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Chemical composition and nutraceutical properties of beehive products: focus on propolis

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PREFACE

This thesis was carried out within the project called "Dottorato di ricerca in apprendistato di alta formazione" founded by Regione Lombardia and shared between University of Pavia and B Natural srl.

The goal of this cooperation was the study of healthy benefits of bee products, especially propolis, and future perspectives of propolis obtained using a standardize extraction method. To achieve these aims, the industrial research was performed in B Natural and the scientific investigations were performed at the University of Pavia.

Nowadays propolis is used in the drug or food manufactures as both single matrix and ingredient in combination with other compounds and mixtures. In current opinion, propolis seems to be safe and less toxic than many synthetic medicines, however its chemical composition is variable and difficult to standardize. To demonstrate the safety and the activity of this matrix several standardized manufacturing processes, quality controls, and good designed clinical trials should be performed.

In light of this, this research combined the deep knowledge of B Natural in propolis extraction and the knowhow of the laboratory of Food Chemistry and Nutraceutical products of the Department of Drug Sciences, Section of Medicinal Chemistry and Pharmaceutical Technology of the University of Pavia to determine the chemical composition of propolis extracts rich in polyphenols and to study the role of the active compounds in biological systems using *in vitro* and *in vivo* studies.

The first scientific work to investigate the chemical composition and properties of propolis was published in 1908 (Hanses, 1908), while the first patent was

posited in 1904 (USA—Composition for treating pins and piano strings). After a century from the first scientific paper, the number of publications on propolis is more than 2,500 papers and 2,800 patents.

Since propolis has been well known since ancient times, it represents a good chance for pharmaceutical and nutraceutical industries. To preserve the inventions and improve the profit deriving from R&D studies, many industries have submitted and obtained several patents.

China, Japan and Russia are the countries owner of the major part of the patents probably because these countries are the main producers and consumers of propolis worldwide. Today, about 40% of patents are Chinese and the first Chinese one was published in 1993. It was founded on the "Process for production mouth freshener". There are about 15% of Japanese patents, and the first appeared in 1988 ("Deodorants controlling mouth odor"). The first patent was obtained in 1968 on Russian "Toothpaste". Russian patents represented about 12% of patents. Brazil deposited its first patent in 1997 on "Dental gel."

Patents found applications in products with medicinal and nutraceutical properties and with dermatological applications and the scientific production on propolis and healthy patents have been improving. Japan imports almost all the propolis used in the country: 10% derives from China and 80% from Brazil. Brazilian propolis has extensive use in foods and beverages in Japan, to maintain or improve human health.

These data confirm the commercial and scientific interests on propolis.

B Natural srl, the European leader in production of propolis extracts, invests many resources in R&D to launch new products, to study their biological activites and to patent improved extraction methods and new applications.

ABSTRACT

Propolis is a natural and resinous product that bees (*Apis mellifera*) collect from gems, exudates and plants, resulting in a heterogeneous mixture of many substances harvested, processed and used by bees to close hive holes and to protect it. The chemical composition of propolis varies according to botanical origins, vegetal sources and the extraction methods. Among the most representative metabolites of propolis there are flavonoids, terpenoids, phenolic acids, phenolic esters and sugars in different proportions. In literature there are hundreds of studies supporting the healthy properties of propolis, such as gastroprotective, hepatoprotective, immunomodulatory, wound healing, antidiabetic and antineoplastic activities. These propolis properties are ascribed to three main activities namely antioxidant, anti-inflammatory and antimicrobial. Despite the great number of investigations, the common scientific approaches to study biological activities of propolis present some limitations due to the high natural variability of propolis and to the different used extracts. Therefore, the results obtained so far are often not comparable each other and are poorly reproducible.

The research of this project on propolis is based on the collaboration between B Natural srl and the University of Pavia to develop a new extraction method to obtain standardized propolis extracts to be studied *in vitro* and *in vivo* for the determination of anti-inflammatory, anti-oxidant and antibacterial activities.

The standardized extraction method was set and posited as patent and the extracts obtained were used for the experiments. In particular it was demonstrated that the molecular mechanisms behind the anti-inflammatory and anti-oxidant activities involved miRNA epigenetic changes *in vitro*. Preliminary results demonstrated *in vivo* the antioxidant activity of propolis chronic administration. Moreover, *in vitro* experiments confirmed the strong propolis activity on a wide variety of bacteria and fungi spp.

In conclusion, the results of this research were obtained from standardized extracts leading to results that can be shared within the scientific community since the extracts are reproducible even starting from different raw materials adding a new approach to study propolis.

