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# PROPOLIS BIOAVAILABILITY AND NUTRACEUTICALS PROPERTIES: PRECLINICAL AND CLINICAL STUDIES

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# Index

## PREFACE

My PhD project was conducted within an industrial programme "Operative National Programme" (PON), developed in collaboration between University of Naples, Federico II (Naples, Italy), B Natural s.r.l (Milan, Italy) and University of Granada (Spain). Thus, the development of this project involved three different partners: University of Naples Federico II, The Company B Natural, and the University of Granada. In each experience, I learned a lot of different analytical and industrial techniques and different ways to study this multifaceted product called propolis.

In these three years, I studied the great and fascinating word of propolis, a bee product. In fact, at the beginning, I faced different difficulties linked to the suppling, the conservation, and the characterization of these multicomponent raw material but, step by step, and with the collaboration of all subjects enrolled in this project, we concluded the planned research project.

Nowadays the bee products are used in different fields of human life. For example, royal jelly is used in the malnutrition or as food supplement for the childhood, pollen is used for the improvement of the protein supplementation, and propolis is used for its biological properties such as antioxidant, anti-inflammatory, antimicrobial activities. The PhD research project was focused on propolis. In view of this, considering the properties of this natural product, the aim of the project was to evaluate the different propolis activities, not only *in vitro* but also performing a clinical trial, because, only with this research approach, we can consider the real efficacy and safety on human health.

**ABSTRACT**

Propolis is a complex mixture, produced by bees, mainly *Apis mellifera* L., to protect the hive against pathogens and intruders, containing resin (about 50%) and other materials (lipophilic material from leaves, mucilage, gum, waxes, about 30%). The remaining part consists of essential oils (about 10%), pollen (about 5%), and other organic substances (about 5%).

The organic compounds are represented by flavonoids, terpenoids, phenolic acids, phenolic esters, and sugars in different proportions. The different activities of the propolis like gastroprotective, hepatoprotective, immunomodulatory, wound healing, antidiabetic and antineoplastic are ascribed at these organic compounds and their antioxidant, anti-inflammatory and antimicrobial activities. Therefore, given the multifaceted aspects of propolis, the aims of this project were:

- to evaluate the *in vitro* antimicrobial activity of a characterized propolis extract obtained by a standardization method (M.E.D.®), developed by B Natural s.r.l.;
- to perform a clinical trial, evaluating the activity on the symptoms of upper respiratory tract infection (URTI) with a propolis oral spray;
- examine the role of propolis on the gut microbiota, with an *in vitro* approach.

The results demonstrated that the standardized propolis extract exerts *in vitro* antimicrobial activity with a high activity against pathogens strains and no activity against eubiotic strains. In addition, propolis oral spray application reduces the time for remission of symptoms in subjects with mild upper

respiratory tract infections and finally, the propolis extract improves the composition and activity of gut microbiota, as demonstrated by the increased production of short chain fatty acids (SCFAs) and the increased number of different eubiotic bacteria strains (through RT-PCR).

In conclusion, we can confirm the efficacy and safety of propolis in different fields on human health applications.

# INTRODUCTION

## PROPOLIS: PHYSICAL AND CHEMICAL PROPRIETIES

Propolis has been commonly used worldwide as a traditional and ethnopharmacological medicine since ancient times. It is a natural resinous substance that bees collect from tree exudates and secretions to build and protect their hive and is composed of resins (40-60%), waxes (20-40%), essential oils (10%), pollen (5%) and other organic compounds (5%) [1,2]. More than 300 compounds of different origins have been identified in propolis [3–6] as fatty acids, phenols, esters, substituted phenolic esters, flavonoids (flavones, flavanones, flavonols, diidroflavonols and calcones), terpens, betasteroids, aromatics aldehydes and alcohols, sesquiterpens, derivates of naphthalene and stilbene [3,7]. Propolis also contains vitamins, including B1, B2, B6, C and E, amino acids of bees' metabolism [3,8] mineral salts, such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe [9] and heavy metals as Cd, Hg, and Pb [10].

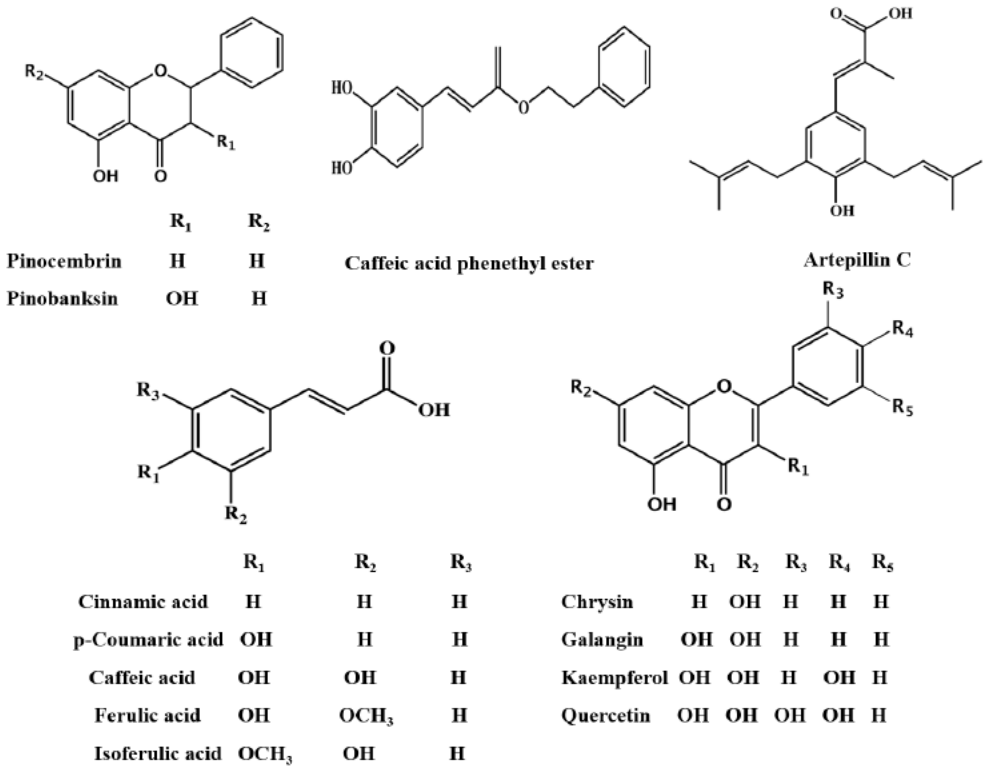
Its composition is very complex and variable, depending on many factors such as geographical origin, types of vegetable sources, bee species, time and season of collection, and postharvest factors as the extraction methods. Each region presents a characteristic flora, and each bee colony seems to have its own preferred source of resin. This explains the wide range of composition, color, and smell of propolis [11]. Since propolis derives from tree resins, it is sometimes classified according to the plant source and/or geographical origin.

These two factors could influence the chemical composition and the biological activities of propolis [12,13].

In Europe, bees collect resin mainly from poplar plants, producing the so called brown propolis. The most popular specie of honeybee, so called European honeybee, is the *A. mellifera.* The poplar tree is common in Europe and in other countries like China, Korea, Croatia, New Zealand, and Africa. Differently, in South America, poplars are rarely cultivated and alternative plants are used, such as *Baccharis dracunculifolia* that has been described as the most important vegetal source of South Brazilian propolis, which is called green propolis for its color [14,15]. Another kind of propolis, recently discovered, is the red propolis from the Northern part of South America. Brazilian green and red propolis are produced by the same bee specie, the Africanized *A. mellifera* [13,16], but they differ each other for the plant sources and, as consequence, for the main polyphenolic species.

Propolis is a lipophilic, hard, and fragile material that when heated, becomes soft, flexible, gummy, and very sticky [17]. The consistency is variable according to the temperature: it is tough and fragile at 15 °C, soft and malleable at about 30 °C, sticky viscous between 30 and 70 °C. The melting point ranges from 96 °C to 100 °C [11]. Propolis is poorly soluble in water and partially soluble in alcohol, acetone, ether, chloroform, and benzene. Only a suitable mixture of solvents with different polarity can dissolve most of its components. The insoluble part is made of vegetal tissues, pollen, debris, and cuticles of silk bees. The color range varies from brown to yellow to green-brown or red-brown to dark red, depending on the botanic and geographic origin [18].

The typical components of temperate propolis are flavonoids without substitutes in the B-ring, such as chrysin, galangin, pinocembrin, pinobaskin (figure 1). Caffeic Acid Phenethyl Ester (CAPE) is one of the constituents of European propolis, but is more present in green propolis, with a number of biological activities such as inhibition of Nuclear Factor kappa B (NF-kB), inhibition of cell proliferation and induction of apoptosis. In tropical regions, however, especially in Brazilian green propolis, prenylated phenylpropanoids, such as artepillin C, and diterpenes are the main components [19].




| | $R_1$ | $R_2$ |
|---|---|---|
| Pinocembrin | H | H |
| Pinobanksin | OH | H |

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Cinnamic acid | H | H | H |
| p-Coumaric acid | OH | H | H |
| Caffeic acid | OH | OH | H |
| Ferulic acid | OH | $OCH_3$ | H |
| Isoferulic acid | $OCH_3$ | OH | H |

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| Chrysin | H | OH | H | H | H |
| Galangin | OH | OH | H | H | H |
| Kaempferol | OH | OH | H | OH | H |
| Quercetin | OH | OH | OH | OH | H |

**Fig.1** The most representative organic compounds in propolis extracts [20]

Several biological properties have been attributed to propolis such as antioxidant, hepatoprotective, anti-tumor, anti-inflammatory and antimicrobial and antiparasitic activities [12,14,21,22]. Some examples include its use to increase the natural resistance to infections, to lower blood pressure and cholesterol levels. It has also been used in colitis and for oral health in toothpastes to prevent and treat caries, gingivitis, and stomatitis [23], in cough syrups, oral pills, pads, ointments, lotions and food supplements against viral diseases, fungal infections, ulcers and burns [24].

**BROWN PROPOLIS**

Brown propolis was used in this research project for the suppling facility and the economic cost in comparison to the other types of propolis.

As regards the chemical profile, brown propolis is a heterogeneous material containing more than 300 compounds [25]. It contains phenolic acids; esters of phenolic acids; flavonoids as flavones, flavanones, flavonols and dihydroflavonols; chalcones and dihydrochalcones; terpenoids; aldehydes, acyclic hydrocarbons and esters of higher alcohols, alcohols, fatty acids, aromatic hydrocarbons, ketones, sterols, sugars and sugar alcohols. In the propolis there are polar (aromatic acids, esters and flavonoids) compounds deriving from poplar exudates and non-polar (fatty acids, their esters and glycerol) from bee metabolism (amino acids, glycerol phosphates); propolis is also contaminated by honey (various sugars) and beeswax [26].

Flavonoids are typical constituents of brown propolis, in particular: pinobanksin, pinocembrin, galangin, chrysin, kaempferol and quercetin that do not present any substitution in B-ring. The aromatic acids in brown propolis are: derivatives of hydroxybenzoic acid (gallic, gentisic, protocatechuic, salicylic and vanillic acids) and derivatives of hydroxycinnamic acid (p-coumaric, caffeic and ferulic acids). They are found also as benzyl-, methylbutenyl-, phenylethyl- and cinnamyl- esters [20].

Phenolic glycosides (sugar conjugates) are poorly identifiable in propolis due to the lipophilic features of the resin and the hydrolysis process occurring during the propolis collection made by β-glucosidase: for these reasons, most of the flavonoids found in propolis are aglycones except for galactose, rhamnose and rutinose. Furthermore, the rate of deglycosilation is strictly linked on the position of the sugar substitution and the structure of the flavonoid [27]. Several authors indicated sugar conjugates as possible specific markers to identify the botanical origin since they are characteristic of brown propolis [28,29]. The sugars most frequently found are glucosides, glucuronides, rutinosides and galactosides linked to quercetin and kampferol derivatives; C-3 and C-7 are the most common sites of glycosylation [29].

The contribute of bees in the propolis chemical modification during harvesting is still unclear. Several authors suggested that no chemical reactions take place during resins collection by bee enzymes, therefore, the chemical profile of plant resins is like the one of propolis. On the contrary, other authors found an increase of some phenol aglycones in the chemical profile of harvested propolis

than the one of plant resins suggesting that bees actively participate in the production of propolis [30].

Waxes and hydrocarbons represent a part of non-polar fractions of propolis secreted by bees and include alkanes, alkenes, alkadienes, monoesters, diesters, aromatic esters, fatty acids, and steroids. In brown propolis, several volatile compounds are present. Essential oils (from 1 to 3%) are responsible for flavor and scent. Most of them originate from the poplar buds or from other exudates [31,32], while others are found only in brown propolis but not in the balsamic fraction of poplar buds [31]. The main components of the polar constituents are mono and sesquiterpenes (β-eudesmol, cadinol, cadinene and its isomeric forms) and non-terpenic aromatic compounds such as benzyl acetate, benzyl benzoate and benzyl alcohol [33,34]. The geographical areas characterized by coniferous plants such as Greece, Croatia and Estonia produce propolis rich in monoterpenes as α- and β-pinene, limonene and eucalyptol [34,35]. Furthermore, the composition of volatile fraction could also depend on beekeeping practices such as the use of thymol: the amount of this molecule is about 70-80% of all volatiles, while it has been found in trace in non-treated hives [33].

Propolis from Sicily and Northwestern Greece, as well as from Croatia and Malta contain mainly diterpenes and almost no phenolics [2,36]. Since the Mediterranean area is rich in *Coniferan spp* (Cupressaceae), it could be possible to identify the botanical origin of propolis analyzing the diterpenic profile [37].

Propolis produced in Iran contains mono- and sesquiterpene esters of benzoic acids with the predominance of flavonoids and caffeate ester [38]. This is principally due to the simultaneous use of *Populus* and *Ferula spp* as vegetal sources. South American propolis (i.e. Uruguayan propolis), shows a chemical profile similar to those of European and Chinese propolis deriving from the same plants [39]. Undoubtedly, poplar-type propolis is the most studied and the best-known type of propolis, both from chemical and biological points of view.

The chemical constituents responsible for its beneficial biological activities, and especially for its antibacterial, antiviral, anti-inflammatory and antioxidant properties, are well documented and ascribable to flavonoids and other phenolic acids [19,21,40]. Moreover, the concentration of polyphenols reflects the quality of the propolis [41].

## M.E.D.® PROPOLIS

Over the last decades, many investigations reported the different activities of propolis. Nevertheless, propolis samples used in these studies show different metabolic profiles with different concentrations of the active metabolites. For this reason, it was very difficult to ascribe the activity of propolis to a compound or a specific group of compounds (like polyphenols, terpenoids ecc) and to compare the results obtained from the different studies.

Thus, B Natural developed a specific extraction method in order to obtain a definite concentration range of the active compounds (polyphenols).

In brief, the extraction process of raw propolis consists of different phases, including several steps for the preparation of the final polyphenol-rich propolis extract. These steps consist of an initial aqueous extraction from dewaxed raw propolis, a series of extractions on the residue using ethanol/water mixtures, with a subsequently increasing concentration of ethanol.

In more details, raw propolis samples were processed as follows:

- **aqueous extraction** aimed to remove waxes and impurities from raw materials, using a 1:1 solvent/propolis ratio, at 80 °C for 10 h and with 100-Watt ultrasounds. After cooling at 8 °C, the solution was filtered with a 30 μm filter;
- **three hydro-alcoholic extractions:** one for each insoluble residue of the preceding extraction step, carried out using different alcoholic degrees and temperatures, from 4 to 36 h, with a fixed 1:1 solvent/propolis residue ratio. Each extraction step was followed by a sample cooling step at about 15 °C, a filtration step with a 30 to 50 μm filter and a concentration step using a rotating evaporator, to obtain a soft mixture.
- **concentration**: the combined extracts were mixed and concentrated to a residual humidity value ranging from 15 to 20% (v/w).

After the concentration, the extract can follow different process: it could be a hydrogliceric products (mixing glycerin and water), dry products (using a spray dry), syrupy products and others like oil, hydroalcolic or glycolic extracts.

All these extracts have a defined concentration of polyphenols ranging from 5 to 25% of phenolic acids, from 75 to 95% of flavonoids, of which flavones and flavanones range from 10% to 40%, flavononones and diidroflavononones from 10% to 40% and glicosilated flavonoids from 20% to 80%. (Patent registration N°0001425516)

In addition, this extract is rich in six active compounds such as: **galangin, crhysin, pinocembrin, apigenin, pinobanksin** and **quercetin** having a relative concentration in the extract of about **25-50% (w/w)**

Using M.E.D.® extraction method, the standardized extracts show a defined composition which results in constant biological activities.

## *CHEMICAL COMPOSITION OF M.E.D.® PROPOLIS*

To demonstrate the reproducible chemical composition of different M.E.D.® propolis extract different analysis were performed. After, M.E.D.® process, the obtained powder was solubilized in different solvents in hydroalcoholic (80% ethanol/20% water), glycolic (98% propylene glycol with the remaining 2% of ethanol), and glyceric (95% and 5% water) solutions, and oil (100% seed oil). It was prepared also a micronized sample called ESIT12.

Despite the production of different extracts, with different types of solvents, the chemical composition remains constant in all of products obtained.

