environmental pollutants [9], and the development of standardized manufacturing procedures is needed. Propolis users, in particular the companies that produce propolis formulations, need to know the characteristic concentrations of its constituents to guarantee a good product quality and a reasonable degree of bioactivity, and current protocols do not take into account the variable composition and pharmacological properties of propolis [41]. Additional tests are needed to fully investigate the biological effects of propolis, not only for those bioactive compounds already described but also for others, considering the chemical diversity of this natural product. Comparative studies on propolis collected from a wide range of countries are thus crucial for linking its provenance to certain bioactivities and hence ensuring that the beneficial properties of propolis are used on a rational base and more effectively by the public.

The analysis of all the biologically important individual components of propolis is often a tedious, time consuming and expensive procedure. It can be avoided in cases when the plant origin and the qualitative composition of the propolis are known. In such case, the rapid and low-cost determination of the quantitative chemical profile of a sample by measuring the concentration of groups of compounds similar in chemical nature (e.g. total flavonoids) is convenient and reasonable. Popova *et al.* [32] developed and validated rapid, low-cost spectrophotometric procedures, which demonstrated that measuring the concentrations of groups of active compounds instead of individual ones could be an adequate approach in the case of propolis. Later on, the same researchers argued that measurement of minimal inhibitory concentration (MIC) should be an obligatory element in propolis quality control, due to the complex synergistic effects of different propolis constituents.

Research on polyphenols (flavonoids and related phenolic acids) has been prompted by their noticeable beneficial effects on health. Flavonoids aroused great interest after they had been found to have effects in inhibiting the copper-catalyzed oxidation of low-density lipoprotein, inhibiting platelet clotting and arachidonate metabolism, reducing liver

injury from peroxidized oil, and having cancer-chemopreventive properties [42, 43]. Several methods have been developed to analyse polyphenols: thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), HPLC-mass spectrometry (HPLC-MS), and capillary electrophoresis (CE). Furthermore, liquid chromatography (LC)-MS technique is able to separate each single component in complex mixtures and to perform their identification and quantification [44]. Due to these several advantages, LC-MS has gained widespread interest and became a reference technique for the determination of pharmacologically interesting compounds in biological matrices. Accurate assessment of the contents of bioactive compounds in extract samples requires the validation of certain analytical parameters such as precision, recovery, linearity and detection limits. Therefore, on-line HPLC-electrospray ionization (ESI)/MS analysis, usually used for commercial pharmaceutical preparations, constitutes an alternative to obtain propolis fingerprints and a reliable identification of a large number of propolis polyphenolic components [44].

4. Biological activities

Independently from the plant source (plant species and geographic origin) and chemical composition, a biological activity of bee glue has always been reported [26, 45], in particular the antimicrobial activity. This is probably the reason why bee glue plays such an important role in the hive: it is a "chemical weapon" against pathogenic microorganisms, a constant threat to the vital sanitary status of this crowded and busy "city", vulnerable to the invasion of an array of enemies and to the proliferation of diseases. In spite of this universal function and due to plant diversity, there are different propolis types, which contain numerous chemical constituents responsible not only for the antimicrobial property but also for other valuable bioactivities [21]. These biological activities include: antibacterial [17, 26, 46, 47], antifungal [26, 48, 49], antiviral [50, 51], antiprotozoan [52-54], antitumour [39, 40, 55, 56], anti-inflammatory [57-59], local-anesthetic [60], antioxidant [61-63], immunostimulating [64-65], cytostatic [15] and hepatoprotective [66, 67].

The active factor(s) in propolis that are responsible for its many biological properties frequently remains to be fully defined and varies with propolis sample, dosage, and the extraction solvents used [68]. Flavonoid and esters of phenolic acids are generally regarded as bioactive compounds but, as a natural mixture of organic compounds, it may well be that the ratio of the combined reagents in propolis is important for its effect.

4.1 Antibacterial

One of the first biological activities to be recognized and probably one of the most important properties of propolis is the antimicrobial activity, especially against bacteria. Several studies have been performed to evaluate this property against a large panel of Gram-positive and Gram-negative bacteria (Table 4), either aerobic or anaerobic, from laboratory collections or isolated from clinical samples, using propolis of different origins and chemical composition and exploring different experimental approaches.