ABBREVIATIONS

1,1-diphenyl-2- picrylhydrazyl (DPPH)

2,2′-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT)

Adenosine Triphosphate (ATP)

American Type Culture Collection (ATCC)

Antioxidant response element (ARE)

Area Under The Curve (AUC)

ATP-binding cassette transporter (ABCA)

Bicinchoninic Acid Assay (BCA)

Body Surface Area (BSA)

Bovine Serum Albumin (BSA)

Brucella Blood Agar (BBA)

Caffeic Acid Phenethyl Ester (CAPE)

Catalase (CAT)

Catechol-O-methyltransferase (COMT)

Cells Presenting the Antigen (APC)

Cytosolic b-glucosidase (CBG)

Dimethyl sulfoxide (DMSO)

DNA copy (cDNA)

Dulbecco's Modified Eagle Medium (D-MEM)

Ethanol Propolis Extracts (EEP)

Fetal Bovine Serum (FBS)

Flavin Adenine Dinucleotide ($FADH_2$)

Glutathione Peroxidase (GPX)

Glutathione Peroxidase 2 (GPX2)

Glutathione Synthetase (GSS)

Glycopeptide-Intermediate Resistant (GISA)

High Density Lipoprotein (HDL)

Human keratinocyte cell lines (HaCaT)

Inducible Nitric Oxide Synthase (iNOS)

Interferon (IFN)

Interleukin (IL)

Manganese Superoxide Dismutase (MnSOD)

Messenger RNA (mRNA)

Methicillin-Sensitive *S. aureus* (MSSA)

MicroRNA (miRNAs)

Minimum Inhibitory Concentration (MIC)

Mitogen-Activated Protein Kinase (MAPK)

Muller Hinton Agar (MHA)

Multi Dynamic Extraction (M.E.D.®)

Natural killer (NK)

Nicotinamide Adenine Dinucleotide (NADH)

Nitric Oxide (NO)

Noncoding RNA (ncRNA)

Nuclear Factor kB (NF-kB)

Nuclear Factor, Erythroid 2 like 2 (NFE2L2)

Oxygen Radical Absorbance Capacity (ORAC)

Penicillin Resistant (Pen-R)

Phloridizin hydrolase (LPH)

Phosphate buffer saline solutions (PBS)

Polymerase II (Pol II)

Quantification cycle (Cq)

Reactive Nitrogen (RNS)

Reactive oxygen (ROS)

RNA-induced silencing complex (RISC)

Room Temperature (RT)

Sabouraud Dextrose Agar (SAB)

Serine/Threonine Kinase 2 (ERK2).

Sulfotransferases (SULT)

Supercritical Fluid Extraction (SFE)

Superoxide Dismutase (SOD)

Thioredoxin Reductase 2 (TRXR2)

Tod Hewitt Agar (THA)

Toll Like Receptor 2 (TLR)

Tumor Necrosis Factor alpha (TNF-α)

Ultra Violet (UV)

Untranslated region (UTR)

Uridine-5’-iphosphate glucuronosyltransferases (UGTs)

INTRODUCTION

Propolis: what is it?

Propolis or bee resin is the generic name used to identify a natural and resinous product that bees (*Apis mellifera*) collect from gems, exudates and plants such as birch, poplar, pine, alder, willow, palm, *Bacchàris dracunculifolia* and *Dalbergia ecastaphyllum* (Park et al., 2004; Daugsch et al., 2008; Castaldo et al., 2002). Propolis is a heterogeneous mixture of many substances harvested, processed and used by bees to close hive holes and to protect it (Burdock et al., 1998). Recent studies have revealed that bee propolis can play an important role in the colony's immunity and in direct defense against pathogens and intruders (Simone-Finstrom et al., 2010; Simões et al., 2004).

The activity of propolis against microorganisms has been used since antiquity (Bankova et al., 2000). Propolis and the other beekeeping products such as honey, royal jelly and pollen have important biological properties, and have been used since 300 a.C. in many traditional medicines (Bankova et al., 1989).

Thus, propolis has become popular as an alternative medicine for improving health and preventing diseases (Teixeira et al., 2010). Several biological properties have been attributed to this natural product such as antioxidant, hepatoprotective, anti-tumor, anti-inflammatory and antiparasitic activities (Burdock, 1998; Banskota et al., 2001; Pontin et al., 2008; Viuda-Martos et al., 2008). 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 (Gómez-Caravaca et al., 2010), in cough syrups, oral pills, pads,

ointments, lotions and food supplements against viral diseases, fungal infections, ulcers and burns (Scheman et al., 2008).