The molecular composition of the different preparations was evaluated by high-performance liquid chromatography-UV-electrospray ionization mass (HPLC-UV-ESI-MS), total polyphenols concentration by Folin-Ciocalteau assay and antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay [42]

The high-performance liquid chromatography equipment was from Jasco (Jasco, Tokyo, Japan) (pump mod. PU-1580, UV detector UV-1570, Rheodyne injector equipped with a 20 μL loop, software Jasco-Borwin rel. 1.5). The phenolic acids and flavonoids from propolis were separated using a 250 × 4.6-mm stainless-steel column Discovery-C18 4 μm 80 Ä (from Sigma-Aldrich). The eluents were (A) 0.5% acetic acid and (B) acetonitrile. Separations were performed at room temperature by solvent gradient elution from 0 min at 50% A/50% B to 60 min at 100% B at a flow rate of 0.8 mL/min. A UV detector set at 260 nm was also used on-line with HPLC equipment. An Agilent 1100 VL series mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) was further used on-line with HPLC equipment. The electrospray interface was set in negative ionization mode with the capillary voltage at 3500 V and a temperature source of 350 ◦C in full scan spectra (200–2200 Da, 10 full scans/s). Nitrogen was used as a drying (9 L/min) and nebulizing gas (11 p.s.i.). Software versions were 4.0 LC/MSD trap control 4.2 and Data Analysis 2.2 (Agilent Technologies, Inc.). Additionally, 2–20 μL of samples were injected at a standardized concentration expressed as the total polyphenol content

evaluated by a spectrophotometric assay according to Folin-Ciocalteau. Phenolic compounds are oxidized by Folin-Ciocalteau reactive (Sigma-Aldrich) and the blue colour produced presents a maximum absorption at 750 nm evaluated by a spectrophotometer against a specific blank. Briefly, 20 µL of the propolis extracts was added to 380 µL of ethanol and 100 µL of the Folin-Ciocalteau reagent. Then, 200 µL of a saturated sodium carbonate solution and 4.3 mL water were added. The tubes were then allowed to stand at room temperature for 60 min before measuring the absorbance at 750 nm against the blank. The total polyphenolic content was expressed as galangin equivalents in % w/w or mg/mL.

The radical scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) molecules was evaluated. Samples were dissolved in a buffer solution at a final concentration of 1 µg/mL or final dilution of 1 µL/mL. For each extract, a series of test tubes were prepared containing between 1 and 1000 µL of these solutions, and the volume completed to 1000 µL with buffer. Finally, 1 mL of a 500 µM DPPH solution was added to each tube. After 30 min of incubation at room temperature in the dark, the absorbance was recorded at 517 nm by a spectrophotometer. Results are expressed in terms of the percentage of decrease with respect to control values (absorbance of 1 mL DPPH solution + 1 mL of buffer) incubated under the same conditions. Readings were made in triplicate. The mean of each result is plotted on a graph constructed by plotting increasing concentrations of the antioxidant trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) vs. Optical Density (OD) values. Propolis samples' antioxidant activity is expressed as µg trolox equivalent.

The molecular composition of the various propolis preparations determined by high-performance liquid chromatography-UV-electrospray ionization mass were indicated in table 1.

| No. | Polyphenols Species | ESIT12 | Oily Extract | Glycolic Extract | Glyceric Extract | Hydroalcoholic Extract |
|---|---|---|---|---|---|---|
| | | % *w/w* | % *w/v* | % *w/v* | % *w/v* | % *w/v* |
| 1 | Phenolic acids (caffeic, coumaric, ferulic, isoferulic) | 9.7 | 0.5 | 0.4 | 0.5 | 0.5 |
| 2 | Quercetin | 0.4 | 0.3 | 0.5 | 1.5 | 0.5 |
| 3 | Pinobanksin 5-methyl ester | 0.6 | 0.7 | 1.0 | 1.4 | 0.9 |
| 4 | Quercetin 3-methyl ester | 1.8 | 1.0 | 2.4 | 5.0 | 2.3 |
| 5 | Pinobanksin | 2.0 | 2.2 | 3.1 | 4.0 | 2.2 |
| 6 | Apigenin | 0.4 | 0.5 | 0.5 | 1.1 | 0.5 |
| 7 | Kaempferol | 1.1 | 1.1 | 1.6 | 3.8 | 1.7 |
| 8 | Isorhamnetin | 1.1 | 1.2 | 1.7 | 3.3 | 1.7 |
| 9 | Luteolin 5-methyl ester | 1.1 | 1.0 | 1.4 | 2.1 | 1.2 |
| 10 | Quercetin 5,7-dimethyl ester | 1.1 | 0.9 | 1.3 | 1.7 | 1.2 |
| 11 | Galangin 5-methyl ester | 1.0 | 0.8 | 1.1 | 1.1 | 0.8 |
| 12 | Quercetin 7-methyl ester | 2.0 | 1.8 | 2.1 | 3.2 | 2.2 |
| 13 | Chrysin | 5.3 | 4.5 | 5.4 | 5.0 | 5.3 |
| 14 | Pinocembrin | 2.0 | 3.1 | 3.1 | 2.9 | 3.1 |
| 15 | Galangin | 5.8 | 5.2 | 5.9 | 7.5 | 6.0 |
| 16 | Pinobanksin-3-*O*-acetate | 6.7 | 8.4 | 8.1 | 9.5 | 8.0 |
| 17 | CAPE | 0.4 | 0.4 | 0.6 | 0.3 | 0.4 |
| 18 | Metoxychrysin | 1.6 | 1.6 | 1.5 | 1.1 | 1.6 |
| 19 | Pinobanksin-3-*O*-propionate | 0.3 | 1.0 | 1.1 | 1.2 | 0.6 |
| 20 | Caffeic acid cinnamyl ester | 0.4 | 0.3 | 0.3 | 0.2 | 0.3 |
| 21 | Pinobanksin-3-*O*-butyrate | 5.4 | 6.5 | 6.7 | 5.7 | 6.7 |
| 22 | Pinobanksyn-3-*O*-pentenoate | 3.0 | 4.1 | 3.8 | 2.6 | 4.2 |
| 23 | Other Pinobanksin derivative | 1.1 | 1.7 | 1.6 | 0.7 | 1.7 |
| 24 | Pinobanksin-3-*O*-hexanoate | 0.3 | 0.7 | 0.4 | 0.3 | 0.5 |
| 25 | Other Pinobanksin derivative | 0.2 | 0.6 | 0.8 | 0.4 | 0.4 |
| 26 | Other Pinobanksin derivative | 4.5 | 6.0 | 5.0 | 3.3 | 5.8 |
| 27 | Other Pinobanksin derivative | 0.8 | 1.2 | 0.9 | 0.5 | 1.0 |
| 28 | Other Pinobanksin derivative | 0.4 | 0.6 | 0.5 | 0.2 | 0.7 |
| 29 | Other Pinobanksin derivative | 1.2 | 4.5 | 1.3 | 0.7 | 3.7 |
| | Total identified polyphenols | 61.7 | 62.4 | 64.1 | 70.8 | 65.7 |
| | Phenolic acids and derivatives | 10.5 | 1.2 | 1.3 | 1.0 | 1.2 |
| | Flavones and flavonols | 22.7 | 19.9 | 25.4 | 36.4 | 25.0 |
| | Flavanones and dihydroflavonols | 28.5 | 41.3 | 37.4 | 33.4 | 39.5 |

**Table 1.** The molecular composition of the various propolis preparations [42]

In the figure 2 were represented the chromatogram with the time elution of all these components.

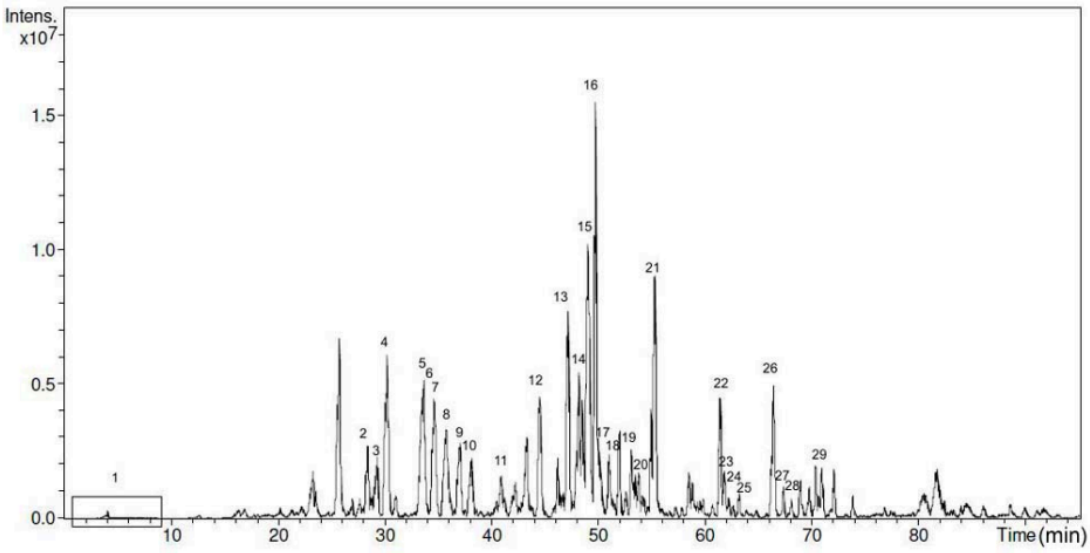



**Fig. 2** Time elution of M.E.D.® propolis compounds [42]

The phenolic contents, measured by Folin-Ciocalteau assay, and the anioxidant activity, using DPPH assay, were indicated in the table 2.

| Propolis Finished Products | Polyphenols Content | Microg Trolox/mg Polyphenols |
|---|---|---|
| ESIT12 (*w*/*w*) | 16.5 ± 0.8% | 74.0 ± 4.2% |
| Oily extract (*w*/*v*) | 24.0 ± 1.4% | 79.4 ± 4.2% |
| Glycolic extract (*w*/*v*) | 81.2 ± 3.7% | 71.7 ± 3.5% |
| Glyceric extract (*w*/*v*) | 26.2 ± 1.6% | 74.4 ± 3.8% |
| Hydroalcoholic extract (*w*/*v*) | 69.7 ± 2.0% | 76.0 ± 4.1% |

**Table 2.** Phenolic contents and antioxidant activity of M.E.D.® propolis extracts [42]

The total polyphenols content was different for the various preparations. This is probably due to the capacity of different solvents to solubilize more species than others when using the same starting raw propolis for all preparations and

a quite similar extractive process. In fact, the well-known high capacity of hydroalcoholic solution, generally 80% ethanol and 20% water, to solubilize a great percentage of polyphenols was also confirmed in this analysis.

## BIOLOGICAL PROPERTIES

Propolis is one of the most widely studied natural substance. Numerous evidences suggest that this product of the hive exerts innumerable pharmacological activities and health properties among which are included the antibacterial, antiviral, antifungal, antiulcer, antioxidant, antiradical, hepatoprotective properties, antitumor, antimutagenic, anti-angiogenic, cito- and chemopreventive, anti-inflammatory and immunomodulatory. In addition, propolis is able to induce muscle contraction at low concentrations and muscle relaxation at high concentrations, has antidiabetic properties, cardioprotective (acting against myocardial damage, performing antithrombotic, antihypertensive and antiarrhythmic functions), local anesthetics and regenerative (cartilage and bone tissue, dental pulp). It also acts as a food preservative [3,5,7,9,12,18,21,33].

Although the different types of propolis, produced from different resin sources and have different chemical composition, typically shows similar biological activities. Many studies, which report the chemical characterization of the studied propolis samples, have tried to determine a relationship between the concentration of each compound in a sample of propolis and its biological activity. This relationship was not easy to establish because positive synergistic

effects may occur among the many components of propolis [43,44]. For this reason, other studies use a different approach, correlating the amount of specific chemical groups with their biological assets [18].

Several mechanisms of action have been proposed for propolis: propolis inhibits bacterial mobility [45]; pinocembrine acts as a quorum sensing inhibitor [46]; galangine blocks the adhesion of Staphylococcus aureus [47]; propolis, both *in vitro* and *in vivo*, inhibits the synthesis of peptidoglycan, more precisely the activity of glucosiltransferase in Streptococcus sorbinus and Streptococcus mutans [9,48–50]. Propolis also reduces the symptoms of peptidoglycan-induced bacterial colitis by primarily inhibiting the production of pro-inflammatory cytokines in macrophages [21,51]. Many researchers have demonstrated the antifungal activity of propolis against *Candida albicans* [52] and other yeasts such as *C. tropicalis* and *C. krusei,* which were equally sensitive to the action of propolis [53]. Combinations of certain antifungal drugs with propolis (10%) increased their activity on *C. albicans* [14,54].

Through *in vitro* and *in vivo* experiments, antifungal activity has also been demonstrated against certain plant fungi [54].

### *ANTI-INFLAMMATORY AND IMMUNOMODULATING ACTIVITIES*

Propolis shows anti-inflammatory and immunomodulatory activities. As an anti-inflammatory agent inhibits the synthesis of prostaglandins, supports the

immune system by promoting phagocytic activity, stimulates cell immunity and induces the healing of wounds of epithelial tissue [55].

The administration of green propolis and artepilline C, its characterizing component, in a mouse model system has been related to the reduction of inflammation mediators, such as neutrophils, prostaglandin E2, nitric oxide (NO), the inhibition of the enzyme nitric oxide inducible synthase (iNos) and NF-kB [56]. Treatment of mice with propolis (200 mg /kg) for 14 days led to inhibition of many interleukins (IL) such as IL-1, IL-6, IL-2, interferon (IFN) and IL-10 in the spleen cells demonstrating anti-inflammatory activity [57]. In addition, it has been suggested that extracts containing 10% crude propolis stimulate antibody production [58]. Propolis can modulate the immune system by inhibiting murine peritoneal macrophages *in vitro* and *in vivo* and stimulating the lithic effect on natural killer cells (NK) against cancer cells and inhibits lymphoproliferation induced by inflammation [59].

Propolis acts directly on immune cells by controlling the activity of mitogen activated protein kinase (MAPK) and serine /threonine kinase 2 (ERK2). In this way, it exerts an important anti-inflammatory effect through the regulation of T cells [60]. Administration of ethanolic propolis extract (at a dose of 200 mg/kg) in mice improves innate immunity through activation of the early stages of immune response via over-regulation of TLR-2 and TLR-4 receptors and pro-inflammatory cytokines (IL-1 and IL-6), the production of macrophages and spleen cells that contribute to the recognition of foreign microorganisms by activating lymphocytes from antigen-presenting cells (APC). The anti-inflammatory property of propolis has also been studied in a clinical study in

relation to chronic stomatitis. This condition is characterized by erythema, edema, and mucous ulcers. All patients treated with propolis gel had complete clinical remission of oral stomatitis [61]. Propolis has also been evaluated as an oral ulcer treatment in other studies and the results showed a statistically significant increase in oral ulcer reduction [61,62].

### *ANTIOXIDANT ACTIVITY*

The antioxidant activity of propolis is mainly due to polyphenols, secondary metabolites of plants involved in many biological functions, such as the protection of plants from ultraviolet (UV) radiation, pathogenic microorganisms, and herbivores [63]; in humans, polyphenols have multiple effects mainly related to their antioxidant power. This effect of polyphenols depends on the number of phenolic rings and others factors, such as the number and location of hydroxyl groups, double bonds in the molecule, the presence of a catechetical group, unsaturation and functional groups capable of chelating metals [64]. In addition to the classic antioxidant action, it is possible to highlight pro-oxidating actions *in vitro* [65]. In fact, on the one hand polyphenols act as antioxidants improving cell survival, on the other hand, they can act as pro-oxidant molecules, inducing apoptosis, necrosis, or inhibition cell proliferation [66]. Phenolic compounds help to maintain a balance between oxidizing substances and antioxidants. Flavonoids and phenolic acids are the main classes of phenolic compounds whose structure-activity relationship (SAR) allows antioxidant function in hydrophilic or lipophilic systems

[3,21]. At mitochondria level, during the process of oxidative phosphorylation, electrons are transferred by cofactors NADH (Nicotinamide Adenine Dinucleotide) and $FADH_2$ (Flavin Adenine Dinucleotide) to oxygen, resulting in the formation of a water molecule.

Electron transport generates a proton gradient to allow the production of adenosine triphosphate (ATP) molecules. However, some electrons react directly with oxygen or nitrogen, resulting in the production of reactive oxygen (ROS) and nitrogen (RNS) species. ROS molecules can also be synthesized with other mechanisms:

1. the activity of certain enzymes such as xanthine oxidase, lipoxygenase and cyclooxygenase [67];
2. during the biotransformation of foreign compounds, toxins or drugs, through the activity of cytochrome P-450 monooxygenase;
3. exposure to environmental factors such as high concentrations of iron salts or UV rays, leading to lipid peroxidation [68];
4. elimination of foreign micro-organisms by the action of macrophages, granulocytes, and neutrophils.

The toxic effect of ROS is related to the lost redox balance: reduced cellular antioxidant capacity and concomitant ability of these molecules to oxidize biological macromolecules, such as lipids, proteins and DNA, resulting in alteration of cell membranes, inactivation of enzymes and receptors, modification of cytoskeleton proteins and damage to the genome. However, the production of ROS is also a physiological event and not only has a negative

impact on the organism. In fact, ROS is also produced during the elimination of pathogens after infection [69]. To counteract the oxidative damage of ROS, sophisticated cellular antioxidant mechanisms exist in humans that include both endogenous and exogenous molecules.

The endogenous antioxidant defenses: iron and copper, proteins such as transferrin, ferritin and lactoferrin; catalase enzymes (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX). While the exogenous antioxidant defenses: fat-soluble vitamins E and A, and polyphenolic compounds.

*In vitro* antioxidant activity of propolis extracts is commonly studied using bleaching with β-carotene, 1,1-diphenyl-2-picrylhydrazyl (DPPH), elimination of free radicals, oxygen radical uptake capacity (ORAC) and 2,2-azine-bis - (3-ethylbenzthiazoline) -6-sulfonic acid (ABTS) and radical cation discoloration tests [70,71]. Propolis extract inhibits lipoxygenases and protects the gastric mucosa from oxidative stress. Brazilian propolis with a dosage of 50 and 250 mg/kg has anti-ulcer action due to the activity of cumaric and cinnamic acids [72]. In addition, propolis has a spasmolytic action in the gastrointestinal tract and protects the stomach from injuries induced by ethanol [73]. Although green propolis is low in flavonoids, in fact, studies have shown scavenger activity of 40% or 57% at a concentration of 500 μg/ml [74]. Antioxidant activity is a very important topic because many syndromes are linked to an imbalance between the antioxidant defense system and the production of free radicals (namely amyotrophic lateral sclerosis, accelerated aging, Alzheimer's disease, cataracts, cardiovascular diseases, and rheumatism) [75].

Oxidative stress and inflammation are closely related phenomena. Indeed, oxidative stress causes inflammation, which in turn induces oxidative stress and causes the appearance of a chronic inflammatory state. Recently, it has been shown that chronic inflammation is a predisposing factor to the onset of certain diseases such as atherosclerosis, neurodegenerative diseases and cancer [76]. The antioxidant activity of flavonoids could be the basis of anti-inflammatory activity because of their structure, their ability to penetrate the lipid cell membrane [77] and to modulate the expression of closely related anti-inflammatory genes [78].