The disc diffusion method is one of the most popular methods used to evaluate this activity. A suspension of a sensitive indicator microorganism is inoculated on agar plates by spreading homogeneously on its surface, and blank paper discs containing the sample to be tested for antimicrobial activity are placed on top. After an adequate incubation period at optimal temperature, antibacterial activity is determined by measuring the diameter of the growth inhibition zones (inhibition halos) in the agar layer surrounding the disc [26]. Some authors argue that this laborious method is unreliable for comparing bioactivities, as results are influenced by the solubility and hence the diffusivity of the individual constituents in agar, proposing the use of another methodology also commonly used for the same purpose: the dilution method. In this procedure, propolis samples are serially two-fold diluted and a fix volume is added to liquid or solid medium, making a concentration series. A bacterial inoculum is subsequently added to each experimental condition and the occurrence of growth is analysed after incubation at optimal conditions. Broth microdilution is considered a good method for a rapid and simultaneous screening of multiple samples; it is suitable for comparing propolis extracts and gives more consistent results. Additionally, it allows the determination of the *minimal inhibitory concentration* (MIC) and the *minimal bactericidal concentration* (MBC) which are, respectively, the lowest concentration that inhibits visible bacterial growth and the lowest concentration that kills bacteria [46, 80]. Ideally, a broad range of operational concentrations should be prepared in order to obtain empirical data suitable for dose-response mathematical modelation. In this way more information could be drawn from the experiment, being possible to estimate other important parameters such as the *inhibitory concentration* IC_{50}, which corresponds to the concentration that induces a 50% reduction in growth. Less common in propolis research but also used to evaluate antimicrobial activity of several compounds is bioautography. Briefly, thin layer chromatography plates where propolis samples were eluted are covered with agar suspensions of the microorganism which sensitivity is going to be tested. Antibacterial activity is visualized as clear areas after proper incubation.

Table 4 Bacteria screened for sensitivity to propolis in antimicrobial tests.

	Gram-positive	References	Gram-negative	References
Aerobic	*Bacillus cereus*	[46, 69]	*Aeromonas hydrophila*	[16]
	Bacillus subtilis	[69]	*Brucella abortus*	[77]
	Enterococcus spp. (*Enterococcus faecalis)*	[46, 69]	*Corynebacterium sp. (C. pseudotuberculosis)*	[16]
	Micrococcus luteus	[70]	*Escherichia coli*	[16-18, 26, 28, 47, 69, 70, 77]
	Nocardia asteroids *Rhodococcus equi*	[71]	*Helicobacter pylori*	[66, 72]
	Staphylococcus aureus	[16-19, 26, 28, 31, 47, 69, 70, 73, 77]	*Klebsiella pneumoniae*	[28, 46]
	Staphylococcus spp. *(S. auricularis, S. capitis, S. epidermidis, S. haemolyticus, S. hominis, S. mutans, S. warnerii)*	[19, 28, 69, 70, 73]	*Salmonella sp (S. enteritidis, S. typhi, S. typhimurium)*	[16, 47, 71, 77-79]
	Streptococcus spp. *(S. cricetus, S. faecalis, S. pneumioniae S. pyogenes, S. β-haemolyticus, S. mutans, S. sobrinus, S. viridians)*	[16, 19, 30, 69, 71, 73, 74]	*Pseudomonas aeruginosa*	[17, 28, 46, 47, 69-71]
			Proteus mirabilis	[71]
			Proteus vulgaris	[28]
			Shigella dysenteriae	[77]
Anaerobic	*Actinomyces naeslundii* *Lactobacillus acidophilus* *Peptostreptococcus micros*	[75]	*Actinobacillus actinomycetemcomitans* *Capnocytophaga gingivalis* *Porphyromonas anaerobius* *Prevotella intermedia*	[76]
			Fusobacterium nucleatum *Porphyromonas gingivalis* *Prevotella melaninogenica*	[75, 76]
			Prevotella oralis *Veillonella parvula*	[75]