Propolis physical properties

Propolis is harvested by bees by a large variety of trees and shrubs. 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 color and smell of propolis (Krell, 1996). Propolis is a lipophilic, hard and fragile material that when heated becomes soft, flexible, gummy and very sticky (Hausen et al., 1987). 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 is between 96 °C and 100 °C (Krell, 1996). Propolis is poorly soluble in water and partially soluble in alcohol, acetone, ether, chloroform and benzene. Only a suitable mixture of solvents with different apolarity can dissolve most of its components (Raoul, 1992). The insoluble part is made of vegetal tissues, pollen, debris and cuticles of silk bees (Debuyser, 1984). The color range varies from brown to yellow to green-brown or red-brown to dark red depending on the botanic and geographic origin (Bankova et al., 2005).

Propolis chemical properties

Propolis typically consists of resin (40-60%), waxes (20-40%), essential oils (10%), pollen (5%) and other organic compounds (5%) (Juliano et al., 2007; Popova et al., 2010b). More than 300 compounds of different origins have been identified in propolis (Marcucci et al., 1995; De Castro et al., 2001; Banskota et al., 2000; Alencar et al., 2007) as fatty acids and phenols, esters, substituted

phenolic esters, flavonoids (flavones, flavanones, flavonols, diidroflavonols and calcones), terpens, betasteroids, aromatics aldehydes and alcohols, sesquiterpens, derivates of naphthalene and stilbene (Aga et al., 1994; Bankova et al., 2000; Marcucci et al., 1995). Propolis also contains vitamins, including B1, B2, B6, C and E, amino acids of bees' metabolism (Marcucci et al., 1995; Attia et al., 2014) mineral salts, such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe (Lotfy, 2006) and heavy metals as Cd, Hg, and Pb (Cvek et al., 2008).

The first studies on the chemical characterization of propolis date back to the beginning of the 20th century (Kuropatnicki et al., 2013). However, the chemical composition may vary according to vegetal sources and harvesting season (de Sousa et al., 2007); for this reason the propolis standardization is complex (Chang et al., 2008).

The propolis compounds have three origins:

1) substances secreted from the plants as wound exudates among other lipophilic materials on leaves and leaf buds, resins, mucilage, gums and lattices which are harvested by bees;

2) substances secreted by bees;

3) materials introduced during the preparation of propolis (pollen, waxes and honey) (Marcucci et al., 1995; Bankova, 2005a).

All these factors contribute to the complex chemical composition of propolis. The plant source and geographic origin influence the main bioactive compounds present in propolis as shown in table 1.

Geographic origin	Plant source	Main bioactive compounds
Europe, North America, nontropic regions of Asia	*Populus spp* most often *P. nigra L*.	Polyphenols
Russia	*Betula verrucosa*	Polyphenols
Brazil	*Baccharis spp*	Prenylated p-coumaric acids, diterpenic acids
Cuba, Venezuela	*Clusia spp*	Polyprenylated benzophenones
Pacific region	Unknown	C-prenylflavanones
Canary Islands	Unknown	Forfuran lignans
Kenya	Unknown	Polyphenols
Greece and Cyprus	Unknown	Flavonoids, terpens

Tab.1. Types of propolis, their origins and chemical compositions. Adapted from Miguel & Antunes, 2011.

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 main constituents of European propolis with a number of biological activities including 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, prenilated phenylpropanoids, such as artepillin C, and diterpenes are the main components (Fernandes-Silva et al., 2013).