### *ACTIVITY ON THE GLYCEMIC BALANCE*

Some studies show that propolis exerts hypoglycemic effects in patients with type II diabetes and contributes to reducing the risk of metabolic syndrome in healthy individuals. Propolis stimulates the activity of damaged pancreatic cells, accelerates tissue regeneration and repair and promotes bone remineralization [79,80].

### *ACTIVITY ON THE CARDIOVASCULAR SYSTEM*

Propolis exerts a cardiovascular protective activity. In fact, it protects the wall of blood vessels whose degeneration can cause arteriosclerosis [81]. A constant administration of polyphenols in the diet reduces the risk of cardiovascular

disorders [82,83]. Propolis can modulate lipid and lipoprotein metabolisms by acting on liver synthesis of triglycerides in rats [81,84].

Propolis also causes the reduction of total cholesterol and the increase of high-density lipoprotein (HDL) in mice. This mechanism involves ABC proteins that are transmembrane transporters involved in the transport of numerous substances through endo and extra cellular membranes (ATP-ABCA), up-regulation of ABCA1 gene expression and associated with an increase in HDL levels [85].

### *ACTIVITY ON RESPIRATORY TRACT*

Bee products have been used empirically for centuries mainly for the treatment of respiratory diseases. Propolis, in fact, can significantly reduce the number and severity of night asthma attacks, improve lung function, and reduce inflammation [86]. Subjects with pharyngitis, treated with an extract containing 75 mg of crude propolis, showed a significant positive trend in symptom relief with a reduction of sore throat, fever, adenomegalies, pharyngeal erythema and exudated [87]. Propolis may be effective in relieving symptoms of allergic rhinitis by inhibiting the release of histamine [88].

Propolis is also able to reduce allergic lung inflammation in the mouse model through the involvement of inflammatory lung cells and the decrease of inflammatory polymorphonucleate cells [89].

## EPIGENETIC ACTIVITY

One of the most studied properties of propolis is its antioxidant capacity. The mains compounds responsible for this activity are phenolic acid and derivates, which show higher radical scavenging activity. In addition, caffeic acid phenethyl ester (CAPE) exerts protective effects on the lipid peroxidation of erythrocyte membranes.

The strong antioxidant activity of propolis suggests that it could be used as an ingredient in the preparation of functional foods and food supplements and may be useful in the prevention and dietary management of patients with chronic diseases caused by oxidative stress.

It has also a good anti-inflammatory propriety. As an anti-inflammatory agent, propolis has been shown to inhibit the synthesis of prostaglandins, activate the thymus, help the immune system by promoting phagocytic activity, stimulate cellular immunity and improve curative effects in epithelial tissues. Based on literature data, CAPE blocks the release of interleukin 1β (IL-1β) through the inhibition of Nuclear Factor kB (NF-kB) activity. Propolis flavonoids and CAPE have been compared to the cyclooxygenase (COX) inhibitor, indomethacin (IM), and the lipoxygenase (LOX) inhibitor, nordihydroguaiaretic acid (NDGA), and were found to have the same effects as IM and NDGA. In addition, a study showed that CAPE inhibits the release of inflammatory cytokines and simultaneously increases the production of anti-

inflammatory cytokines, such as IL-10 and IL-4. The same research showed that CAPE decreases the infiltration of inflammatory cells, such as neutrophils and monocytes [90].

Regarding epigenetic mechanisms, microRNAs (miRNAs) play an important role in the regulation of gene expression. They are a class of endogenous non-coding RNA, consisting of about 22 nucleotides, which can regulate gene expression at the post-transcriptional level. They exert their functions by binding complementary sequences on messenger RNA (mRNA) targets, interfering with the translation process and preventing or altering gene expression.

There are some studies on the epigenetic effects of propolis. In fact, the ability of propolis to modulate the expression of microRNA and mRNA involved in anti-inflammatory and oxidative process could be a good step forward to elucidate the propolis mechanism of action, that is yet unknown.

So, the expression levels of miRNAs, mRNAs and proteins associated with oxidative stress and inflammatory responses in treated human keratinocyte HaCat cell lines was investigated, with chemically characterized green and brown hydroglyceric propolis extracts. This cellular line was selected because it represents the most abundant cell type in the epidermis and is highly present in the oral cavity (the main route for administration of propolis products).

For the determination of propolis non-cytotoxic concentrations, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed with increasing concentrations of propolis extracts, ranging from

0.19 to 25 mg/mL, for 24 h. The highest non–cytotoxic concentration that did not cause a decrease in cell viability greater than 30%, was 3.125 mg/mL. Thus, HaCat cells were treated with 0.78, 1.56 and 3.125 mg/mL of propolis extracts for 24 h.

RNA was extracted from treated and untreated (control sample) cell cultures for subsequent RT-PCR assays. The results indicated that miR-19a-3p and miR-203a-3p, which target mRNA coding for TNF-α, were significantly upregulated by propolis. A significant increase in the expression levels of miR-19a-3p was registered following treatment with all tested concentrations of both green and brown propolis when compared to the control sample. The levels of miR-203a-3p only increased in cell cultures treated with brown propolis, at all tested concentrations, when compared to the control sample. Green propolis did not induce any significant changes in the expression level of miR-203a-3p.

As far as miR-27a-3p is concerned, it regulates NFE2L2 expression. A significant increase was registered at the two lowest concentrations for both green and brown propolis treatments compared to the control sample. The expression levels of another miRNA, miR-17-3p, which targets mRNA coding for three mitochondrial antioxidant enzymes (GPX2, MnSOD and TRXR2) were significantly decreased only by brown propolis treatments at the two lowest concentrations tested compared to the control sample.

The determination of the expression levels of mRNAs and proteins, which are validated targets for the studied miRNAs, was performed. For miR-19a-3p and

miR-203a-3p, it was investigated changes in the expression levels of mRNA coding for TNF-α. As expected, brown propolis was found to induce a decrease in the expression levels of mRNA in all cultures treated, when compared to the control sample. Conversely, green propolis did not induce any significant changes in mRNAs coding for TNF-α. These results suggest that to decrease the expression levels of mRNAs coding for TNF-α, an increase in both the miRNAs, 19a-3p and 203a-3p, is needed. TNF-α protein concentrations confirmed the expression levels of mRNAs. Significant decrease in expression levels were measured at all tested concentrations for brown propolis compared to the control sample. For the green propolis treatments, TNF-α concentrations did not change significantly, which also correlates with the mRNA expression levels registered.

For miR-27a-3p, it was investigated changes in the expression levels of mRNA coding for NFE2L2. As expected, it was found that mRNA expression levels dropped for the two lowest concentrations in cells treated with brown propolis, in response to the overexpression of miR-27a-3p at these concentrations. Green propolis did not induce any significant changes in mRNAs coding for NFE2L2. As far as NFE2L2 is concerned, brown propolis treatment induced a decrease in the concentration of the protein in HaCat cells at all concentrations tested. In agreement with the mRNA expression levels, the green propolis treatment did not generate any significant changes in the concentration level of the protein, compared to the control sample.

For the mRNA targets of miR-17-3p, involved in the regulation of mitochondrial antioxidant enzymes, namely MnSOD, GPX2 and TRXR2, the

mRNAs coding for GPX2 were the only ones showing significant increases, and then only in cells treated with brown propolis, and at all concentrations tested.

So, after this research, the antioxidant and anti-inflammatory effects attributed to green and brown propolis could be ascribed to modulation of the levels of certain miRNAs. An interesting aspect lies in the different capacities, shown by the two types of propolis tested, to induce changes in the expression levels of miRNAs. Brown propolis, which is richer in flavonoids than in hydrocinnamic acid derivatives, was active on all miRNAs tested, while the treatment with green propolis caused changes in the expression levels of only two of the miRNAs, miR-19a-3p and miR-27a-3p. These results could suggest that brown propolis has greater epigenetic activity, probably due to the higher contents of flavanone and flavone. The same considerations can be made with regards to their ability to induce changes in the expression levels of mRNAs. In this case, brown propolis has also been shown to possess a superior modulatory capacity; it is able to modify the expression levels of mRNAs coding for TNF-α, NFE2L2, GPX2, TNF-α and NFE2L2 protein levels [91].

## ORAL BIOAVALABILITY

In last year's different studies were focused on the bioavailability of propolis. The bioavailability representing the amount of propolis and/or its components able to be absorbed into a living system and to reach the sites in which they may exert their biological effects through systemic circulation. This parameter

can be affected by many factors (i.e., the food matrix, possible interactions with other compounds, environment, chemical structure, and concentration) and it is therefore difficult to evaluate. Until now, propolis shows low bioavailability, which in turn, reduces propolis therapeutic effects. The difficulty in determining the bioavailability of propolis has prompted researchers to focus their studies on single components of propolis, instead of the combined substance. Considering the importance of bioavailability to evaluate the *in vivo* antioxidant capacity of propolis it was performed an *in vivo* study where European propolis (brown propolis) was administered under acute and prolonged treatment. In addition, the *in vivo* antioxidant activity was evaluated monitoring the concentration of three antioxidant enzymes (SOD-1; catalase, CAT; glutathione synthase, GSS).

The propolis dosages to treat the experimental animals were calculated considering the polyphenol maximum dosage admitted for humans and the estimated average intake of polyphenols occurring in propolis (ca. 3 mg/kg of polyphenols). The extrapolation of animal dose to human dose was performed through normalization to body surface area (BSA) using the following formula: animal dose = HED x Human Km /Animal Km (where human Km factor is 37 for a human, and animal Km factor is 3 for a mouse).

To evaluate *in vivo* the bioavailability of brown propolis M.E.D.® extract and its antioxidant effects, according to the major compliance with mice, a dry extract was chosen. This extract was analyzed using HPLC-UV-DAD-MS to evaluate the chemical profile and the number of total polyphenols.

In this sample the number of total polyphenols was 7.21 mg/g (HPLC-UV-DAD-MS vs Gal) as reported in table 3.

| | *M/Z* | RT MS | % (w/w) |
|---|---|---|---|
| **Quercetin** | 301 | 12 | 0,36 |
| **Apigenin** | 269 | 17.1 | 0,83 |
| **Pinobanksin** | 271 | 20.4 | 1,01 |
| **Chrysin** | 253 | 40.2 | 13,81 |
| **Pinocembrin** | 255 | 47 | 1,97 |
| **Galangin** | 269 | 47.9 | 16,26 |
| **Total polyphenols** | | | 7,21 |

**Tab 3**. Total polyphenols content [92]

To study the bioavailability of brown propolis. Before the identification of galangin in blood salmples, a calibration curve was prepared adding galangin to plasma samples of untreated animals at different known concentrations.

HPLC-UV-DAD-MS analysis showed that galangin was not found in plasma of treated mice but was identified its metabolite Galangin-glucuronide.

To quantify the galangin glucuronide the calibration curve of galangin previously reported was used. Neither galangin nor its metabolite (galangin glucuronide) were found in the plasma samples after sampling at 30 sec and 2 min from the acute propolis administration. A peak of galangin glucuronide corresponding to the concentration of 4.29 µg/ml was found in plasma at 5 minutes after administration, followed by a plateau between 10 and 25 minutes. Then, the concentration decreases gradually until 45 min after which galangin glucuronide was not detected anymore

The chronic treatment showed that propolis metabolites did not accumulate in the blood since it was not possible to detect any component of the propolis or its metabolites within the plasma. To verify if galangin or its metabolites were accumulated in the liver, HPLC-PAD-ESI-MS was performed under the same conditions used for plasma samples and none polyphenols molecules were detected.

At the end of this study, the authors confirm that in poplar brown propolis, the main pharmacologically active constituent is galangin. The results demonstrated that galangin was absent in all plasma samples of mice after both types of treatments at any time of sampling. Since several studies, in both humans and animals, demonstrated that circulating flavonoids are mostly present as glucuronides [93], the quantification of galangin glucuronide was carried out. This molecule was found in plasma samples after 5 minutes of acute administration of propolis extract (HPLC-PAD-ESI-MSn) until 45 minutes. Galangin and its metabolite (galangin glucuronide) were found after chronic treatment neither in plasma samples nor in liver tissues. These results suggested that these molecules did not accumulate in these tissues. To evaluate the physiological effects of chronic administration of propolis, antioxidant enzymes were quantified. The results showed that mice treated with 250 mg/kg had a statistically significant increase in the concentration of SOD compared to control group. This result could indicate that chronic administration of brown propolis increases the endogenous antioxidant defenses. For CAT and GSS enzymes there were no statistically significant differences comparing treated mice with control group.

These experiments suggest that propolis was absorbed and immediately metabolized and chronic administration of propolis was able to increase the endogenous antioxidant defenses [92].

## AIM OF THE PhD RESEARCH PROJECT

Based on literature data, propolis has anti-inflammatory, antioxidant, and antimicrobial activity. Thanks to these biological proprieties, it could be utilized for treating different pathologies such as oral cavity infections, the alteration of intestinal permeability, the functionality of gut microbiota and many others.

So, the aims of this project were:

- to evaluate the *in vitro* antimicrobial activity (**antibacterial, antimycotic and antiviral**) of a characterized propolis extract obtained by the standardization method M.E.D.®;
- to perform a **clinical trial**, evaluating the activity on the symptoms of upper respiratory tract infection (URTI) with a propolis oral spray;
- examine the role of propolis on the **gut microbiota**, first with an *in vitro* approach, evaluating the production of short chain fatty acids (SCFA) and activity on microbiota (RT-PCR), and after with a clinical trial.

We started with the evaluation of antimicrobial activity *in vitro*, highlighting the activity on different bacteria pathogenic strains and mycosis. After we obtained a good result on the activity on two different kind of Herpes viruses HSV-1 and HSV-2.

On the basis of these first result we started a clinical trial to evaluate the efficacy of a propolis spray on the symptoms of Upper Respiratory tract

infection (URTIs). The results indicated that the use of propolis decrease the time of symptoms disappearing respect to the placebo.

In the last year of my doctorate, in Granada (Spain), we started the evaluation of propolis on gut microbiota. First of all, we performed the digestion-fermentation assay to reproduce the real process of absorption of the product. After we evaluated the SCFA production by gut microbiota, and the results highlighted the production of acetate, butyrate, and propionate. At the end the activity of propolis on gut microbiota, evaluated by RT-PCR, indicated the increase of different eubiotic strain like *Lactobacillus* and *Bifidobacterium,* and the decrease of some pathologic strains respect to the placebo product. After these results we started to perform a trial that which is still going on.

## ANTIBACTERIAL AND ANTIMYCOTIC ACTIVITY

### *Introduction*

The aim of our study [94] was to demonstrate the chemical and biological reproducibility of poplar-type propolis extracts obtained using a M.E.D.® method and mixing a combination of poplar-type propolis of different geographical origins in order to highlighted the antibacterial and antimycotic activity. We used high-performance liquid chromatography coupled with UV detection and mass spectrometry (HPLC–UV–MSn) to compare the chemical composition of nine hydroalcoholic propolis extracts and three non-ethanolic M.E.D.® propolis. Moreover, it was evaluated the antimicrobial activity and used as a validation method of the extractive process M.E.D.® to show the biological reproducibility of M.E.D.® propolis.

The work described in this chapter was also previously published in the article entitled "Multi Dynamic Extraction: An Innovative Method to Obtain a Standardized Chemically and Biologically Reproducible Polyphenol Extract from Poplar-Type Propolis to Be Used for Its Anti-Infective Properties" *Materials (Basel).* **2019**, *12*, 3746 by Zaccaria, V.; Garzarella, E.U.; Di Giovanni, C.; Galeotti, F.; Gisone, L.; Campoccia, D.; Volpi, N.; Arciola, C.R.; Daglia, M.. *Materials (Basel).* **2019**, *12*, 3746.

### *Materials and methods*

Three poplar-type raw propolis samples were obtained from European regions (Italy (E1), Spain (E2), and Turkey (E3)), three different Southern American

regions (Uruguay (sA1), Mexico (sA2), and Argentina (sA3)), and the last three poplar-type raw propolis samples were collected from distinct Asian regions (Mongolia (A1), Kazakhstan (A2), and North China (A3)). Samples (20 mg each) and were dissolved in 2 mL of 70% ethanol. It was mixing and through sonication process for 30 min, polyphenols were extracted at 70 °C in a water bath for 2 hours. After centrifugation at 10,000 RPM for 10 min, hydroalcoholic propolis extracts were analyzed by RP-HPLC–PDA–ESI–MSn.

The determination of the chemical composition and the antibacterial activity of M.E.D.® propolis, was investigated using three mixtures (mix A, mix B, and mix C) combining a European, an American, and an Asian poplar-type raw propolis sample (Eu +Am+ As) and obtaining three M.E.D.® propolis A, B, and C extracts, following Multi Dynamic Extraction as described before.

In brief, raw propolis mixtures were submitted to the M.E.D.® extraction process, comprising several steps. We started with an initial aqueous extraction from dewaxed raw propolis, followed by a series of extractions on the residue using an ethanol/water mixture, and after each extraction being carried out on the residue from the previous extraction using a higher percentage of ethanol. The combined extracts were mixed and concentrated by distillation to a residual humidity value ranging from 15 to 20% (w/w). The concentrated extracts were then analyzed by RP-HPLC–PDA–ESI–MSn and submitted to an antimicrobial assay.

The evaluation of the inhibitory effects of M.E.D.® propolis extract against different microorganisms were performed using the broth dilution method according to the procedures of the Clinical and Laboratory Standards Institute (CLSI), to determine the minimum inhibitory concentration (MIC), defined as the lowest concentration of an antimicrobial agent that can inhibit the growth of microorganisms. Each dry M.E.D.® propolis extract (A, B, or C) was resuspended in 50% (v/v) ethanol/water to obtain a final concentration of 50 mg/mL. To compare and to evaluate the effective activity a blank sample was prepared. Then, the three samples were serially diluted 1:2 in 50% ethanol, and 0.8 mL of each dilution were mixed with 7.2 mL of the specific agar culture medium, previously equilibrated at 70 °C, to finally cover a polyphenol concentration range between 0.007 mg/mL and 0.872 mg/mL. Once mixed by vortexing, agar culture medium was added to each M.E.D.® propolis extract at different concentrations and then poured into a 6 mm Petri plate, and a cell suspension from a frozen vial was plated at about 5 x $10^3$ CFU/spot. As a positive control, some plates were prepared with the culture medium containing 0.8 mL of the blank stock.