Data from numerous studies concerning antibacterial properties of propolis support the fact that propolis is active mainly against Gram-positive bacteria and either displays much lower activity against the Gram-negative ones or is inactive at all [11, 17, 26, 45-47, 49, 58, 77, 80]. Such results can be seen in the work of Kujumgiev *et al.* [26] who tested propolis samples from different geographic regions (tropical and temperate zones) against *Staphylococcus aureus* and *Escherichia coli*. All the extracts displayed significant antibacterial activity against *S. aureus* but none was active against *E.coli*. Although it can be argued that only two species were tested, it is also relevant that all the 12 samples tested, from so different origins, showed the same effect. Grange and Davey [46] reported that ethanol extracts from propolis (EEP) completely inhibited the growth of *S. aureus*, *Enterococcus* spp. and *Bacillus cereus*, partially inhibited *Pseudomonas aeruginosa* and *E. coli* growth and had no effect on *Klebsiella pneumoniae*. Sforcin *et al*. [47] investigated the antibacterial activity of Brazilian propolis collected during the four seasons and observed that low concentrations inhibited the growth of Gram-positive bacteria and that higher concentrations of EEP were needed to inhibit Gram-negative bacteria. Interestingly, no significant differences were registered on the survival curves of *S. aureus* and *E. coli* after incubation with propolis collected at different times. It could be expected that propolis produced in different seasons, and therefore from potentially different plant species or plant organs, exhibit significant chemical variation but, as climatic diversity is also an important factor, it may be the case that the sampling area might not reveal a significant climatic variation during the year and so it may have a more or less constant flora.

A variety of studies have been performed with bacteria of odontological relevance, which is one of the most prominent areas of propolis application. Dental plaque is related with colonization of oral microorganisms and the accumulation of extracellular polysaccharides that are synthesized from sucrose by glucosyltransferase of *Streptococcus* spp. [14]. Park *et al.* [30] tested Brazilian propolis samples collected in various regions and noticed that all the samples inhibited the enzyme activity. Also, several periodontal disease-causing anaerobic bacteria are susceptible to propolis aqueous-ethanolic extract, again the Gram-negative being the most resistant to propolis action [44, 75, 76]. Some authors suggested that the increased resistance of Gram-negative bacteria could be due to the presence of plasma membrane efflux pumps that would prevent intracellular entry of propolis constituents, or promote their extrusion from the cell, or even because propolis contains many plant-derived resin constituents which are secreted to protect plants from Gram-positive pathogens mostly [81]. Varroa mites are parasites that can destroy the hive and which harbours predominantly Gram-positive bacteria [82], a fact that could be another ecological rationale for this "antiseptic" activity against Gram-positive microbes.

Propolis antibacterial activity has been mainly correlated with flavonoids, with galangin, pinocembrin and pinostrobin being recognized as the most effectives [64]. However, there are reports of propolis samples containing only traces of flavonoids but displaying an antibacterial action [23]. Ferulic and caffeic acid, prenylated coumaric acid and benzophenone derivatives or diterpenic acids are also bioactive compounds [1, 2, 26, 28, 32, 83] but the exact

mechanism of antimicrobial action still remains to be elucidated and has been subject of only a few publications. Through electron microscopy and micro-calorimetric assay, it was shown that EEP interferes with the division of *Streptococcus agalactie* through the formation of pseudo-multicellular forms, cytoplasm disorganization, protein synthesis inhibition and cell lysis [84]. Many bacteria are effectively killed by flavonoids. However, the primary targets of flavonoids, the eicosanoids, do not appear to be formed by bacteria as the involved enzymes are only present in eukaryotic cells. Prokaryotic do however contain metalloenzymes, such as phosphatases, and the heavy metal atoms form a strong ligand complex with flavonoids. The bactericidal effect of flavonoids may therefore be originated from a metabolic perturbation in ion channels as a result of impairment in phosphorylation/dephosphorylation reactions [70, 83]. Caffeic acid, benzoic acid, and cinnamic acid probably act on the microbial membrane or cell wall sites as well, causing functional and structural damages [11, 83]. Some diterpenes and phenolic compounds possess activity against *Helicobacter pylori* [72] a Gram-negative bacteria that causes peptic ulcer disease and gastric cancer.

Until now, no single propolis component has shown to possess antibacterial activity higher than that of the total extract [85]. Some authors attribute the highly complex and variable composition of propolis as a reason for its antimicrobial activity and the data gathered so far suggests that it can be linked to multiple targets, with several constituents acting in synergy [2, 51, 73, 83, 84]. Propolis affects cytoplasmic membrane, inhibits bacterial motility and enzyme activity, exhibits bacteriostatic activity against different bacterial genera and can be bactericidal in high concentrations [83]. The antibacterial efficacy of EEP increases at higher temperature (37 ºC) and at acidic pH (pH 5.0) [86]. Propolis has a multiple action against many virulence factors of Gram-positive bacteria of clinical interest: staphylococcus's virulence factor coagulase is completely suppressed, lipase strongly reduced and a dose-dependent prevention of biofilm formation is evident in the presence of EEP [73]. Although *in vivo* observations are still missing, this reduction of microbial virulence factors is undoubtedly an interesting target in the treatment of Gram-positive infections. Synergism between EEP and antibiotics with a clear reduction of MIC values for several strains has also been reported [73, 78, 80).