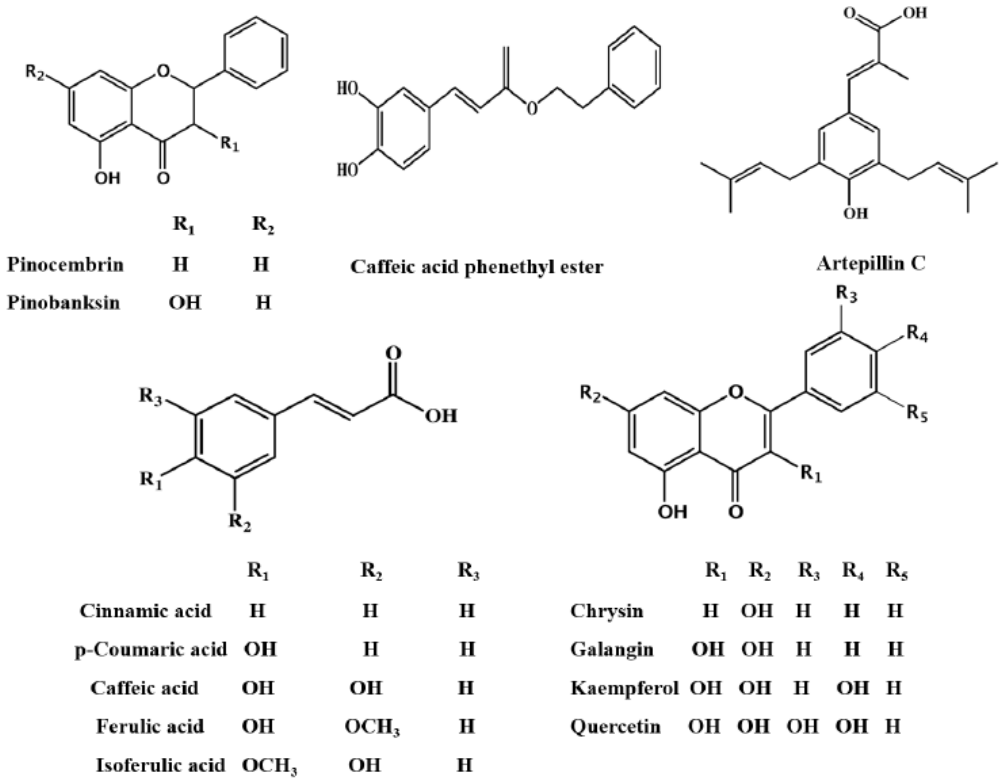

Fig. 1. Representative chemical components in propolis (Huang et al., 2014).

Botanical origin of propolis

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 (Kosalec et al., 2004; Burdock et al., 1998; Teixeira et al., 2005).

In Europe bees collect resin mainly from poplar plants, producing the so called brown propolis. Conversely, in the tropics, poplars are rarely cultivated and alternative plants are used as resin source such as *Baccharis dracunculifolia* that has been described as the most important vegetal source of Brazilian South propolis, which is called green propolis for its color (Leitão et al., 2004; Sousa et al., 2007).

Brazilian Green Propolis

Brazil is characterized by temperate, subtropical and tropical areas with a wide biodiversity (Salomão et al., 2004). For this reason, twelve different types of propolis have been recognized according to their composition and botanical origin: five from the southern, six from the northeast and one from the south-east (Bankova et al., 2000; 2010; Park et al., 2002). It has been suggested that *Hyptis divaricata* is the source of northeastern propolis resin, *B. dracunculifolia* of south-east propolis and *Populus nigra* of southern propolis. Other sources of Brazilian green propolis are: *Araucaria heterophylla*, *A. angustifolia, Clusia maggiore* and *Eucalyptus citriodora* (Banskota et al., 1998; Salatino et al., 2005). Due to the Brazilian biodiversity, it is not realistic to correlate a given type of propolis with only a certain type of plant resin source. Instead, it is reasonable to consider that some plants are used by bees as predominant source

of a certain type of propolis. This observation is in agreement with different chemical and physical profiles found analyzing propolis (Salatino et al., 2005).

In recent years, a new type of propolis from the northern part of the country has been identified, not included in the previous twelve classes: red propolis, which has only been studied since 2006 (Trusheva et al., 2006), characterized by a high presence of isoflavones (Silva et al., 2008; Daugsch et al., 2008). Although Brazilian green and red propolis are collected from the same bee specie, the Africanized *A. mellifera* (Teixeira et al., 2005; Daugsch et al., 2008), they differ each other for the plant sources and, as consequence, for the main polyphenolic species, respectively: *B. dracunculifolia* is rich in prenylated phenylpropanoids and *Dalbergia ecastophyllum* is rich in isoflavonoids (Huang et al., 2014).

Resins belonging to *B. dracunculifolia* have been found in large quantities and with a relative stable chemical profile both in dry and rainy seasons (Park et al., 2004). In Brazilian green propolis, the most common components are polypropylene prenilated, especially prenilated cinnamic acids, which are also studied for their antimicrobial activity and toxicity against cancer cells (Salatino et al., 2005; Salomao et al., 2004) (figure 2). Among these, there is artpillin C (3,5-diprenyl-4-hydroxycinnamic acid), a low molecular weight molecule, absent in European, North American, New Zealand, Argentine and Chilean propolis (Kumazawa et al., 2004). Green propolis contains a high concentration of artepillin C from 2% to 12% according to harvesting season (Marcucci et al., 2001; Jorge et a., 2008; Park et al., 2004). Prenilated cinnamic acid has proved to be characteristic of green propolis and it may also be present as esters, such as 3 prenyl-cinnamic allyl esters (Negri et al., 2003). Although

not abundant, there are also flavonoids in green propolis as isosacuratenin and trace of kaempferol (Gardana et al., 2007; Park et al., 2002).