***Results***

All the extracts obtained from different European regions (Eu1, Eu2, Eu3), Southern American regions (Am1, Am2, Am3), and Asian regions (As1, As2, As3) were submitted to hydroalcoholic extraction. The extracts were analyzed by means of RP-HPLC–PDA–ESI–MSn. The main flavonoid species, flavonols (galangin, quercetin), flavones (chrysin, apigenin), and flavonones (pinocembrin, pinobanksin), were identified based on their UV and mass

spectra. After the initial identification of quercetin, apigenin, pinobaskin, chrysin, pinocembrin, and galangin, they were using an on-line HPLC–UV according to methods described above.

| Polyphenols | Eu1 | Eu2 | Eu3 |
|---|---|---|---|
| 1-Quercetin | 1.4 ± 0.6 | 0.8 ± 0.2 | 0.7 ± 0.4 |
| 2-Pinobanksin | 1.5 ± 0.1 | 1.0 ± 0.2 | 1.5 ± 0.3 |
| 3-Apigenin | 1.6 ± 0.3 | 1.1 ± 0.3 | 1.2 ± 0.3 |
| 4-Chrysin | 18.3 ± 0.3 | 21.4 ± 0.2 | 24.1 ± 0.4 |
| 5-Pinocembrin | 2.8 ± 0.3 | 4.8 ± 0.1 | 3.1 ± 0.2 |
| 6-Galangin | 12.6 ± 0.1 | 12.0 ± 0.1 | 12.0 ± 0.2 |
| Sum of percentages | 38.2 | 41.1 | 42.6 |
| - | **Am1** | **Am2** | **Am3** |
| 1-Quercetin | 0.5 ± 0-6 | 0.5 ± 0.1 | 0.9 ± 0.5 |
| 2-Pinobanksin | 1.0 ± 0.2 | 0.9 ± 0.2 | 3.0 ± 0.8 |
| 3-Apigenin | 1.5 ± 0.9 | 0.9 ± 0.3 | 3.5 ± 1.2 |
| 4-Chrysin | 30.3 ± 3.3 | 22.2 ± 1.1 | 28.6 ± 0.6 |
| 5-Pinocembrin | 4.4 ± 0.4 | 1.8 ± 0.3 | 13.9 ± 1.1 |
| 6-Galangin | 15.4 ± 0.6 | 11.5 ± 0.4 | 9.4 ± 1.3 |
| Sum of percentages | 53.1 | 37.8 | 59.3 |
| - | **As1** | **As2** | **As3** |
| 1-Quercetin | 0.4 ± 0.4 | 0.4 ± 0.5 | 0.9 ± 0.4 |
| 2-Pinobanksin | 1.0 ± 0.2 | 1.8 ± 0.4 | 10.0 ± 2.0 |
| 3-Apigenin | 2.2 ± 0.8 | 2.0 ± 1.7 | 1.2 ± 0.1 |
| 4-Chrysin | 25.0 ± 2.5 | 24.4 ± 0.8 | 19.6 ± 1.4 |
| 5-Pinocembrin | 2.0 ± 0.1 | 2.4 ± 0.1 | 1.7 ± 0.2 |
| 6-Galangin | 16.1 ± 0.6 | 15.9 ± 0.5 | 12.0 ± 1.0 |
| Sum of percentages | 46.7 | 46.9 | 45.4 |

**Tab 4.** Different polyphenols origin concentration (% w/w)

The results (table 4) showed that while the total polyphenol contents of the Asian hydroalcoholic propolis extracts were similar (mean value: 46.3% w/w, standard deviation: 0.8), and the same happened with the total polyphenol content of the American propolis samples (ranged from 37.8 to 59.3 mean

value: 50.1%, standard deviation: 11.1), and the total polyphenol contents of the European propolis samples (ranged from 38.2 to 42.6 mean value: 40.6%, standard deviation 2.2). Evaluating the relative percentage of each main flavonoid compound, the analysis of variance (ANOVA) was used to investigate whether the concentration of each compound was statistically different between the hydroalcoholic propolis extracts, considering their different origins.

The results, reported in table 5, showed that **the relative percentages of the polyphenols were often statistically different, confirming that the high variability of propolis raw materials of different origin leads to propolis extracts with different compositions when using common extraction methods (i.e., hydroalcoholic extraction)** (figure 5)

| Comparisons | Significance | | | | | |
|---|---|---|---|---|---|---|
| | Quercetin | Pinobanksin | Apigenin | Chrysin | Pinocembrin | Galangin |
| EU 1 vs EU 2 | Yes * | No ** | No | No | Yes | No |
| EU 1 vs EU 3 | Yes | No | No | Yes | No | No |
| EU 1 vs AM 1 | Yes | No | No | Yes | Yes | Yes |
| EU 1 vs AM 2 | Yes | No | No | No | No | No |
| EU 1 vs AM 3 | No | No | Yes | Yes | Yes | Yes |
| EU 1 vs AS 1 | Yes | No | No | Yes | No | Yes |
| EU 1 vs AS 2 | Yes | No | No | Yes | No | Yes |
| EU 1 vs AS 3 | No | Yes | No | No | No | No |
| EU 2 vs EU 3 | No | No | No | No | Yes | No |
| EU 2 vs AM 1 | No | No | No | Yes | No | Yes |
| EU 2 vs AM 2 | No | No | No | No | Yes | No |
| EU 2 vs AM 3 | No | No | Yes | Yes | Yes | Yes |
| EU 2 vs AS 1 | No | No | Yes | No | Yes | Yes |
| EU 2 vs AS 2 | No | No | Yes | No | Yes | Yes |
| EU 2 vs AS 3 | No | Yes | No | No | Yes | No |
| EU 3 vs AM 1 | No | No | No | Yes | Yes | Yes |
| EU 3 vs AM 2 | No | No | No | No | Yes | No |
| EU 3 vs AM 3 | No | No | Yes | Yes | Yes | Yes |
| EU 3 vs AS 1 | No | No | Yes | No | No | Yes |
| EU 3 vs AS 2 | No | No | No | No | No | Yes |
| EU 3 vs AS 3 | No | Yes | No | Yes | Yes | No |
| AM 1 vs AM 2 | No | No | No | Yes | Yes | Yes |
| AM 1 vs AM 3 | No | No | Yes | No | Yes | Yes |
| AM 1 vs AS 1 | No | No | No | Yes | Yes | No |
| AM 1 vs AS 2 | No | No | No | Yes | Yes | No |
| AM 1 vs AS 3 | No | Yes | No | Yes | Yes | Yes |
| AM 2 vs AM 3 | No | No | Yes | Yes | Yes | Yes |
| AM 2 vs AS 1 | No | No | Yes | No | No | Yes |
| AM 2 vs AS 2 | No | No | Yes | No | No | Yes |
| AM 2 vs AS 3 | No | Yes | No | No | No | No |
| AM 3 vs AS 1 | No | No | Yes | No | Yes | Yes |
| AM 3 vs AS 2 | No | No | Yes | No | Yes | Yes |
| AM 3 vs AS 3 | No | Yes | Yes | Yes | Yes | Yes |
| AS 1 vs AS 2 | No | No | No | No | No | No |
| AS 1 vs AS 3 | No | Yes | Yes | Yes | No | Yes |
| AS 2 vs AS 3 | No | Yes | No | Yes | No | Yes |

**Tab 5.** Relative percentages of the polyphenols statistically different (*) obtained from different regions.

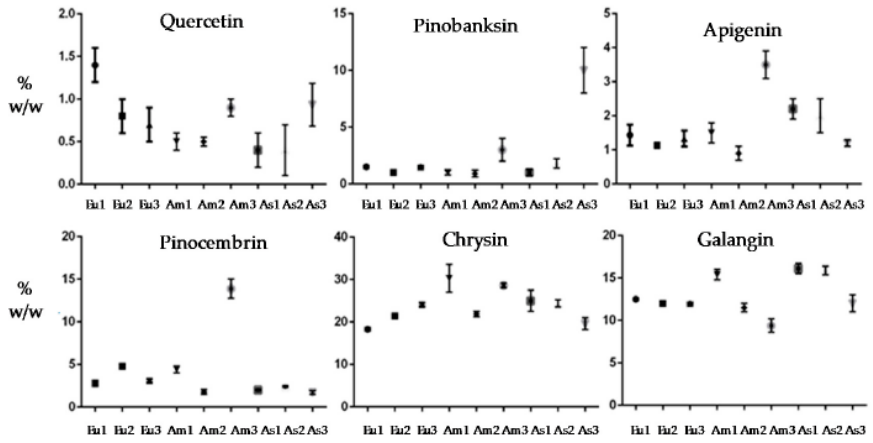



**Fig 5.** Statistical polyphenols differences between classic extraction method.

In light of this we combined the different raw propolis material to obtain three mixtures of European, American, and Asian poplar-type propolis (Eu + Am + As) and each mixture was submitted to the M.E.D. ® extraction process obtained the extracts A, B, and C, respectively. After the identification of quercetin, apigenin, pinobaskin, chrysin, pinocembrin, and galangin based on their UV and mass spectra, they were quantified by means of HPLC–UV analyses (table 6).

| Polyphenols | M.E.D. Propolis A | M.E.D. Propolis B | M.E.D. Propolis C |
|---|---|---|---|
| 1-Quercetin | 1.1 ± 0.05 | 1.2 ± 0.10 | 0.9 ± 0.06 |
| 2-Pinobanksin | 1.2 ± 0.40 | 0.8 ± 0.11 | 1.6 ± 0.36 |
| 3-Apigenin | 1.2 ± 0.30 | 1.0 ± 0.20 | 1.4 ± 0.04 |
| 4-Chrysin | 23.2 ± 0.60 | 22.0 ± 0.71 | 22.0 ± 1.02 |
| 5-Pinocembrin | 1.17 ± 0.04 | 1.4 ± 0.06 | 1.4 ± 0.04 |
| 6-Galangin | 13.4 ± 0.15 | 14.7 ± 0.11 | 14.3 ± 0.10 |

**Tab 6.** Quantification of polyphenols in three M.E.D.® propolis extracts (A, B and C) (% w/w)

The chromatograms acquired at 260 nm for each M.E.D.® propolis (A, B and C) extract are reported in figure 6.

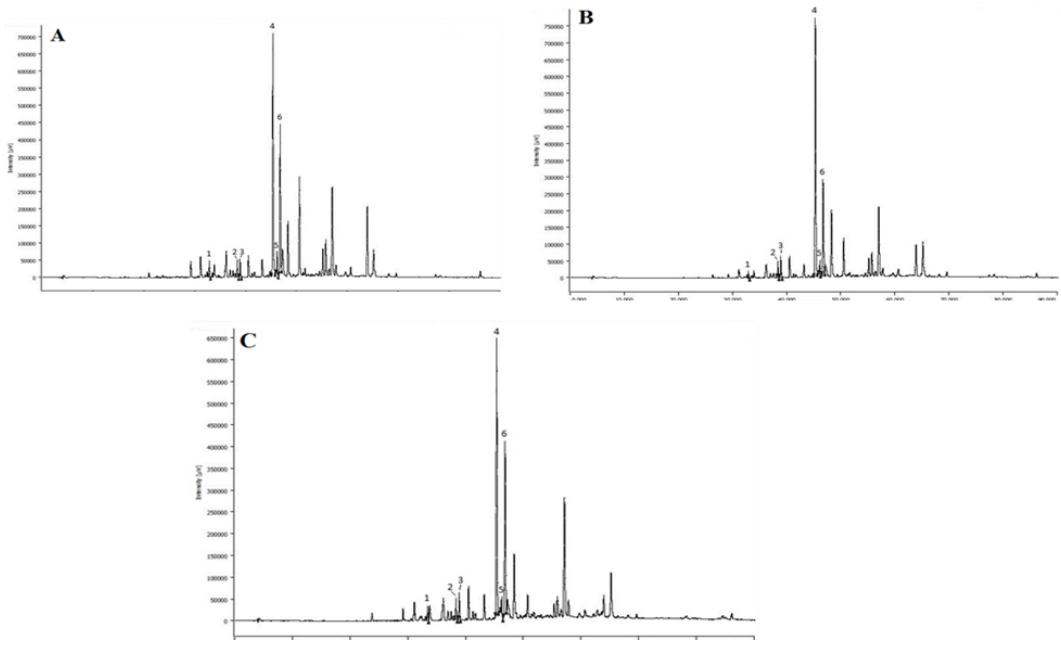



**Fig 6.** Chromatograms acquired at 260 nm for each M.E.D.® propolis (A, B and C) extract.

The numbers represent the polyphenols quercitin (1), pinobanksin (2), apigenin (3), chrysin (4), pinocembrin (5) and galangin (6).

The relative percentages of each flavonoid were tested with ANOVA, showing that no differences were found in flavonoid composition between the non-ethanolic M.E.D. propolis extracts (figure 7).

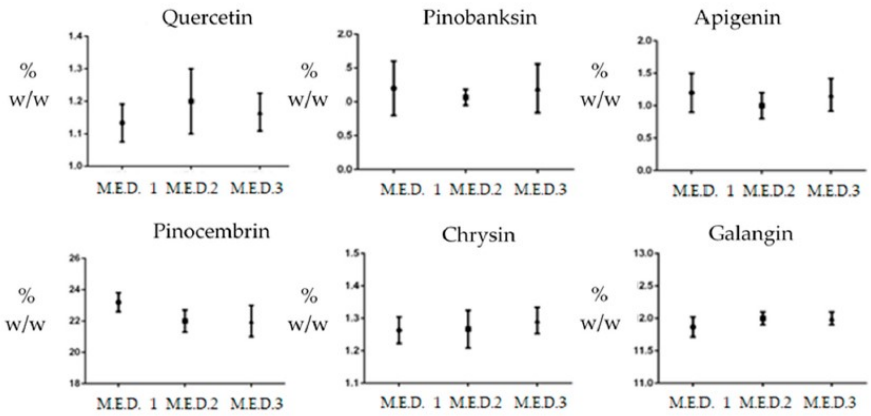



**Fig 7.** Statistical polyphenols differences between M.E.D.® extraction method.

After that it was evaluated the antimicrobial activity of the three M.E.D. propolis extracts (A, B and C) first tested against microorganism strains representing the major families: Gram-positive or Gram-negative bacteria and fungi. As expected, MIC values showed that the three M.E.D.® propolis extracts exerted antimicrobial activity, confirming literature data. Low MIC values (ranging between 20 and 156 µg/mL) were found against *Aspergillus niger*, *Streptococcus pneumonia* penicillin-susceptible, *Moraxella catarrhalis*, *Atopobium vaginae*, and *Neisseria gonorrhoeae*. Moderate activity was found against *Staphylococcus spp* and *Gardnerella vaginalis*, (MIC value = 312 µg/mL). Slight effects were registered on the growth of *Candida spp* and

*Clostridium spp*, shown by MIC values above 1250 µg/mL; while no activity was detected against *Bacteroides fragilis* and *Lactobacillus spp* (table 7).

The results obtained by our experiments gave comparable MIC values for each extract obtained using the M.E.D.® method against the same microorganisms, despite the different geographical origins of the three samples.

| Microbial Strain | MIC (µg/mL) | | | |
|---|---|---|---|---|
| | CODE | A | B | C |
| *Staphylococcus aureus* MSSA ATCC25923 | L1280 | 312 | 312 | 312 |
| *Staphylococcus epidermidis* ATCC12228 | L147 | 312 | 312 | 312 |
| *Escherichia coli hyperpermeable* | G1640 | 312 | 625 | 625 |
| *Moraxella catarrhalis* | L3292 | 39 | 78 | 78 |
| *Streptococcus pneumoniae* penicillin-susceptible | L44 | 20 | 39 | 39 |
| *Candida albicans* ATCC24443 | L4120 | 1250 | 1250 | 1250 |
| *Candida albicans* ATCC90028 | L3023 | 1250 | 2500 | 2500 |
| *Candida parapsilosis* ATCC90018 | L3022 | 2500 | 2500 | 2500 |
| *Candida kruzei* | L2280 | 2500 | 2500 | 2500 |
| *Aspergillus niger* ATCC10535 | L53 | 78 | 156 | 156 |
| *Bacteroides fragilis* ATCC25285 | L1011 | 5000 | >5000 | >5000 |
| *Propionebacterium acnes* ATCC25746 | L1016 | >5000 | >5000 | >5000 |
| *Clostridium difficile* | L1365 | 2500 | 2500 | 2500 |
| *Clostridium difficile* ATCC17858 | L4013 | 5000 | 2500 | 2500 |
| *Atopobium vaginae* | ND736 | 156 | 156 | 156 |
| *Lactobacillus gasseri* | ND787 | 5000 | >5000 | >5000 |
| *Lactobacillus acidophilus* | ND786 | >5000 | >5000 | >5000 |
| *Neisseria gonorrhoeae* | L1600 | 156 | 156 | 156 |
| *Neisseria gonorrhoeae* | L1601 | 156 | 78 | 78 |
| *Gardnerella vaginalis* | L1629 | 312 | 312 | 156 |
| *Gardnerella vaginalis* | L1630 | 312 | 312 | 312 |

**Tab 7.** Antimicrobial activity of the three M.E.D. propolis extracts (A, B and C)

### *Discussion and conclusions*

In this study, the chemical composition and antimicrobial activity were investigated. The extracts show the presence of the main flavonoid species,

flavonols (galangin, quercetin), flavones (chrysin, apigenin), and flavonones (pinocembrin, pinobanksin).

The chemical compositions of M.E.D.® propolis extracts were similar, even if the single propolis raw materials acquired from different areas had varying compositions, according to the analysis of variance. Not only chemically, but also biologically, the three M.E.D. propolis extracts were shown to be repeatable. In fact, they had similar antibacterial efficacy against a variety of bacteria and fungi. M.E.D.® propolis extract has the lowest MIC against Gram-positive bacteria, has good action against some Gram-negative bacteria, and has weak activity against fungus, according to our research on poplar-type propolis. Because of the diversity in the raw material, propolis extracts demonstrate poorly reproducible chemical composition and biological properties when extracted using commonly used procedures (e.g., hydroalcoholic extraction).