4.2 Antifungal

The evaluation of antifungal activities of propolis extracts is normally performed using the methods already described for bacteria. The disk diffusion assay has been used to assess the sensitivity of several yeasts and fungi to ethanol extracts of different propolis samples. Although antibacterial activity is more relevant than the antifungal properties of propolis, many studies have reported the susceptibility of clinically important yeasts belonging to *Candida* genera [26, 49, 80] such as *Candida albicans* [16, 18], as well as the sensitivity of some filamentous fungi, mainly dermatophites [14]. Longhini and co-workers [87] showed that propolis has antifungal activity in dermatophytes even in small concentrations, present low toxicity and so that it can be used topically. Various yeast species isolated from onychomycosis, including *Saccharomyces cerevisiae* and *Trichosporon* sp. are susceptible to propolis [88]. European propolis samples have a fungicidal effect against *Candida, Microsporum, Mycobacteria, Trichophyton, Fusarium* and other dermatophytes [14]. An antifungal activity of propolis was also observed in some plant fungi *in vitro*. In the case of Brazilian EEP, which was tested against *Candida* sp., it was shown that *C. tropicalis* was the most susceptible followed by *C. albicans*, *C. guilliermondii* and *C. parapsilosis* [89].

The fungicidal effect was associated with the presence of flavonoids [70] and other phenolic components such as for antibacteria properties. Differences in antifungal activity of propolis extracts can again be attributed to the differences in chemical composition and concentration of propolis compounds [17, 89]. As for antibiotics, a synergistic effect with conventional antimycotic drugs was observed [80].

4.3 Antiprotozoan

Another important biological property already ascribed to propolis is the antiprotozoan activity. This property is evaluated by an *in vitro* growth inhibitory effect on a culture of parasites after incubation in the presence of different concentrations of propolis. Population density is estimated upon centrifugation and the growth inhibitory effect is determined comparing the values obtained for cultures treated and untreated with propolis.

Several publications reported the effect of European propolis on protozoa that cause diseases in humans and animals such as trichomoniasis, toxoplasmosis, giardiasis, Chagas disease, leishmaniasis and malaria. Indeed, antiprotozoan activity has also been reported on *Giardia lamblia*, *Trichomonas vaginalis*, *Toxoplasma gondii*, *Leishmania donovani* and *Trypanosoma cruzi* [90, 91]. Propolis preparations were classified as a good coccidiostat against *Chilomonas paramecium* [92]. An antiprotozoan activity of EEP was reported against *G. duodenalis* [52], as well as an inhibitory effect in trophozoite adherence and a reduction of flagella beating frequency in great part of trophozoites.

Antiprotozoan activity was verified in experimental *Trypanosoma cruzi*-infected mice treated with 50 mg/kg body weight/day of Bulgarian EEP, leading to a decrease in parasitemia [53], and in experimental animals infected with *Eimeria magna*, *E. media* and *E. perforans* [92]. As *T. cruzi* is the etiologic agent of Chagas disease, an endemic parasitosis that infects 16–18 million people in Latin America [93], these results are quite enthusiastic. Propolis also seems to have a prophylactic effect against malaria in endemic areas in Brazil [94].

4.4 Antiviral

The antiviral activity is normally assessed with the cytopathogenic effect (CPE) reduction assay. Confluent cell monolayers are prepared in plastic plates, infected with virus and incubated for the required period of time at an appropriate temperature, after which cells are microscopically observed for a cytopathogenic effect.