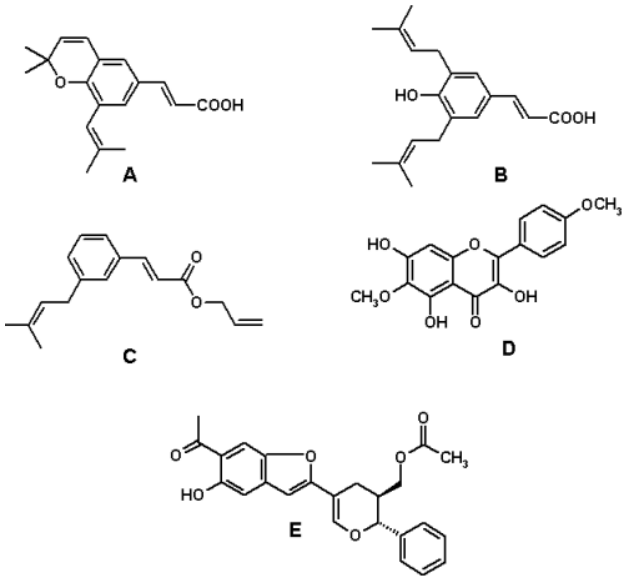

Fig.2 Structures of phenolic compounds isolated from green propolis.
(A) 2,2-Dimethyl-8-prenylchromene; (B) 4-hydroxy-3,5-diprenyl cinnamic acid (artepillin C); (C) 3-prenyl cinnamic acid allyl ester; (D) kaempferide; (E) propolis benzofuran A. (Salatino et al., 2005).

Some other compounds of Brazilian green propolis are mono and sesquiterpenes which contribute to the characteristic resinous odor and antimicrobial activity; clerodane diterpenoids contribute to the anticancer activity; non-volatile sesquiterpenes such as dehydrocostus lactone (Negri et al., 2003); triterpenoids, some of which are largely present in plants and long chain fatty acid esters (Furukawa et al., 2002). Many studies on chemical composition of different samples of green propolis show a variation in the

proportion of mevalonate derivatives (terpenoids, sesquiterpenoids, and triterpenoids) and shikimate derivatives (phenolic, prenylated or not) (Salatino et al., 2005). This variation could influence the physical properties such as texture and color of propolis. In fact, the soft appearance increases as the level of triterpenoids increases and the amount of shikimic derivatives decreases (Valko et al., 2006). Only few studies have been carried out on volatile compounds which varies according to flora, season and bee species (Gasparro et al., 1998, Morton et al., 1987; Tsai et al., 2005).

Brown Propolis

Poplar propolis is also known as brown propolis. The poplar tree is common in Europe, and the name of this plant is used to indicate the common type of propolis rich in flavonoids and phenylpropanoids. However the propolis collected from many other countries than Europe could have similar chemical profiles to the poplar one; in particular those collected from China (Ahn et al., 2007), Korea, Croatia (Kosalec et al., 2003), Taiwan (Chen et al., 2003; 2004; Huang et al., 2007), New Zealand (Markham et al., 1996) and Africa (Hegazi et al., 2002). The reason of this similarity is the common presence of flavonoids even in plants native of these other countries (Li et al., 2010) (table 2). The principal sources of propolis from temperate zone are Populus spp (*P. alba*, *P. tremula* and *P. nigra*). In Europe, there are other important plant sources of resins such as *Betula pendula*, *Quercus,* Acacia spp, *Aesculus hippocastanum, Alnus glutinosa,* Ulmus, Picea, Fraxinus, Pinus spp and *Salix alba* (Salatino et al., 2005; Rai et al., 2012).

The most popular specie of honeybee, so called European honeybee, is the *A. mellifera*. Honeybees collect lipophilic plant substances from buds, lattices, mucilage, leaves, branches and barks to produce propolis, and melt them with beeswax in the hive. In the temperate zone, this harvesting takes place from spring to late summer in the warm part of the day. Only few honeybees collect resins (about 10 mg of resins per bee per flight) (Meyer, 1956).