However, because propolis extracts are used in medications, foods, and cosmetics, it's critical to have non-ethanolic propolis extracts with a consistent chemical composition and the assurance of same biological qualities in the end product. We are the first to disclose the antibacterial activity of three combinations of poplar-type propolis prepared using the M.E.D.® extraction method from different geographical locations with the same polyphenol concentration.

This study also found that M.E.D.® propolis extracts have noteworthy actions against diverse pathogen bacteria strains and antibiotic resistant bacteria such

*Streptococcus pneumoniae* clindamycin/erythromycin resistant and, to a lesser extent, other *Staphylococcus* strains.

In addition, the inactivity of M.E.D.® propolis extract against *Bacteroides fragilis* and *Lactobacillus spp*. is a crucial consideration in the context of its application in gastrointestinal diseases.

In conclusion, despite the geographical origins, the findings of our trials yielded comparable MIC values for each extract prepared utilizing the M.E.D.® approach against the same bacteria.

## ANTIVIRAL ACTIVITY OF DIFFERENT EXTRACTS OF STANDARDIZED PROPOLIS PREPARATIONS AGAINST HSV.

### *Introduction*

In this study [95] we aimed to investigate the antiviral activity of different extracts of Standardized Propolis Preparations (M.E.D.®) with glycol, ethanol, glycerol, and soya oil, against herpes simplex type 1 (HSV-1) and type 2 (HSV-2) viruses.

MTS (3-(4,5-dimethylthiazol-2-yl)- 5- (3-carboxymethoxyphenyl)- 2- (4-sulfophenyl)-2H-tetrazo- lium) on human immortalized keratinocyte (HaCaT) cell line was used to evaluate the cytotoxicity of each extract in vitro ($CC_{50}$). A quantitative real-time PCR approach was used to assess the effective concentration ($EC_{50}$) that can kill 50% of cells infected with HSV-1 and HSV-2, and the antiviral activity of the extracts was measured by selective index values (SI: $CC_{50}/EC_{50}$). Acyclovir was utilized as a positive control to compare the efficacy of the extracts against the viruses.

Standardized M.E.D.® propolis extract preparations as glycolic (98% propylene glycol), ethanolic (80% ethanol/20% water), glycerol (95% and 5% water) and soya oil (100% oil) extracts were provided by BNatural s.r.l.

Total polyphenol content was evaluated by Folin-Ciocalteu assay.

The work described in this chapter was also previously published in the article entitled “Multi Dynamic Extraction: An Innovative Method to Obtain a Standardized Chemically and Biologically Reproducible Polyphenol Extract from Poplar-Type Propolis to Be Used for Its Anti-Infective Properties”

*Materials (Basel).* **2019**, *12*, 3746 by Zaccaria, V.; Garzarella, E.U.; Di Giovanni, C.; Galeotti, F.; Gisone, L.; Campoccia, D.; Volpi, N.; Arciola, C.R.; Daglia, M.. *Materials (Basel).* **2019**, *12*, 3746.

***Materials and methods***

The extracts were sterilized with Millipore 0.22 μm filters, and 1 ml aliquots were kept at 4°C until employed in cell culture. Before diluting with medium, each extract was warmed for 1 hour at room temperature and vortexed. Glyceric and soya oil extracts were diluted to 3.5 mg/ml in Dulbecco's Modified Eagle Medium, whereas ethanol and glycolic samples were prepared at 1 mg/ml (DMEM).

250 mg of acyclovir (acyclovir sodium, Zovirax) was dissolved in 10 ml sterile saline solution (sodium chloride intravenous infusion BP, 0.9 percent w/v) to obtain a concentration of 25 mg/ml, which was then diluted to prepare 20 μM working stock aliquots that were stored at -20°C until use.

Herpes simplex type 1 (MacIntyre, #0810005CF, Zeptometrix) and herpes simplex type 2 (MS, #0810006CF, Zeptometrix) viruses, immortalized human keratinocytes (HaCaT) cell line, and herpes simplex type 2 (MS, #0810006CF, Zeptometrix) viruses were utilized (cell line and viruses were obtained from the Genetic and Bioengineering Department of Yeditepe University, Istanbul, Turkey).

The MTS (3-(4,5-Dimethylthiazol-2-yl)- 5- (3-carboxymethoxyphenyl)- 2- (4-sulfophenyl)-2H-tetrazolium) technique was used to assess the toxic and non-toxic concentrations of the compounds using the immortalized HaCaT cell line.

HaCaT cells were sown in a 96-well plate as 5x103 cells (100 l/well) and incubated for 24 hours at 37°C with 5% CO2. Following incubation, medium was aspirated, and diluted M.E.D.® propolis extracts of various concentrations were applied to cells and plates, which were then incubated for 72 hours at 37°C with 5% CO2. After this time, the old media was discarded, and 100 l of MTS solution (10% MTS in DPBS/glucose media) was given to the cells and incubated for 2 hours. The absorbance of colored dye was measured at 490 nm using a microplate reader (Bio-Rad, Tokyo, Japan), and the absorbance values were quantified by comparing treated and control cells.

The 50 percent tissue culture infectious dose ($TCID_{50}$) approach, which involves microscopic observation of cytopathic effect (CPE) or counting viral plaque in a culture plate, is the most used method for estimating viral titers. However, because the traditional $TCID_{50}$ approach is time consuming and prone to errors, we adopted the colorimetric MTS method to determine viral titers with minor changes in our investigation [96].

HaCaT cells were seeded at 3x104 cells per well (200 l/well) on a 96-well plate and incubated for 24 hours at 37°C with 5% CO2. The following day, cells were examined under a microscope (Zeiss Axio Vert.A1, Köln, Germany) for morphology and confluency, and different virus dilutions were prepared on ice in log scale (log 2) from 10-2 for HSV-1 diluted virus stock to 10-1 for HSV-2 diluted virus stock to obtain a 50% infectivity point for virus inoculation. Old media were aspirated and properly washed three times with DPBS after the creation of viral dilutions.

Diluted virus solutions were inoculated into cells at a rate of 50 µl/well and incubated for 2 hours at 37°C with 5% CO2. Unbound viruses were aspirated after incubation, and 200 l of viral medium (DMEM with 2% FBS and 1% PSA) was added to each well and incubated for 72 hours at 37°C with 5% CO2. Following incubation, the medium was aspirated, and 200 l of viral media containing 10% MTS was added to the cells. After 3 hours of incubation, absorbance was measured at 490 nm using a Bio-Rad microplate reader, and the absorbance was quantified by comparing treated cells to controls. This experiment was used to evaluate the viral titration capable of inducing a 50% virus infection in HSV-1 and HSV-2.

Viral DNA was obtained from infected and treated cells for qRT-PCR analysis using the Roche high pure viral DNA isolation Kit (#11858874001, Switzerland). In 48-well plates, HaCaT cells were seeded at 1x105 cells (400 l/well) and incubated for 24 hours at 37°C and 5% CO2. The next day, the media was aspirated from the well and cleaned three times with DPBS. Injected cells were inoculated with 150 µl of previously produced working viral stock and incubated for 2 hours at 37°C with 5% CO2. Meanwhile, the various M.E.D.® propolis extract samples were prepared at various dilutions in DMEM with 2% FBS and 1% PSA at a cytotoxic concentration of 1%. Working stock of acyclovir was also warmed at room temperature to prepare concentrations ranging from 0.4 to 5 µM.

Unbound viruses were sucked out after incubation, and the cells were treated with 400 l M.E.D.® propolis extract samples and acyclovir. As a control, 20% of DMSO-treated cells were utilized. Supernatants were collected and

centrifuged at 5,000 rpm for 30 minutes to remove cell debris after 72 hours of incubation at 37°C and 5% CO2. Viral DNA was extracted and kept at -20°C until usage according to the technique. For HSV-1 and HSV-2, these procedures were repeated three times with varied concentrations of each sample and acyclovir.

After DNA isolation with a quantitative HSV-1 and an HSV-2 Kit (R-gene HSV-1 #71015, HSV-2 #71016, bioMérieux, Lyon, France), real-time PCR with TaqMan 5'-nuclease technology was used to do the qRT-PCR. HSV-1 has a targeted sequence in the US7 gene with an amplified fragment size of 142 base pairs, while HSV-2 has a targeted sequence in the US2 gene with an amplified fragment size of 177 base pairs. With the data from qRT-PCR (virus amount expressed in copies/ml), the EC50 values of the individual samples were determined. The SI, a widely accepted parameter used to express the *in vitro* efficacy of a compound in the suspension of virus replication, was calculated based on $CC_{50}$ values for HaCaT cell lines and $EC_{50}$ values for HSV-1 and HSV-2, as illustrated: SI = cytotoxic concentration 50 ($CC_{50}$)/effective concentration 50 ($EC_{50}$).

***Results***

The cytotoxic effects of the four different M.E.D.® propolis extract and acyclovir on HaCaT cells were evaluated by increasing their concentrations. On HaCaT cells, the glycolic extract had a severe cytotoxic effect at 800 µg/ml and a mild effect at 400 g/ml. At 200 µg/ml, there was no cytotoxic effect, but at 100 and 50 µg/ml, there was a proliferative effect. As a result, the minimum

cytotoxic concentration was determined to be 200 μg/ml (MCC or maximum non-toxic dose). Glycol solvent was found to be non-toxic to HaCaT cells at the maximum concentration of M.E.D.® propolis extract.

At 1000 μg/ml, the ethanolic M.E.D.® propolis extract sample had a severe cytotoxic effect, and at 500 μg/ml, it had a moderate cytotoxic effect. Proliferation was examined at 150 μg/ml and 100 μg/ml, with no cytotoxicity reported. Ethanol had a minimal proliferative effect on HaCaT cells when it was fixed at 200 μg/ml as MCC. At 3000, 2500, and 2000 μg/ml, M.E.D.® propolis extract in glycerol had a moderate cytotoxic impact, whereas at 1300 μg/ml there was no effect, and proliferation was recorded at 1000 and 900 μg/ml. Glycerol was shown to be non-toxic at a concentration of 1300 μg/ml. At 2500 and 2000 μg/ml, the soya oil extract was cytotoxic, while at 800 g/ml, there was no cytotoxicity. The proliferative effect was observed for the soya oil solvent tested at the highest concentration of M.E.D.® propolis extract, and 800 μg/ml was evaluated as MCC. Acyclovir showed cytotoxic effect producing cell death over 50% at 20 μM and causing limited cytotoxicity between 10 and 1 μM on HaCaT cells. No significant effect was measured at 0.8 μM, and this concentration was determined as MCC.

Each suspension data graph yielded a cytotoxic concentration 50 ($CC_{50}$), which is the concentration of a substance capable of killing half of the cells in an uninfected cell culture. The $CC_{50}$ of glycol extract was 593 μg/ml, 375 μg/ml for ethanolic sample, 1,723 μg/ml for M.E.D.® propolis extract in glycerol, and 1,664 μg/ml for soya oil preparation. For HaCaT cells, the $CC_{50}$ of acyclovir was determined to be 15.9 μM.

Based on previously determined non-toxic concentrations, antiviral activity of the various M.E.D.® propolis extracts and acyclovir for HaCaT cells were assessed for HSV-1 (figure 8) and HSV-2 (figure 9) viruses. In treated cells, virus inhibition was measured in copies/ml.

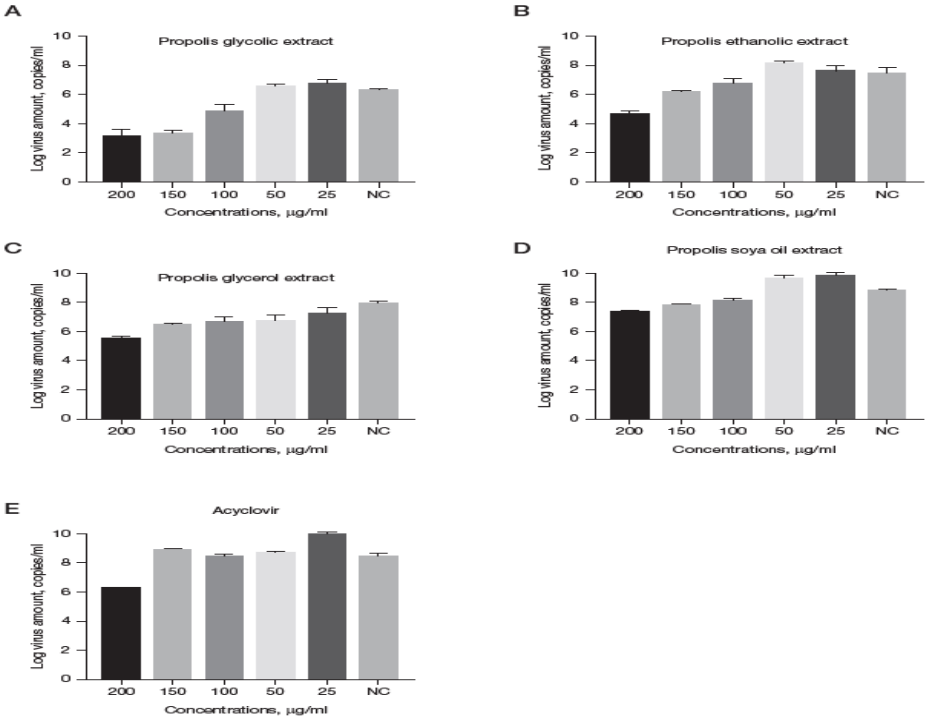



**Fig. 8** Antiviral activity of different M.E.D.® propolis extracts and acyclovir on HSV-1.

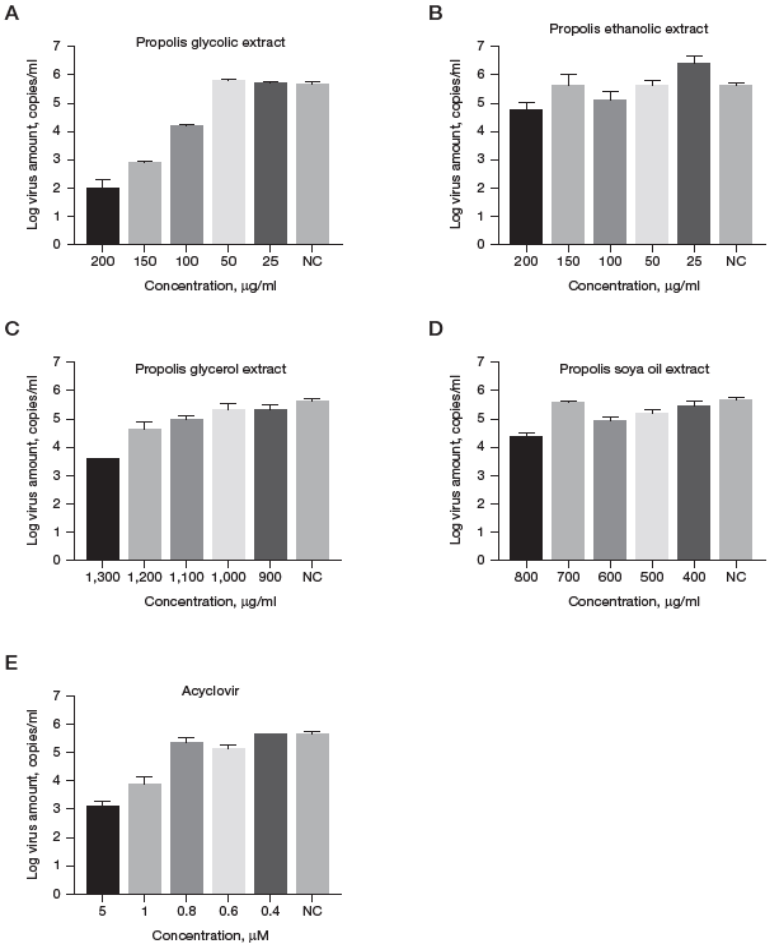



**Fig. 9** Antiviral activity of different M.E.D.® propolis extracts and acyclovir on HSV-2.

The $EC_{50}$, or the concentration of a substance capable of inhibiting virus reproduction by 50% as compared to an untreated virus-infected control, was also determined. Tables 8 and 9 show the $EC_{50}$ values determined for each M.E.D.® propolis extract and acyclovir for both viruses.

**The SI values of the various M.E.D.® propolis extracts, calculated by $CC_{50}$ and $EC_{50}$ data are shown in tables 8 and 9 for HSV-1 and HSV-2, respectively**. SI values of glycol, ethanol, glycerol, soya oil extracts and acyclovir were determined as 6.8, 4.1, 2.2, 3.3 and 6.3, respectively, for HSV-

1. SI values of glycol, ethanol, glycerol, soya oil extracts and acyclovir were determined as 6.4, 7.7, 1.9, 4.2 and 2.9, respectively, for HSV-2.

| | HaCaT $CC_{50}$, µg/ml | $EC_{50}$, µg/ml | $EC_{50}$, µg/mg of polyphenols | SI ($CC_{50}/EC_{50}$) |
|---|---|---|---|---|
| Extract | | | | |
| Glycolic | 593.0 ±3.9 | 86.6 ±5.0 | 4.3 ±0.2 | 6.8 |
| Ethanolic | 375.0 ±2.4 | 90.9 ±6.7 | 5.5 ±0.4 | 4.1 |
| Glyceric | 1,723.0 ±1.5 | 768.0 ±6.7 | 7.5 ±0.1 | 2.2 |
| Soya oil | 1,664.0 ±3.1 | 501.0 ±7.4 | 16.7 ±0.22 | 3.3 |
| Control | | | | |
| Acyclovir | 15.9 ±8.6[a] | 2.5 ±7.3[a] | | 6.3 |

**Tab 8**. $EC_{50}$, $CC_{50}$ and SI values of different M.E.D.® propolis extracts and aciclovir on HSV-1.

| | HaCaT $CC_{50}$, µg/ml | $EC_{50}$, µg/ml | $EC_{50}$, µg/mg of polyphenols | SI ($CC_{50}/EC_{50}$) |
|---|---|---|---|---|
| Extract | | | | |
| Glycolic | 593.0 ±3.9 | 92.1 ±3.9 | 4.6 ±0.2 | 6.4 |
| Ethanolic | 375.0 ±2.4 | 49.0 ±5.4 | 2.9 ±0.3 | 7.7 |
| Glyceric | 1,723.0 ±1.5 | 904.1 ±4.9 | 8.9 ±0.1 | 1.9 |
| Soya oil | 1,664.0 ±3.1 | 396.1 ±4.8 | 13.2 ±0.2 | 4.2 |
| Control | | | | |
| Acyclovir | 15.9 ±8.6[a] | 5.5 ±4.7[a] | | 2.9 |

**Tab 9.** $EC_{50}$, $CC_{50}$ and SI values of different M.E.D.® propolis extracts and aciclovir on HSV-2.

As a result, a glycolic M.E.D.® propolis extract possesses a greater antiviral activity than acyclovir for both HSV type 1 and type 2, while glycolic, ethanolic and soya oil preparations were found to have greater activity than acyclovir for HSV-2.