There are few data available concerning antiviral effects of propolis but the studies performed have shown that propolis from various geographic regions displays significant antiviral activity, acting at different levels and interfering with the replication of some viruses [14]. The *in vitro* effect of EEP was evaluated on several DNA and RNA viruses including herpes simplex type 1, herpes simplex type 2, adenovirus type 2, vesicular stomatitis virus and poliovirus type 2 [50, 51]. The results provided evidence that propolis is very active *in vitro* against poliovirus and herpes viruses, whereas vesicular stomatitis virus and adenovirus are less susceptible. The inhibition of poliovirus propagation was clearly observed. Besides this effect on virus multiplication, a virucidal action on the enveloped viruses herpes simplex and vesicular stomatitis virus was also detected. Virus inactivation was time and dose-dependent. It was reported that propolis affected the replication of influenza viruses A and B, vaccinia virus and Newcastle disease virus. Harish and co-workers [95] found that propolis suppressed HIV-1 (human immunodeficiency virus) replication. Antiviral activities of propolis samples from Egypt reduced the infectivity mean titers of the Bursal Disease virus and Reo-virus [96], and propolis samples from different geographic origins showed antiviral activity against Avian influenza virus [26].

Flavonoids and aromatic acids derivatives are responsible for the antiviral activity of propolis extracts [11]. Some flavonoids (baicalin) have inhibitory effect on HIV infection and replication as showed by *in vitro* studies. In the inhibition of Amazon parrot herpes virus, König and Dustmann [97] verified that luteolin was more active than quercetin, but remarkably less than caffeic acid. Some esters of substituted cinnamic acids found in propolis also proved to have antiviral properties: isopentyl ferulate significantly inhibited the infectious activity of influenza virus A and 3-methylbut-2-enyl caffeate showed strong inhibition on herpes simplex virus type 1 growth [50, 51]. Vaccinia and adenovirus were more sensitive than polio and parainfluenza virus to the antiviral effect of caffeic acid, which displays a weak activity against influenza virus.

This broad antimicrobial activity of propolis targeting a wide spectrum of phylogenetic different taxa and life forms, from virus thru prokaryotes to single cell eukaryotes and to more complex multicellular organisms, like the protozoa and filamentous fungi, is consistent with an important non-specific function of ecological relevance: the protection of hive community from potential microbial infections, that could rapidly propagate in such a densely colonized and confined space, with potential devastating results.

4.5 Antioxidant activity and associated biological properties

Free radicals are highly reactive species that can damage cellular components, such as proteins, nucleic acids and lipids, and are implicated in a variety of diseases. Their reactivity is usually neutralized in the body by antioxidant enzymes and nutrient-derived antioxidant molecules, which protect humans from deleterious oxidative processes. Propolis is notable for its antioxidant properties, only surpassed by those of green tea, and is more active than the rest of the beehive products in what concerns this property [44]. The antioxidants present in propolis [98-101] play a great role in its immunomodulatory properties [102]. It was reported that propolis increases the cellular immune response through the increase of mRNA for interferon-γ and activates the production of cytokines [99].

One of the most common used techniques to evaluate antioxidant potential is based on the depletion of free radicals by the addition of scavenger compounds. Measurements of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical consumption are related to the intrinsic ability of a substance or a complex mixture to donate hydrogen atoms or electrons to this reactive species in a homogeneous system.

The relatively strong antioxidant effects exhibited by EEP from different geographic origins (Argentina, Australia, China, Hungary and New Zealand) were correlated with high polyphenol and flavonoid contents, particularly kaempferol and phenethyl caffeate [103]. Similar results were obtained with Turkish propolis and with samples from various regions of Korea [63]. DPPH free radical-scavenging activity of Korean EEP was higher than that of butylated hydroxytoluene (BHT), used as control. In general, synthetic antioxidants showed better antioxidant properties than EEP, but at higher concentrations the reducing power of EEP and of artificial antioxidants were similar. Ferulic acid, quercetin, caffeic acid, prenylated compounds, apigenin and also galangin, *p*-coumaric and CAPE were identified as bioactive compounds responsible for antioxidant potential in different propolis samples [44, 98-101]. Oyaizu *et al.* [62] reported that α-tocopherol is contained in almost all propolis samples and correlates with its antioxidative effect.

4.6 Antitumor and cytotoxic activity

Antitumor activity, including cytotoxicity, was reported for EEP [39, 40, 55, 56]. Different methods allow determination of cytotoxic effects *in vitro* but, normally, cells are maintained in appropriate medium and then cultured in the presence of different concentrations of propolis extracts. Cellular viability is then assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide] or Trypan Blue Exclusion assays.