Brown propolis is composed by resin and vegetal balsam for the 50%, waxes for 30%, essential and aromatic oils for 10%, pollen for 5%, and other substances for 5% including amino acids, vitamins and mineral salts (Huang et al., 2014). As regards the chemical profile, the brown propolis is a

heterogeneous material containing more than 300 compounds (De Groot et al., 2014). In particular 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 (De Groot, 2013).

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 (Huang et al., 2014).

Phenolic compounds	Geographical origin
Flavonols	
Quercetin	China, Serbia, Italy, Slovenia
Kaempferol	Serbia, Italy, Slovenia
Isorhamnetin	China, Serbia, Italy
Kaempferide	Serbia, Italy, Slovenia
Bis-methylated quercetin	Serbia, Italy
Quercetin-methyl ether	China, Serbia, Italy
Flavanonols	
Pinobaskin	China, Serbia, Italy, Slovenia, Germany
Pinobaskin-5-methyl-ether-3-O-acetate	Serbia, Italy
Pinobaskin-5-methyl-ether	Serbia, Italy
Pinobaskin-3-O-acetate	China, Serbia, Italy, Slovenia, Germany
Pinobaskin-3-O-propionate	Serbia, Italy
Pinobaskin-3-O-buyrate	China, Serbia, Italy
Pinobaskin-3-O-pentenoate	China, Serbia
Pinobaskin-3-O-pentanoate	Serbia, Italy
Pinobaskin-3-O-hexanoate	Serbia, Italy
Flavones	
Luteolin	Serbia, Italy, Slovenia
Apigenin	China, Serbia, Italy, Slovenia
Chrysin	China, Serbia, Italy, Slovenia, Germany
Metoxyl-chrysin	Serbia, Italy
Flavanones	
Naringenin	Serbia, Italy, Germany
Liquiritigenin	Serbia, Germany
Pinostrobin	Serbia, Germany
Pinocembrin	China, Serbia, Italy, Slovenia, Germany

Tab. 2 List of flavonoid compounds commonly present in the brown type of propolis. Adapted from Ristivojević et al., 2015a.

Phenolic glycosides (sugar conjugates) are poorly identifiable in propolis due to the lipophilic character of the resin and the hydrolysis process occurring during the propolis collection made by β-glucosidase: for these reasons the majority of the flavonoids found in propolis are aglycones with the exception of 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 (Zhang et al., 2012). Several authors indicated sugar conjugates as possible specific markers to identify the botanical origin since they are characteristic of brown propolis (Ristivojević et al., 2015b; Falcão et al., 2013). 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 (Falcão et al., 2013).

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 similar to 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 (Peev et al., 2009).

Waxes and hydrocarbons represent 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. In particular essential oils (from 1 to 3%) are responsible for flavor and scent. Most of them originate from the poplar buds or from other exudates (Jerković et al., 2003; Milos et al., 2002), while others are

found only in brown propolis but not in the balsamic fraction of poplar buds (Jerković et al., 2003). The main components of these oils 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 (Bankova et al., 2014; Borcic et al., 1996). Areas characterized by coniferous plants such as Greece, Croatia and Estonia produce propolis rich in monoterpenes like α- and β-pinene, limonene and eucalyptol (Borcic et al., 1996; Kaškoniene et al., 2014). 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 (Miguel et al., 2013).

Propolis from Sicily and northwestern Greece, as well as from Croatia and Malta contain mainly diterpenes and almost no phenolics (Popova et al., 2010b; Trusheva et al., 2003; Melliou et al., 2004). Since the Mediterranean area is rich in Coniferans spp (Cupressaceae), it could be possible to identify the botanical origin of propolis analyzing the diterpenic profile (Popova et al., 2010a).

The propolis produced in Iran contains mono- and sesquiterpene esters of benzoic acids with the predominance of flavonoids and caffeate ester (Trusheva et al., 2003; Tukmechi et al., 2010). This is principally due to the simultaneous use of Populus and Ferula spp as vegetal sources.

South American propolis, in particular the Uruguayan one, presents a chemical profile similar to those of European and Chinese propolis deriving from the same plants (Kumazawa et al., 2006). 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 in particular ascribable to flavonoids and other phenolic acids (Banskota et al., 2001; Bueno-Silva et al., 2013, Nijveldt et al., 2001). Moreover, the concentration of polyphenols reflects the quality of the propolis (Zhang et al., 2014).

EXTRACTION METHODS

Propolis cannot be used in drugs or food supplements as crude material since it should be purified from undesired and inert materials preserving in the meantime the active components. In literature many extraction methods using different solvents are reported: water, absolute ethanol, ethanol-water mixtures (80, 90, and 96%), glycerol, methanol, hexane, acetone, dimethyl sulfoxide (DMSO) and chloroform (Gómez-Caravaca et al., 2006; Miguel et al., 2010; Netı´kova' et al., 2013; Sforcin & Bankova, 2011) (figure 3).