***Discussion and conclusions***

The $EC_{50}$ values for HSV-1 and HSV-2 viruses were determined using a qRT-PCR method, which is widely accepted as the best and most validated method for assessing a drug's antiviral properties. HSV-1 and HSV-2 are known to have a variety of differences in how they interact with their host cells. HSV-2 infection of cells is more effectively suppressed by polyanionic compounds than HSV-1 infection, which is more effectively prevented by polycationic substances. These discrepancies between the two viruses, as well as the distinct types of M.E.D.® propolis extracts utilized in this investigation, can be attributed to viral differences. The $EC_{50}$ range of acyclovir (Zovirax) for HSV-1 between 0.02 and 13.5 μg/ml and HSV-2 between 0.01 and 9.9 μg/ml was approved by the US Food and Drug Administration (FDA), supporting our findings. Propolis in combination with acyclovir had previously been demonstrated to have better antiviral efficacy than acyclovir alone.

When compared to acyclovir, the ethanolic extract has a SI that is roughly comparable, whereas the glycolic, glyceric, and soya oil extracts have lower values. Ethanolic, glycolic, and soya oil, on the other hand, are more effective against HSV-2 than acyclovir, except for glyceric extract, which has low antiviral characteristics. Extracts or compounds with a SI value of 1 in the initial screen (tested at log dilution) are considered sufficiently active to support further testing in the primary screen [97].

As a result, for further research, glyceric extract can be considered an effective product against HSV-2. This is even more relevant when we consider that the

findings of sensitivity testing vary greatly based on numerous of factors, which also explains why our results differ. Nonetheless, apart from the soya oil preparation, the $EC_{50}$ values determined in μg/mg of polyphenols demonstrate that the two extracts, glycolic and ethanolic, have very similar values.

This shows that the overall amount of polyphenols is a crucial parameter to consider when considering propolis (and, in general, extracts with polyphenols as active ingredients) as an antiviral product. The ability of propolis samples to precisely suspend viral DNA polymerase throughout the intracellular replication cycle when new viral DNA is generated is also supported by the finding that M.E.D.® propolis extracts have greater SI values than acyclovir.

In conclusion, different extracts of Standardized Propolis Preparations (M.E.D.®) with glycol, ethanol, glycerol and soya oil, were found having potent antiviral activity with different capacities against HSV-1 and HSV-2 viruses.

**EFFICACY OF A STANDARDIZED POLYPHENOL MIXTURE EXTRACTED FROM POPLAR-TYPE PROPOLIS (M.E.D.® propolis) FOR REMISSION OF SYMPTOMS OF UNCOMPLICATED UPPER RESPIRATORY TRACT INFECTION (URTI): A MONOCENTRIC, RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL.**

***Introduction***

The term Upper Respiratory Tract Infections (URTIs) is commonly used to describe acute infections of mucosa lining the upper respiratory tract caused by bacteria and viruses. Cough, sore throat, runny nose, nasal congestion, headache, low-grade fever, facial pressure, sneezing, malaise, and myalgias are the most common clinical signs of a URTI. In the past, management of presumptive bacterial URTIs has focused on advising antibiotic drugs to avoid complications. While most cases of uncomplicated URTIs recover spontaneously without therapy after about 7–10 days, management of presumptive bacterial URTIs has focused on advising antibiotic drugs to avoid complications. If symptoms persist, nonsteroidal anti-inflammatory drugs with antipyretic, analgesic, and anti-inflammatory properties, topical and systemic steroids to reduce mucosa swelling, dextromethorphan and codeine as centrally acting cough suppressants in adults, and antibiotics in the case of detected bacterial infections are the most common pharmacological treatments. Due to the significant side effects (AEs) associated with these medications,

complementary and alternative treatments are commonly employed in the treatment and prevention of URTIs.

In our investigations, M.E.D.® propolis resulted to exert antioxidant and anti-inflammatory activities through an epigenetic mechanism of action, modifying the expression level of microRNAs and mRNA targets coding for antioxidant enzymes and pro-inflammatory cytokines. More recently, it was discovered that oral administration of M.E.D.® propolis causes quick absorption and metabolism of galangin, as well as promoted adaptation of the antioxidant first line defense system in experimental animals (adult male mice C57BL/6).

Because remission of URTI symptoms is the most common reason for outpatient visits among adults in the first days of URTI and, to our knowledge, no clinical trials have been conducted to show the beneficial effects of propolis in reducing URTI symptoms in adults, and because propolis has anti-inflammatory activity, which is the primary cause of URTI symptoms, the aim of this study is to evaluate the effectiveness of a local treatment of URTI symptoms in adults[98].

The work described in this chapter was also previously published in the article entitled “Multi Dynamic Extraction: An Innovative Method to Obtain a Standardized Chemically and Biologically Reproducible Polyphenol Extract from Poplar-Type Propolis to Be Used for Its Anti-Infective Properties” *Materials (Basel).* **2019**, *12*, 3746 by Zaccaria, V.; Garzarella, E.U.; Di Giovanni, C.; Galeotti, F.; Gisone, L.; Campoccia, D.; Volpi, N.; Arciola, C.R.; Daglia, M.. *Materials (Basel).* **2019**, *12*, 3746.

***Materials and methods***

M.E.D.® propolis, vegetal glycerine (10%), and natural flavors (< 1%) constitute the propolis oral spray. The M.E.D.® propolis used in this study is a hydro-alcoholic (6:4 v/v) solution obtained by the extraction of poplar-type raw propolis selected and worked in accordance with the Dynamic Multi Extraction patented method by B Natural s.r.l. (Corbetta, MI, Italy). According to the manufacturer's specifications, the propolis oral spray complies with European specifications for contaminants and microbiologic limits.

Placebo consists of a hydro-alcoholic (6:4 v/v) solution containing vegetal glycerin (10%), natural flavours (<1%) and commercial caramel color (E150) at a concentration so that the overall acceptable daily intake of the dietary colorant resulted to be less than 300 mg/kg of body weight /day (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2011). B Natural (Corbetta (MI) developed propolis and placebo oral spray, which were packaged in 20 mL mouth spray bottles for oral use and were indistinguishable in aspect, color, and flavor. Total polyphenol content (TPC) of propolis oral spray was determined through Folin-Ciocalteau's method, using galangin as polyphenolic standard compound. Propolis oral spray was analyzed in triplicate and the concentration of total polyphenols was calculated in terms of galangin equivalents.

A monocentric, randomised, double-blind, placebo-controlled clinical trial was performed by Samnium Medical Cooperative (Benevento, Italy) to evaluate the effects of propolis oral spray on an adult population suffering from

uncomplicated forms of mild URTI diagnosed through a check-up by physicians and a throat swab.

The study was double-blind, both for the investigating physician and for the enrolled subjects. Before giving their written agreement, the participants received oral and written information about the study.

Protocol, letter of intent of volunteers, and synoptic documents regarding the study were submitted to the Scientific Ethics Committee of ASL Benevento, Italy). The study was approved by the Committee (protocol number 152,869 of 18/12/2019) and carried out in accordance with the Helsinki declaration of 1964 (as revised in 2000). This study is listed on the ISRCTN registry (www.isrctn.com) with ID ISRCTN17594930 (doi.org/10.1186/ISRCTN17594930).

The clinical trial duration was 8 weeks. Participants underwent four visits (baseline t0, after 3 days (t1), after 5 days (t2), and after the follow-up of 15 days (t3)) in an outpatient setting. At baseline visit (t0) information on the sociodemographic, clinical and symptomatologic characteristics of the subjects was collected and reported in the case report form (CRF). In particular, the following URTI symptoms (presence/absence) were registered: sore throat, muffled dysphonia and swelling and redness of throat. Moreover, throat swabs were collected from physicians and transported to UNILAB SANNIO (San Giorgio del Sannio, Benevento, Italy) for microbiological analysis.

The enrolment was carried out by family practitioners from the Samnium Medical Cooperative (Benevento, Italy), who worked under the direction of the

study's primary investigator. At the end of the baseline visit, the randomization sequence was generated by a statistician using STATA 16 software (Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC) and the randomization list was kept hidden. Subjects were assigned to each treatment groups (propolis or placebo) by simple randomization (1:1 allocation ratio). It was not used stratification or blocking. Using progressively numbered, opaque, sealed, and stapled envelopes, the allocation sequence was kept hidden from the physician recruiting and evaluating subjects. The associated envelopes were only opened until all baseline evaluations had been completed by the enlisted participants. According to the allocation sequence, both interventions were assigned a number.

The participants received propolis spray and a placebo which had same packaging, color, and taste. During the baseline visit, the placebo group received 2–4 sprays of a dye E150 hydro-alcoholic solution. The treatment was then repeated three times each day in the subject's home for five days.

The propolis group was submitted to the same treatment with propolis oral spray. Clinical visits were carried out at t1 (after 3 days of treatment) and t2 (after 5 days of treatment) to evaluate the persistence of the symptoms (sore throat, muffled dysphonia and swelling and redness of throat) and health status. After the visits, the remission of symptoms was registered by the physicians on the CRF. After 5 days of treatment, subjects were followed up for 15 further days (t3). At the end of this follow-up period, a final throat swab was performed for the participants that were found to still be positive to bacterial infections. All data were compiled in the CRF by physicians.

The number of participants recruited in this study (in December 2019) was 146, although the number of subjects involved was reduced to 122 due to not meeting the inclusion criteria. The subjects (58 in propolis group and 64 in placebo group) were recruited by the Samnium Medical Cooperative (Benevento, Italy). Subjects of both sexes, aged 18–77 years, were enrolled in December 2019 and were considered eligible for enrolment if they suffered from at least one of the following URTI symptoms: sore throat, muffled dysphonia and swelling and redness of throat. In addition, subjects were recruited only if these symptoms appeared the same day of the first baseline visit (t0). Pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding women, patients with allergies, cystic fibrosis, congenital or acquired immunodeficiency syndrome, history of asthma, serious renal disorders, cancer, cardiovascular diseases, systemic chronic disease, and those considered unsuitable for the participation by the physician were excluded from the study. In addition, other exclusion criteria were the use of antibiotics and anti-inflammatory drugs within 72 h prior to enrolling in the study, and the use of immunological drugs within 4 weeks before the enrolment.

As sociodemographic characteristics, the age and gender of the participants were registered in the CRF. The primary endpoint was the remission of symptoms associated with URTIs, assessed at baseline (t0) and after visits at t1 (after three days) and t2 (after five days). A URTI diagnosis was made by the physician based on one or more URTI symptoms (sore throat, muffled dysphonia, swelling and redness of throat). As a secondary outcome, the

persistence of positive throat swabs after the follow-up at 15 days was evaluated, to ascertain the incidence of the presence of pathogen strains resistant to antibiotics at the end of the follow-up (t3).

Susceptibility tests were performed to determine the susceptibility of bacteria to antibiotics, at t0 (baseline visit) and t3 (after the follow-up of 15 days), both for the placebo and propolis group. To evaluate the incidence of the presence of pathogen strains resistant to antibiotics, the protocol provides for the determination of susceptibility to antibiotics of the bacteria occurring in the biological material taken from throat swab using the Kirby-Bauer method. The medium used was Mueller-Hinton (MH) agar, the commonly used microbiological grown medium for antibiotic susceptibility tests. To prepare the inoculum, 4–5 colonies grown on the primary isolation medium were suspended in 4–5 ml of Tryptic Soy Broth (enrichment broth), incubating for 2–6 h. Then, a bacterial suspension adjusted at the 0,5 MacFarland standard (see Clinical Laboratory Standards Institute document) was inoculated with a sterile swab on the MH agar surface. The antimicrobial-impregnated disks were placed on the agar surface, using sterile tweezers and the plates were incubated at 37 ◦C for 24 h. The results were evaluated by diameter zone of inhibition around the disks.

The sample size calculation was made using three 1-β power values equal to 0.95 and a significance level $\alpha = 0.05$. The sample size was determined to be 134 participants, allowing for a 15% drop out rate. Descriptive statistics were used to characterize all survey items, using mean and standard deviation for the continuous variables, and numbers and frequency distributions for the

categorical variables. Secondly, a univariate analysis was conducted by using $\chi^2$ tests on 2 x 2 contingency tables to compare respiratory symptoms between treated and untreated subjects. We used Yates's correction when at least one cell of the table has an expected count smaller than 5. Thirdly, a multivariate logistic regression analysis was then conducted to determine the extent to which independent variables predicted the outcome of interest. We performed four logistic models, with the following outcome variables: remission or remission of all symptoms (Model 1), sore throat (Model 2), swelling and redness of throat (Model 3) and muffled dysphonia (Model 4) at three days. Independent variables included into the models were the following: age, gender, oral application of propolis (all Models), positive medical history for sore throat (Models 1, 3), positive medical history for muffled dysphonia (Models 1, 2, 3), positive medical history for swelling and redness of throat (1, 2), pharyngeal swab positivity (Models 1, 2, 3). The subject age was treated as a continuous variable, whereas all other predictors were treated as two-levels factors. The results of the logistic regression models were presented as Odds Ratios (ORs) with 95% confidence intervals (95% CIs). All reported values were based on two-tailed tests and were considered statistically significant at $p = 0.05$ or less. All data were coded and analyzed using Stata software, version 15 (Stata Corp. Stata 2017. Stata Statistical Software. Release 15. College Station, TX: StataCorp LLC.).

There were no toxicity tests conducted as part of this study. Nonetheless, adverse events were observed throughout the intervention period for the evaluation of tolerance and safety of the intervention (propolis oral spray

administration) through spontaneous reporting of adverse events (AEs) by participants to their respective physicians. The principal investigator examined all subject data at the end of the intervention period to determine the presence or absence of AEs.

***Results***

The study flowchart is reported in figure 10 according to the CONSORT PRO reporting guideline [99]. The number of participants involved in this study was 122. The treated group consisted of 58 (29 male and 29 female) subjects treated with propolis oral spray. The suggested daily dose was 2–4 sprays (corresponding to 0.8–1.6 ml of propolis oral spray and 12–24 mg/ml of polyphenols from M.E.D.® propolis) repeated three times/day for 5 days.

The shipment was inventoried upon arrival at the trial center, ensuring that the information on the packing slip (inside and outside containers) matches exactly with what was sent to the site, including the amount, batch numbers, manufacturing date, expiry date, name of manufacturer, quantity, and storage conditions. Both interventions were kept in a locked cabinet in a closed room at room temperature, with only study staff having access to them. Only essential research employees had access to the storage facility. The following records were meticulously kept: an entry and exit logbook, as well as a dietary supplement accountability logbook. The monitor reviewed drug accountability on a regular basis and verified that final drug reconciliation with the sponsor was completed.

The placebo group comprised of 64 patients (25 males and 39 females) who were not given any treatment. There were no significant differences in the sociodemographic features of the participants in the two groups. There was no difference between the two groups at the beginning. We discovered a statistically significant difference between treated and untreated participants after the treatment. **Subjects treated with propoli showed a higher remission of symptoms than subject in the placebo group.**

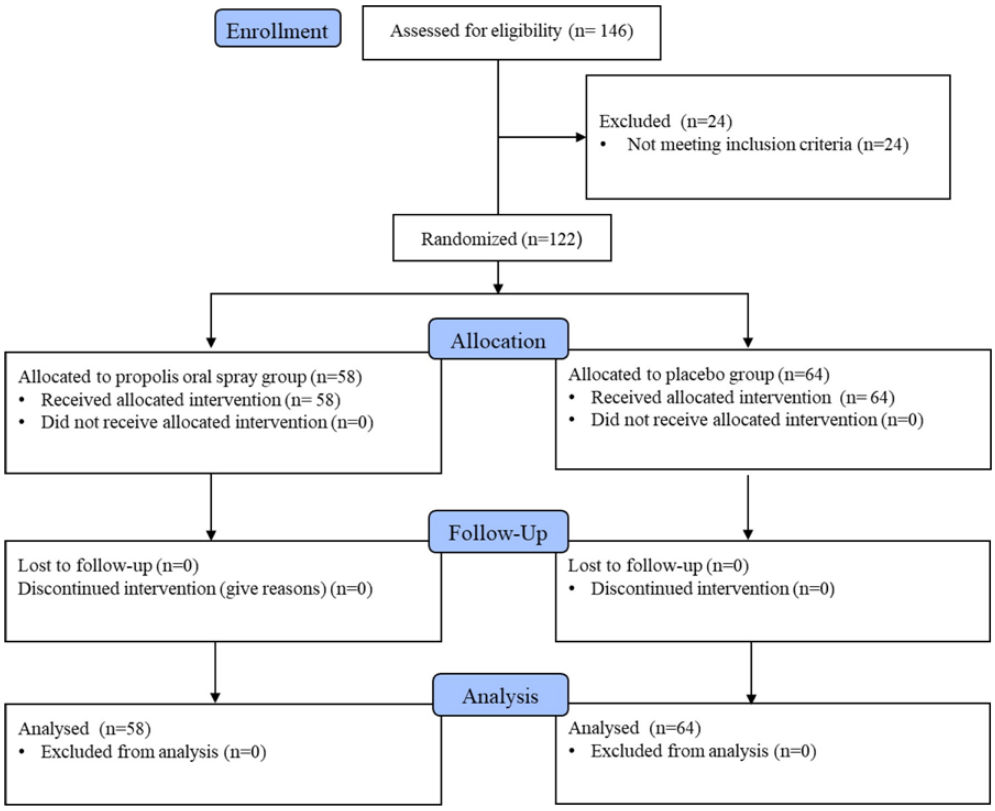



**Fig. 10** The CONSORT flow diagram.

The baseline characteristics of the subjects for each group are summarized in table 10. Mean ± standard deviation (SD) of subject age was 44 ± 14 years,

and 68 of 122 (55.7%) were female. Moreover, in table 10, the number of subjects and the relative percentage reporting sore throat, muffled dysphonia, swelling and redness of throat and positive/negative throat swab are reported. After 5 days all the subjects in the study had recovered from all symptoms, as expected considering that the first inclusion criterion was the diagnosis of uncomplicated forms of mild URTI.

| Characteristics | Number of observations | % | Treated (n=58) | Untreated (n=64) |
|---|---|---|---|---|
| Gender | | | | |
| Male | 54 | 44.3 | 29 | 25 |
| Female | 68 | 55.7 | 29 | 39 |
| Age | 44 ± 14 (18-77) * | | 44±14 (18-77) * | 44 ± 5 (18-77) * |
| Positive medical history for sore throat | | | | |
| Yes | 102 | 83.6 | 46 | 56 |
| No | 20 | 16.4 | 12 | 8 |
| Positive medical history for muffled dysphonia | | | | |
| Yes | 17 | 13.9 | 10 | 7 |
| No | 105 | 86.1 | 48 | 57 |
| Positive medical history swelling and redness of throat | | | | |
| Yes | 40 | 32.8 | 22 | 18 |
| No | 82 | 67.2 | 36 | 46 |
| Throat swab | | | | |
| Positive | 15 | 12.3 | 7 | 8 |
| Negative | 107 | 87.7 | 51 | 56 |

**Tab 10.** Characteristics of study population.

Figure 11 shows the distribution of the three different symptoms in the treated and untreated groups after three days of watchful waiting.