Some new compounds responsible for these properties such as diterpenic acids were isolated from propolis. Clerodane-type diterpenes PMS-1 exhibited cytotoxicity towards human lung carcinoma HLC-2 and human cervical

carcinoma HeLa cells [66]. Coniferyl aldehyde, betuletol, kaempferide and ermanin isolated from Brazilian propolis showed potent cytotoxicity towards human HT-1080 fibrosarcoma and murine colon 26-L5 carcinoma cells, having effective dose (ED_{50}) values of 10 μg/ml [15]. The new prenylflavanones propolin A and propolin B from Taiwanese propolis exhibit cytotoxic properties towards human melanoma, C6 glioma, and HL-60 cell lines, inducing apoptosis with DNA fragmentation [104]. Propolin H isolated from Taiwanese propolis samples inhibited the proliferation of human lung carcinoma cell lines through induction of G1 phase cell cycle arrest [105]. CAPE has been identified as one of the major active compounds in propolis with chemopreventive and antitumor properties [40] without being cytotoxic to normal cells [106]. A direct relationship between the cytotoxic effects of CAPE and the induction of DNA fragmentation and apoptosis was established by Su *et al*. [56]. In what concerns flavonoids, the assessment of its activity against HeLa cells revealed the following susceptibility order: quercetin had the strongest antitumor activity, followed by rhamentin and galangin [107].

Natural resistance to tumour development has been associated with the cytotoxic activity of natural killer (NK) cells [107], a lymphocyte subpopulation that shows lytic activity principally towards several types of tumour and virus-infected cells. Sforcin *et al*. [108] found an increase of NK activity in spleen cells of propolis-treated animals.

4.7 Other bioactivities

Many other biological and pharmacological properties of propolis have been described in several studies [11], including tissue regenerative properties, anti-inflammatory effects, immunogenic properties, liver detoxifying action, hepatoprotective activity, choleretic and antiulcer action *in vitro*. The hepatoprotective effect appears to be due to the presence of dicaffeoylquinic acid derivates and flavonoids [66]. Cardioprotective [110], neuroprotective [111] and radioprotective [112] effects were also reported. Propolis seems to lower cholesterol levels and blood pressure making possible its use in the prevention and treatment of atherosclerosis [28]. Furthermore, Maruyama and collaborators [113] suggest that EEP of Brazilian green propolis and its main constituents may be useful for prevention of hypertension. Propolis has anaesthetic activity with effects similar to those of cocaine [60]. It also kills the ectoparasitic mites *Varroa destructor* [82], which attack honeybees causing the varroatosis disease.

5. Propolis applications and future challenges

One of the most ancient applications of propolis is in odontology to reduce the incidence of dental caries [30, 74] and in dermatology due to its capacity of wound healing and promotion of tissue regeneration [43]. Propolis is also claimed to have beneficial effects on otorhinolaryngologie and in gynecological diseases [11] as well as in stomatology [55] and geriatry. The various uses of propolis in clinical trials show that its therapeutic efficacy lies mainly in diseases caused by microbial contaminations.

The broad spectrum of propolis biological properties, the long history of its use and safety profile, together with the results that are being observed in preclinical studies, provide a rationale for several applications in human and veterinary medicine as well as in pharmacology, which must be studied in clinical settings. Propolis synergism with antibiotics could allow the reduction in the dose of selected antimicrobials, potentiating the antimicrobial therapy. This is particularly important in the light of the widespread emergence of antibiotic resistant strains and of opportunistic pathogens, which demand for novel therapeutic agents and strategies, but the exact propolis compounds that are involved in the mechanisms of synergistic interaction with other drugs must be identified.

The increasing interest towards natural therapies, effective and healthy pharmacological compounds is obviously a stimulus for propolis research. However, new challenges are emerging in this field. As already referred, propolis chemical composition and pharmacological activity may vary widely from region to region [12], and potential medical applications of propolis have led to the need of standardization, and of origin and quality control. Moreover, bioactive(s) compound(s) remain to be identified or fully defined in many propolis types and studied samples. Subsequent studies will be needed in order to isolate and identify the main active compound(s) through bio-guided fractionation, to confirm if individual isolated compound(s) can reproduce propolis effects and, finally, to disclose the molecular targets and mechanisms underlying the biological activities.

Propolis research continues to surprise scientists and remains a fascinating subject for further studies and applications. The continuous discovery of new compounds demonstrates that the search for new promising bioactive compounds and biological activities is still a hot topic. But propolis research should not be restricted to this applied perspective of natural products; the comprehensive understanding of this bee-made product at the ecological level, its roles in hive integrity and sanitary conditions, and eventually in bees health and disease control, is no less stimulating.

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