Water	Methanol	Ethanol	Chloroform	Dichloromethane	Ether	Acetone
Anthocyanins, starches, tannins, saponins, terpenoids, polypeptides, and lectins	Anthocyanins, terpenoids, saponins, tannins, xanthoxyline, totarol, quassinoids, lactones, flavones, phenones, polyphenols, polypeptides, and lectins	Tannins, polyphenol, polyacetylenes, terpenoids, sterols, and alkaloids,	Terpenoids, flavonoids	Terpenoids, tannins, polyphenols, polyacetylenes, sterols, and alkaloids	Alkaloids, terpenoids, coumarins, and fatty acids	Flavonols

Fig. 3 Different solvents used for the extraction of propolis (Wagh, 2013).

However, the most used is ethanol because the obtained extract shows low concentrations of waxes and high concentrations of biologically active compounds (Pietta et al., 2002).

According to this extraction method, the propolis should be kept overnight in freezer (−20 ˚C). After this step, the frozen propolis is milled (particle size of about 10–80 μm) and incubated with 70% ethanol (1:30 w:v) for 24 h at room temperature (RT). Additionally, it is possible to use sonication for 20 min in an ultrasonic bath at 20 ˚C. The resulting suspension must be filtered at RT using a paper filter. The propolis residue is re-extracted repeating the above mentioned steps (Popova et al., 2004). Then, the propolis concentrated extract is finally

obtained using the evaporation of solvents. This method is simple and effective, although presents some disadvantages such as strong residual flavor and limitations of application in cosmetics and pharmaceutical industry since the use of ethanol is not suitable for the treatment of some diseases in ophthalmology, otorhinolaryngology, pediatrics and in alcohol intolerance. Therefore, the production of non ethanolic propolis extracts is an important scientific issue since little is known on the production of propolis aqueous or oily extracts. The most important problem is the low solubility of actives in these solvents, in fact the amount of phenolic compounds is 10-fold lower than in ethanolic extracts (Mello et al., 2010; Moura et al., 2009; Ramanauskienè et al., 2011). Water dissolves less than 10% of propolis weight (Bankova et al., 2016).

To overcome these disadvantages of solvents extraction, it is possible to prepare propolis extracts using the supercritical fluid extraction (SFE) method. It is used for the preparation of high valuable products since SFE uses low temperatures, reduces the energy consumption and removes the extraction solvent which is then recycled. Another method was reported by Stahl et al. (1988) using supercritical CO_2 at 600 bar and 40 °C to extract the resins and to separate the insoluble flavonoids. Moreover, to obtain propolis extracts rich in volatile compounds different methods can be used: hydrodistillation (Clevenger), static and dynamic head-space, distillation-extraction (Likens-Nikerson), steam distillation, solvent extraction (including ultrasound-assisted and microwave-assisted extraction) and solid-phase microextraction (Torto et al. 2013; Bankova et al., 1998).

The method used determines the chemical composition of propolis volatile constituents. Despite these compounds represent about 1% of propolis weight,

they are responsible for its specific flavor and some activities (Bankova et al., 2014).

PROPOLIS BIOLOGICAL ACTIVITIES

A large body of evidence suggests that propolis exerts many pharmacological activities and healthy properties such as antibacterial, antiviral, antifungal, antiulcer, antioxidant, antiradiation, hepatoprotective, antitumor, antimutagenic, anti-angiogenic, cyto- and chemopreventive, anti-inflammatory, wound healing, immunomodulating (immunostimulating and immunosuppressive in autoimmune diseases), muscle contracting at low concentration, muscle relaxing at higher concentration, anti-diabetic, cardioprotective (antimyocardial injury, antithrombogenic, antihypertensive, antiarrhythmic), local anesthetic, regenerative (cartilaginous and bone tissue, dental pulp) and food preservative activities (Fidalgo et al., 2011; Mathivanan et al., 2013; Lofty, 2006; Marcucci, 1995; Burdock, 1998; Castaldo et al., 2002 Sforcin et al. 2001; 2007; Banskota et al. 2001; Bankova, 2005c; 2014).