At t0, 8 persons in the treatment group and 7 people in the placebo group had a positive throat swab. At t1, 17 percent of propolis-treated patients had at least

one symptom, compared to 72 % (RR: 2.93, CI: 1.95–4.42) of untreated subjects. Similar outcomes were seen when single symptoms were remitted. In terms of a sore throat, it was discovered in roughly 16 % of participants treated with propolis oral spray, compared to 68 % in the untreated group. (RR: 2.64, 95% confidence interval: 1.77–3.94). Moreover, 10% of subjects (*vs*. 71% in the untreated group) and 18% of subjects (*vs*. 83% in the untreated group) showed symptoms of muffled dysphonia (RR: 3.15, CI: 0.96–10.34) and swelling and redness of the throat (RR: 4.9, CI: 1.71–14.05), respectively.

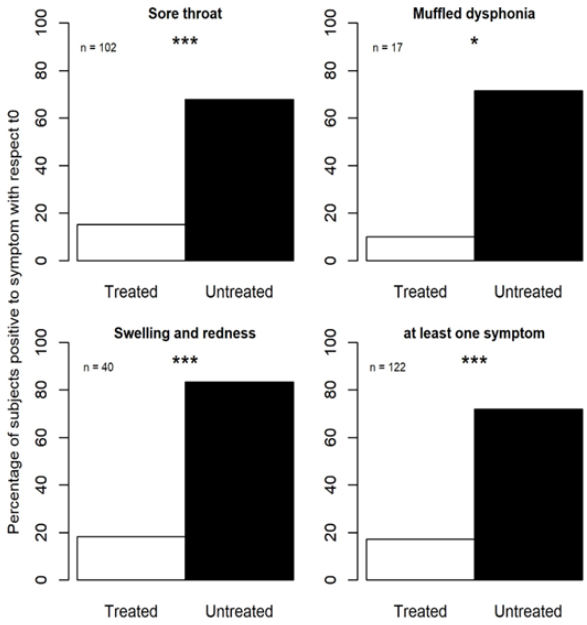



**Fig. 11** The number of patients with URTI symptoms at t0; n is for the whole sample including treated and untreated subjects; *: p<0.05, ***: p<0.001.

All the participants with bacterial URTIs, in both the treated and untreated groups, had a negative throat swab at the end of the study (which ended in

January 2020). Because there were only 15 participants with bacterial infections, no statistical analysis of propolis treatment vs placebo was possible.

No individuals reported any adverse events (AEs) related to the administration of propolis during the five-day treatment period, including the absence of oral mucosal allergies, and the primary investigator assessed that the application of propolis oral spray was well tolerated.

***Discussion and conclusions***

Mild uncomplicated URTIs are acute bacterial or viral infections that cause inflammation of the upper airways and cause a variety of symptoms that usually resolve without the use of drug after about a week. URTIs, however, are a leading cause of missed work and daily activities, according to statistics. Propolis has long been thought to be an effective treatment for URTIs, although scientific data is lacking. Furthermore, because of the considerable variability and low reproducibility of propolis' chemical makeup, it is impossible to link the content of bioactive chemicals to its efficacy. To demonstrate the effects of a poplar-type propolis extract with a standardized polyphenol content in the remission of URTI symptoms, a monocentric, double-blind, placebo controlled clinical trial was done in the current investigation. The results of this clinical study clearly show that the application of propolis oral spray assists in three days remission of the most common symptoms of URTIs, in comparison to a placebo group showing a statistically lower incidence of symptom remission after three days of watchful waiting. This means that symptoms resolution,

which in the studied adult population commonly occurs after 5 days, was two days early.

This work has limitations and strengths. The main limitation is represented by the low number of patients with URTIs of bacterial origin, shown by the low number of patients with positive throat swabs at t0, which prevents the assessment of significant differences between the treated and untreated groups after the follow-up period, as all patients showed a negative throat swab.

On the other hand, the major strength of this study are that, 1) to the best of our knowledge, it was the first double blind, controlled interventional study of the effects of a propolis oral spray on the symptoms of uncomplicated form of mild URTIs, as the other published studies were retrospective or non-controlled studies; and 2) it was the first clinical in which a propolis with a well-known content of polyphenols (M.E.D.® propolis) was studied. Moreover, it is the first one to involve an adult population for which a more rapid course of URTIs means a shorter sickness leave and a faster return to work and normal daily activities. After 5 days, all participants recovered from the symptoms, while most of the subjects who received propolis application recovered from the symptoms after 3 days. Therefore, propolis oral application reduces the time for disappearance of symptoms.

In conclusion, propolis oral spray can be used to alleviate both bacterial and viral moderate uncomplicated URTI symptoms in a shorter period of time without the use of symptomatic medication, resulting in a faster recovery.

## ACTIVITY OF A STANDARDIZED POLYPHENOL MIXTURE EXTRACTED FROM POPLAR-TYPE PROPOLIS (M.E.D.® PROPOLIS) ON HEALTHY AND DISEASED HUMAN GUT MICROBIOTA

### *Introduction*

The aim of this study was the evaluation of the impact of M.E.D.® propolis on gut microbiota utilized fecal material obtained from healthy and diseased subjects using a simulated *in vitro* digestion-fermentation process designed to replicate natural digestion in the human oral, gastric, and intestinal chambers. We also evaluated the antioxidant activity and the polyphenols concentration, of the M.E.D.® propolis extract, after the digestion-fermentation and the production by gut microbiota of short chain fatty acids (SCFAs) after the fermentation process. The work described in this chapter was submitted for publication in Biomedicine and Pharmacotherapy and the manuscript is currently under revision.

### *Materials and methods*

Propolis (PROPOLIS DRY EXTRACT ESIT 12® produced by BNATURAL s.r.l.) was subjected to *in vitro* simulated oro-gastro-intestinal digestion and fermentation to simulate physiological human intestinal processes as described by Pérez-Burillo et al [100]. 5 grams of sample were weighed in triplicate for digestion in falcon tubes. The *in vitro* digestion was composed of the oral phase (with α-amylase 75 U/mL, 5 minutes at 37ºC, pH 7.0), the gastric phase (with pepsin 500 U/mL, 2 hours at 37°C, pH 3.0) and the intestinal phase (with bile

salts 10 mM and pancreatin 100 U/mL, 2 hours at 37°C, pH 7.0). The same procedure was followed for the blank sample. After this period two fractions were obtained: the supernatant and the solid fraction. Aliquots of the supernatant were taken for further study (as the fraction available for absorption in the small intestine), while the solid fraction was used for *in vitro* fermentation. For the *in vitro* fermentation, 500 mg of the solid digestion residue, plus 10% of the final digestion volume, were taken for both propolis and blank samples. This process was carried out following a protocol described by Pérez-Burillo, S et al. [100], using fecal material from different donors: 3 healthy adults (Body Mass Index [BMI]=21.3), and 12 children (5-10 years old): 3 healthy children (BMI=16), 3 obese children (BMI=27), 3 celiac children and 3 children with a food allergy. All donors had not taken antibiotics in the last 3 months. To carry out the *in vitro* fermentation, a pull was made with the feces of the donors separated by group to reduce inter-individual variability. The samples were incubated at 37°C in oscillation for 20 hours. At the end of the process two fractions were obtained: a solid residue (fraction not available for absorption and excreted by feces) and a supernatant (fraction available for absorption in the large intestine). Aliquots of the supernatant were taken for further analysis.

The antioxidant capacity was studied in two stages: the supernatant obtained from *in vitro* digestion and the supernatant obtained from *in vitro* fermentation. The sum of both was considered the total antioxidant capacity. The respective blanks (chemicals, enzymes, and inoculum) were considered to correct the antioxidant capacity values of each method. The assays performed were the

$TEAC_{FRAP}$ assay (Trolox equivalent antioxidant capacity referred to reducing capacity) and the $TEAC_{DPPH}$ assay (Trolox equivalent antioxidant capacity against DPPH radicals).

Moreover, for the analysis of polyphenols, Folin-Ciocalteu assay (Determination of total phenolic content) was performed.

SCFAs were determined in fermentation supernatant. After the fermentation process, the supernatant was centrifuged, filtered through a 0.22 μm nylon filter, analyzed by means of a HPLC system and the analysis was performed in duplicate.

Instead, for the evaluation of the activity of propolis on gut microbiota, DNA extraction was performed using a NucliSENS easy MAG platform (Biomerieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/μL in 10 mM Tris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in Klindworth et al. (13). Primer sequences were Forward 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3′ and Reverse 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3′. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131-1096). After 16S rRNA gene amplification,

amplicons were multiplexed, and 1 mL of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to the manufacturer's instructions in a 2 × 300 cycle paired-end run (MiSeq Reagent kit v3MS-102-3001).

By mixing a standardized polyphenol mixture extracted from poplar-type propolis with arabic gum used as a high molecular weight carrier in spray drying and adding sucralose and silicon dioxide excipients, the propolis sample was prepared in a water-dispersible powder delivery form. The blank sample comprised of arabic gum in the same delivery form as sucralose and silicon dioxide. These samples were put through a simulated in vitro digestion and fermentation process, which was supposed to mimic natural digestion in the oral, gastric, and intestinal chambers of humans. The antioxidant profiles of propolis samples were determined before and after digestion and fermentation. Furthermore, by comparing the propolis sample to a blank sample, the ability of digested-fermented propolis to affect gut microbiota composition and create SCFAs was assessed.

***Results***

To evaluate how digestion and fermentation affect propolis, it was measured total polyphenol content and antioxidant properties before and after oro-gastro-intestinal digestion and fermentation. A Folin-Ciocalteu assay was used to assess the total polyphenol content in propolis before and after digestion (oro-gastro-intestinal) and fermentation, and two different methods were used to

estimate the antioxidant capacity of digested and fermented samples (FRAP, which measures $Fe^{3+}$ reduction, and DPPH, which measures antiradical activity). The Folin-Ciocalteu assay showed that the total polyphenol content of M.E.D.® propolis extract before the digestion process corresponds to 70.0 g of gallic acid equivalents/kg of propolis (corresponding to 111.2 g of galangine equivalents/kg of propolis). After the oro-gastro-duodenal process, propolis polyphenols underwent almost total degradation, with a total polyphenol content corresponding to 15.0 g of gallic equivalents /kg of propolis. As far as the fermentation process is concerned, the total polyphenol content decreased to 2.5, 2.9, 3.7, 2.1, and 0.9 g of gallic acid equivalents/kg of propolis, when gut microbiota from healthy adults, and healthy, allergic, obese, and celiac children were used for the fermentation, respectively.

The radical scavenging capacity of M.E.D.® propolis extract was tested against DPPH, a stable nitrogen synthetic radical, and was expressed as $TEAC_{DPPH}$. The DPPH assay showed that before the digestion-fermentation process, the propolis sample exerted radical scavenging ability corresponding to 158 g of Trolox /kg of propolis. After oro-gastro-duodenal process, $TEAC_{DPPH}$ decreased to a value corresponding to 8 g of Trolox /kg of propolis. Finally, $TEAC_{DPPH}$ decreased to 7.2 g of Trolox/kg of propolis and 6.9 g of Trolox/kg of propolis after fermentation with the gut microbiota isolated from fecal material of healthy adults and healthy children, respectively. $TEAC_{DPPH}$ resulted to correspond to 7.5 g of Trolox /kg of propolis, 4.6 g of Trolox /kg of propolis, and 8.5 g of Trolox /kg of propolis, after fermentation with fecal materials from allergic, obese, and celiac children, respectively.

The reducing power expressed as $TEAC_{FRAP}$ was determined through a FRAP assay. Before the digestion-fermentation process, $TEAC_{FRAP}$ was 142 g of Trolox /kg of propolis. After the digestion process, it decreased to 16 g of Trolox /kg of propolis. After the fermentation $TEAC_{FRAP}$ decreased to 7.3 g of Trolox /kg of propolis with fecal materials from healthy adults, 9.2 g of Trolox /kg of propolis for healthy children, 9.3 g of Trolox /kg of propolis for allergic children, 9.1 g of Trolox /kg of propolis for obese children, and 9.5 g of Trolox /kg of propolis for celiac children.

| **Assay** | ***In vitro* Digestion** | ***In vitro* Fermentation** | | | |
|---|---|---|---|---|---|
| | | **Healthy adults** | **Healthy children** | **Allergic children** | **Obese children** | **Celiac children** |
| FOLIN-CIOCALTEU (g of gallic acid equivalents/kg of propolis) | $15.0 \pm 2.3^{a}$ | $2.5 \pm 0.4^{b}$ | $2.9 \pm 0.3^{b}$ | $3.7 \pm 0.6^{b}$ | $2.1 \pm 0.2^{c}$ | $0.9 \pm 0.1^{d}$ |
| DPPH (g of Trolox/kg of propolis) | $8.0 \pm 1.7^{a}$ | $7.2 \pm 1.4^{a}$ | $6.9 \pm 1.1^{a}$ | $7.5 \pm 1.2^{a}$ | $4.6 \pm 0.5^{b}$ | $8.5 \pm 1.3^{a}$ |
| FRAP (g of Trolox/kg of propolis) | $16.0 \pm 2.1^{a}$ | $7.3 \pm 1.0^{b}$ | $9.2 \pm 1.8^{b}$ | $9.3 \pm 1.7^{b}$ | $9.1 \pm 1.6^{b}$ | $9.5 \pm 1.8^{b}$ |

Different letters within the same row indicates statistically significant differences ($p < 0.05$).

**Tab 11.** Antioxidant capacity of propolis after *in vitro* digestion-fermentation in healthy and diseased subjects.

SCFAs are metabolites produced by gut microbiota, mainly following the fermentation of dietary fibers and carbohydrates. After the digestion-fermentation process of the propolis sample and the blank sample, SCFAs were determined in the fermentation supernatant by a chromatographic method coupled with UV detection (HPLC-UV). The SCFAs identified in the samples

were acetic, propionic, and butyric acids, while succinic, isovaleric and pentanoic acids were not detected at concentrations within the limit of detection (LOD) of the applied method. Acetate, propionate, and butyrate were quantified, and the concentrations (mM) determined in the supernatant obtained from propolis fermentation were compared with blank concentrations. The results are reported in table 12**.**

| Subjects | Sample | Acetic acid | | Propionic acid | | Butyric acid | |
|---|---|---|---|---|---|---|---|
| | | (mM) | Increase | (mM) | Increase | (mM) | Increase |
| Healthy adults | Propolis | 316.3 ± 10.3[a] | 119% | 381.2 ± 12.8[a] | 832% | 29.3 ± 0.4[a] | 142% |
| | Blank | 144.4 ± 4.7 | | 40.9 ± 0.9 | | 12.1 ± 0.1 | |
| Healthy children | Propolis | 223.5 ± 7.6[b] | 33% | 198.7 ± 6.6[b] | 904% | 15.9 ± 0.2[b] | 47% |
| | Blank | 167.7 ± 5.4 | | 19.8 ± 0.3 | | 10.8 ± 0.1 | |
| Allergic children | Propolis | 335.9 ± 11.9[b] | 3226% | 694.6 ± 21.7[c] | 7289% | 10.2 ± 0.2[c] | 9% |
| | Blank | 10.1 ± 0.2 | | 9.4 ± 0.1 | | 9.4 ± 0.1 | |
| Obese children | Propolis | 135.5 ± 3.9[c] | 692% | 921,3 ± 30.9[d] | 7% | 113.2 ± 3.9[d] | 116% |
| | Blank | 17.1 ± 0.3 | | 859.3 ± 29.6 | | 52.4 ± 1.8 | |
| Celiac children | Propolis | 112.9 ± 3.9[d] | 5842% | 235.5 ± 7.3[e] | 1194% | 7.1 ± 0.3[e] | 145% |
| | Blank | 1.9 ± 0.1 | | 18.2 ± 0.2 | | 2.9 ± 0.1 | |

Different letters within the same column indicates statistically significant differences ($p < 0.05$).

**Tab.12** SCFA concentrations (mM) in healthy and diseased subjects.

The results suggest that propolis significantly increased the production of SCFAs in comparison to the blank sample, exerting a booster effect on SFCA producing bacteria. Generally, the increase in butyric acid production is lower than the increase induced for acetic and propionic acid concentrations by gut microbiota obtained from healthy and diseased subjects. In particular, in the presence of propolis, fecal bacteria of food allergic and celiac children significantly increased the production of acetic and propionic acids compared

to the blank sample, while butyric acid concentration significantly increased after the treatment of propolis with fecal materials obtained from celiac children but did not increase significantly after the treatment of propolis with bacteria from allergic children.

The Principal Component Analysis (PCA) was carried out for the exploratory analysis of SCFAs (figure 12).

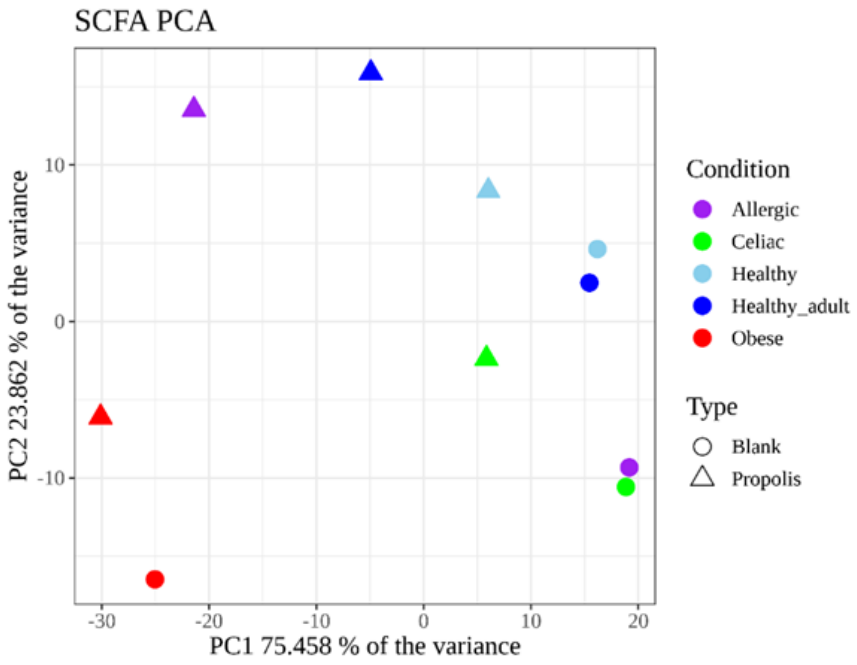



**Fig.12** Principal Component Analysis (PCA) for the exploratory analysis of SCFA

**Addition of propolis alters the production of SCFAs.** The figure shows a similar production of SCFAs between the healthy children and the healthy adults, as well as between celiac and allergic children. The obese children however, showed a rather different SCFA profile than the others.

After the fermentation, supernatants were submitted to antioxidant assays and to the determination of SCFAs, while the solid residues of each sample were analyzed using RT-PCR for the determination of gut microbiota after treatment with the propolis sample, compared with the blank sample. Different kinds of analysis were performed to evaluate the alteration of gut microbiota, such as the Principal Coordinates Analysis (PCoA) with the Bray-Curtis dissimilarity index, which was carried out for the exploratory analysis of 16S rRNA sequencing data (figure 13)

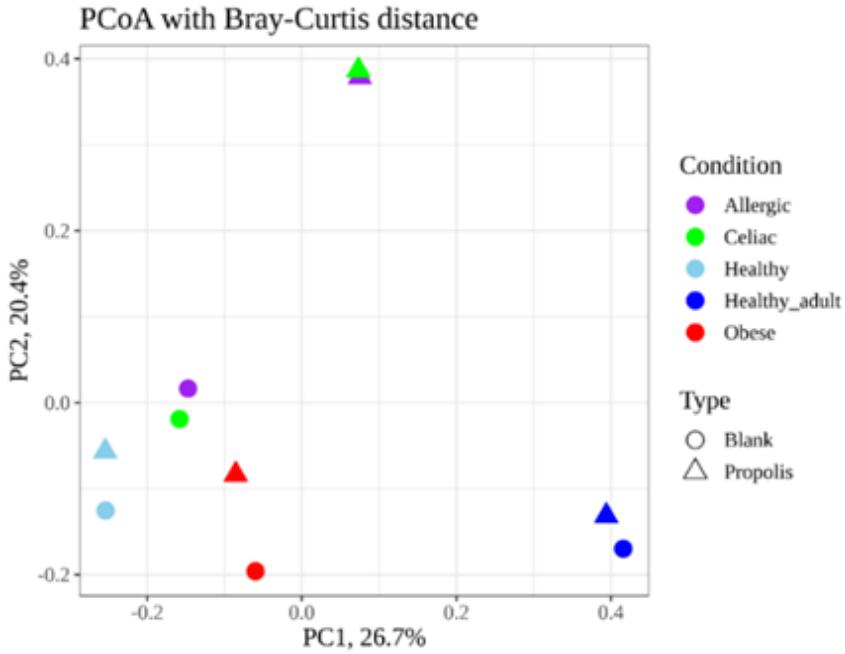



**Fig. 13** Principal Coordinates Analysis (PCoA) with the Bray-Curtis dissimilarity index for the exploratory analysis of 16S rRNA sequencing data.

As can be observed from the PCoA, variations in microbial communities were greater between samples than because of adding propolis, except in the case of celiac and allergic children. In both cases, the addition of propolis caused a deeper change in the microbial community than for the other groups.

A Coinertia analysis was carried out as an interpretative method. This method tries to find associations between two sets of variables, in this case the microbial community structure and SCFA. It shows a correlation between the metabolites PCA and the microbial PCoA. The strength of the association found through Coinertia analysis is measured via the RV coefficient. It is a number between 0 and 1, with higher values representing a stronger association (figure 14).

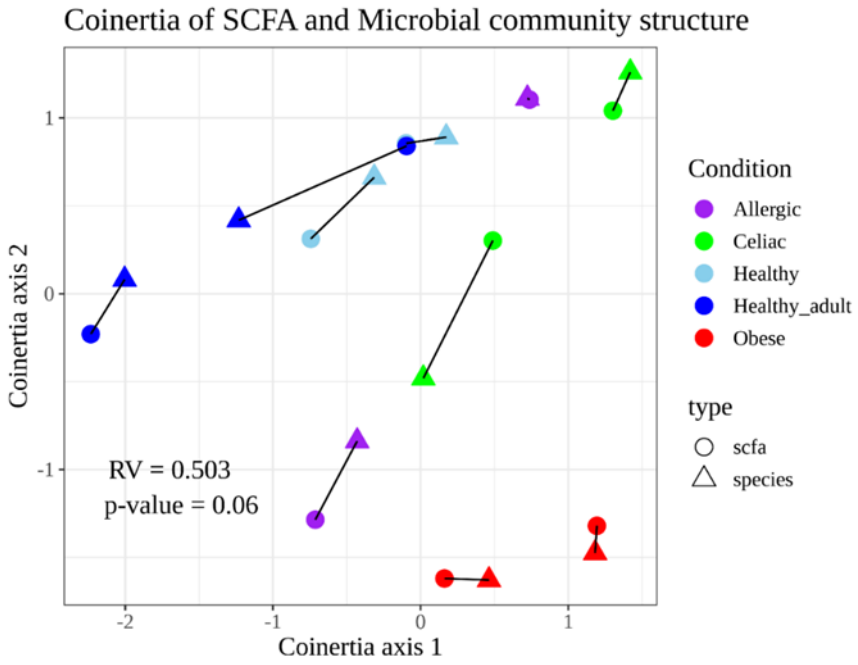



**Fig. 14** The Coinertia analysis to find associations between the microbial community structure and SCFA.

There are two sets of samples for each group: SCFAs vs microbial population for blanks, and SCFAs vs microbial population for propolis. As it can be seen in figure 14, there is a mildly strong, though not statistically significant, correlation (RV = 0.503; p = 0.06) between the SCFA-based PCA and the genus- based PCoA ordinations, since the distance between the same samples (depicted with an arrow) is usually less than between different samples. **This indicates that SCFAs and gut microbial composition analyses support each other, and when an arrow is shorter for one set of sample respect another set, it means that the correlation of PCA and PCoA, is bigger in the first than in the second.**

The figure 15 represents the increase or decrease of specific bacteria strains after administration of propolis. A log2 fold increase of each bacterium with respect to the blank was calculated as in the following example: log2 (Bacteroides with propolis / Bacteroides blank), or in words, only those bacteria with a log2 fold increase greater than 2 were considered. A green signal means that the propolis increased the relative abundance of that specific bacteria, whereas a red signal means a decrease in the relative abundance of specific bacteria with propolis.

The minimum increase or decrease, that we take into consideration, is fourth time; even if all the microbial population increase or decrease if we use, or not, the propolis.

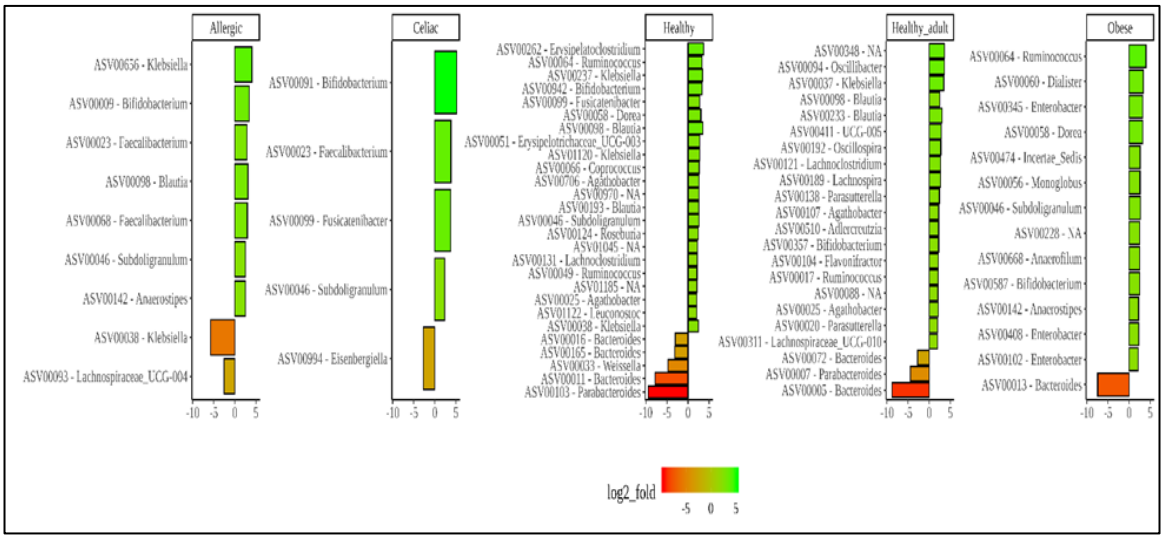



**Fig.15** Alteration of specific bacteria strains after propolis administration.

### *Discussion and conclusions*

The antioxidant profile of a standardized polyphenol combination isolated from poplar-type propolis (propolis sample) was determined after it was put through a simulated oro-gastro-intestinal digestion. The digested sample was then submitted to fermentation using fecal material from five different donors: healthy adults, and healthy, obese, celiac and food allergic children. Then the digested-fermented propolis sample was analyzed to determine its bioactivities, such as antioxidant properties, ability to modify gut microbiota (gut microbial composition) and activity in terms of SCFA production and compared with those of the blank sample submitted to the same digestion-fermentation process.

Our findings revealed that fermentation causes further polyphenol degradation, with slight variations depending on the origin of the gut microbiota used to perform the fermentation. This is likely due to the different ability of microorganisms found in feces to use propolis polyphenols as substrates for their metabolism. Regarding the antiradical activity of propolis and the reducing power of propolis before digestion, **our results confirmed the high antioxidant potential reported by a large body of evidence.**

As far as SCFAs are concerned, fermentation of dietary polysaccharides (that are not otherwise digested) is the pivotal role of gut microbiota, where enzymes derived from microflora digest soluble fibers into SCFAs (acetate, propionate, and butyrate). SCFAs are absorbed in the intestine and used as energy by the host. They exert regulatory functions on gut physiology, metabolism, and immunity and act as regulators of energy intake and inflammation.

As expected, our results showed that the total concentration of SCFAs is higher in the supernatants of blank samples obtained from the fermentation induced by fecal materials of healthy adults and children (197.4 mM and 198.3 mM, respectively), while it is lower in the supernatants corresponding to allergic and celiac children (28.9 mM and 23.0 mM, respectively). In addition, as shown by PCA, SCFA production by healthy adults and children is similar, as is the SCFA production by other subjects (celiac and allergic children), while obese children showed a different trend. In particular, the concentrations of propionic acid and butyric acid, determined in the supernatant obtained from the fermentation process of a blank sample induced by the gut microbiota of obese children, are 20 to 40 times higher for propionic and about 5 times higher for

butyric, in comparison with the concentration determined in healthy subjects. With regards to the influence of propolis on SCFA production**, propolis exerted a beneficial effect leading to an increase in total SCFA production**, especially in allergic and celiac children. These findings suggest that different bacteria strains are impacted by propolis in different ways, producing SCFAs to varying degrees. This increase could be due to the increased relative abundance and/or activity of SCFA-forming bacteria, as well as the synthesis of SFCAs utilising propolis components as substrates. While propolis had a large booster impact on the production of SCFAs when interacting with the gut microbiota of obese children, it did not cause a significant increase in propionic acid concentration. The gut microbiota is important for human health and the prevention of a variety of diseases, including inflammatory bowel disease, celiac disease, obesity, and metabolic disorders. Many endogenous and external variables determine the composition of the gut microbiota (i.e., age, body mass index, healthy status, environmental factors, diet, stress, drugs). Microbiota community structure in fermented propolis was assessed by comparison to blank samples using high-throughput sequencing of the 16S rRNA gene to assess the influence of propolis on gut microbiota composition. The results suggest that propolis fermented with gut microbiota obtained from healthy subjects (i.e., adults and children) and obese children decreased the abundance of *Bacteriodes* and increased the level of a variety of beneficial microrganisms including *Firmicutes* (*Ruminococcus*, *Dorea*, *Roseburia*) and Actinobacteria (*Bifidobacterium spp*.). Although it is not entirely correct to speak about beneficial and harmful gut microrganisms, because some species

could be beneficial or harmful depending on the habitat, which may determine different behaviors, the modifications of gut microbiota composition induced by propolis, especially when the obese child microbiota is considered, suggest that propolis could improve dysbiosis. In fact, a large body of evidence supports that dysbiosis consists in an overall reduction of beneficial bacterial species (i.e. lactobacilli and bifidobacteria) which use fiber and increase the production of SCFAs, such as butyrate (which is beneficial for human colonocytes, enhancing intestinal epithelia cell barrier function and immune function), and an increase in Bacteroides and other putrefactive bacteria which generally result from a high fat diet rich in animal foods, which produce metabolites (i.e. ammonia, amines, and phenols) that negatively affect gastrointestinal and systemic health. At the genus level, propolis fermented with the gut microbiota of obese children resulted in the increase of some genera such as *Dialister, Subdoligranulum* and *Anaerostipes*. As far as the level of *Dialister* is concerned, high levels are associated with lower BMI and weight reduction. In our study, further beneficial bacteria belonging to the *Subdoligranulum* genus were increased by propolis fermented by gut microbiota of obese children. Many studies have shown a positive association between high *Subdoligranulum* abundance and a healthier metabolic status (i.e., fecal microbiota richness, high HDL-cholesterol and adiponectin levels, and low-fat mass, adipocyte diameter, and low levels of leptin, an adipokine produced by adipocytes, and inflammation markers such as CRP and IL-6). When propolis was fermented by the gut microbiota of obese children, it increased the level of genus *Anaerostipes*, which is one of the butyrogenic

bacteria in the healthy microbiota that decreases in type 2 diabetes patients. As far as the influence of propolis on the gut microbiota composition of celiac children is concerned, the most interesting result is an increase in levels of *Bifidobacterium,* and of the genera of *Faecalibaterium,* which belongs to the Firmicutes Phylum and the Ruminococcaceae Family*,* and F*usicatenibacter*, belonging to Firmicutes Phylum and Lachnospiraceae Family. With the increasing *Bifidobacteria*, propolis leads to a microbiota profile and SCFA production similar to those of healthy children. Moreover, the increase in the levels of butyrate producers (i.e., *Faecalibaterium* and F*usicatenibacter*) can reduce the chronic inflammation caused by these autoimmune disorders.

Finally, propolis fermentation had a modulatory influence on gut microbiota in both healthy and diseased patients, increasing the concentration of SCFAs, indicating an increase in the development of SCFA-producing bacteria. Therefore, **all the results suggest that propolis might contribute to gut health and could be a candidate for use as a prebiotic ingredient of food supplements or bioactive products in drugs, with the aim of prevention and treatment against chronic disorders.**

## CONCLUSIONS

My project started at the end of 2018 with the first approach to this natural and heterogeneous product called propolis. At the beginning the extraction process was optimized in order to obtain a standardized product with a constant of polyphenols concentrations. During the PhD course, I spent a period in B Natural company in Milan (Italy) and I saw and performed the process and the analysis regarding the Multi Dynamic Extraction (M.E.D.®). This allowed me to obtain in each propolis extract sample a specific range concentration of six pholyphenols (galangin, crhysin, pinocembrin, apigenin, pinobanksin and quercetin) having a relative concentration in the extract of about 25-50% (w/w).

On this extract we performed different investigations, starting from the evaluation of all the component present in the extract through the UV, HPLC and Mass Spectrometry, to the biological properties: antibacterial, antimycotic and antiviral.

**These biological studies highlighted the optimal propolis antimicrobial activity** (especially on the bacteria strains involved in the developing of pathologies and not on the beneficial strains like Lactobacilli and Bifidobacteria).

After the *in vitro* evaluation of the antimicrobial properties, with a Cooperative of physicians, we performed a clinical trial, to evaluate the propolis activity in the remission of the symptoms of upper respiratory tract infections (URTIs). Also, in this case the results highlighted the beneficial effect of the standardized propolis oral spray. In fact, **propolis oral application reduces the time for**

**remission of symptoms** and can be used to improve both bacterial and viral mild uncomplicated URTI symptoms in a lower number of days without the use of symptomatic treatment leading to a more immediate resolution.

At the end of my doctorate, in 2020/21, I spent six months in the CIBM (Centro de Investigacion Biomedica) in Granada (Spain). There, I evaluate the activity of propolis standardized product (ESIT 12®) on the gut microbiota. We evaluated the production on SCFAs (metabolites involved in many activities like immunomodulatory, antioxidant, anti-inflammatory and others) and the improve or decrease of the bacteria strain that compose the gut microbiota.

A great production of SCFAs like acetate, butyrate, and propionate respect to the control sample was found.

Regarding gut microbiota there was a decreasing of different kind of pathologic strain, but, the most important aspect was the increasing of Lactobacillus and Bifidobacterium, the probiotic strain.

In conclusion, the obtained results confirm the beneficial effect and safety of propolis for different aspects of human life: starting from the prevention and maintaining of physiological homeostasis, to the treatment of the mild form of upper respiratory tract infections.

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