Although propolis has different chemical compositions and could be made starting from different resin sources, it shows similar biological activities (Bankova, 2005c; Seidel et al., 2008). Many studies also report the chemical characterization of the used propolis samples. The determination of a relationship between concentration of each compound in a propolis sample and the biological activity found is critical and not easy to be established because positive and negative synergic effects can occur among propolis components (Boisard et al., 2015; Bonvehi et al., 1994; Chen et al., 2008). For this reason other studies use a different approach, correlating the amount of specific

chemical groups with their biological activities (Bankova, 2005c). In fact, it was recently demonstrated that the combination of polyphenolic species is essential for the biological activity of propolis (Boisard et al., 2015).

Antimicrobial activity

The *in vitro* antimicrobial activity of propolis against several bacterial strains has been reported by a large number of scientific publications (Chee et al., 2002, Ota et al., 2001). Several studies demonstrated that the high flavonoid content could be responsible to antibacterial activity (Cowan, 1999; Takaisi et al., 1994), while other researches attributed this activity to cinnamic acids, aromatic molecules, diterpene acids and phenolic compounds (Bankova et al., 1996; Burdock, 1998; Boukraa Sulaiman, 2009; Ramanauskiene & Inkènienè, 2011). However, the mechanism at the bases of propolis antimicrobial activity is complex and it could be due to a synergism between phenolic compounds and other resin molecules against different bacteria, fungi, molds and parasites (Kujumgiev et al., 1999; Popova et al., 2017). In light of this, the relationship between the chemical composition and antibacterial activity of propolis constituents is still unclear (Boisard et al., 2015; Yang et al., 2011).

Ethanol propolis extracts (EEP) are more active against gram-positive pathogens than gram-negative bacteria (Grange & Davery, 1990). The EEP antibacterial spectrum is wide: propolis is very active against *Staphylococcus aureus* (Trusheva et al., 2010) and shows good results against *Streptococcus mutans* and *S. sobrinus* (Kim et al., 2011), *Helicobacter pylori*, micrococci (Farnesi et al., 2009), *Bacillus subtilis, B. cereus, Enterrococus faecalis, Listeria monocytogenes, Proteus mirabilis, B. larva, P. vulgaris, Salmonella enterica typhi* and *typhimurium* (Orsi et al., 2005), *Pseudomonas aeruginosa,*

Escherichia coli, *S. faecalis* and *L.innocua* (Marcucci et al. 1995; Erkmen & Ozcan, 2008; Pavilonis et al., 2008).

Different mechanisms of action have been proposed:
propolis inhibits bacterial mobility (Mirzoeva et al., 1997); pinocembrin acts as quorum sensing inhibitor (Savka et al., 2015); galangin blocks the adhesion of *S. aureus* (Cushnie et al., 2007); propolis, both *in vivo* and *in vitro*, inhibits peptidoglycan synthesis, more precisely the glucosyltransferase production and activity in *S. sorbinus* and *S. mutans* (Parolia et al., 2010; Duarte et al., 2006; Koo et al., 2000; Lotfy et al., 2006; Ikeno et al., 1991); propolis reduces the symptoms of bacterial peptidoglycan-induced colitis by inhibiting mainly the production of pro-inflammatory cytokines in macrophages (Fitzpatrick and Wang, 2001, Banskota, 2001).
Many researchers demonstrated propolis antifungal activity against *Candida albicans* (Santos et al., 2008; Dias et al., 2007; Borrelli et al., 2002) as well as against some other yeasts such as *C. tropicalis* and *C. krusei* that are equally sensitive (Vardar-Unlu et al., 2008). Combinations of certain antimycotic drugs with propolis (10%) increased their activity on *C. albicans* (Martins et al., 2002; Pontin et al., 2008). Through *in vitro* and *in vivo* experiments, the antifungal activity was also shown against some plant fungi (Martins et al., 2002).

Anti-inflammatory and immunomodulatory activities

Propolis shows anti-inflammatory and immunomodulatory activities. As anti-inflammatory agent, propolis has been shown to inhibit the synthesis of prostaglandins, support the immune system by promoting phagocytic activity, stimulate cellular immunity and induce epithelial tissue wound healing (Casaroto et al., 2010). The administration of green propolis and artpillin C in mouse model was correlated with the decrease in inflammatory mediators such as neutrophils, prostaglandin E2 and nitric oxide (NO); and with the inhibition of inducible nitric oxide synthase (iNOS) and NF-kB activity (Paulino et al. 2006).

Treatment of mice with propolis (200 mg/kg) for 14 days has led to the inhibition of interleukin (IL) –1, IL-6, IL-2, interferon (IFN), and IL-10 in spleen cells suggesting propolis anti-inflammatory activity (Missima et al., 2009; 2010). Moreover, it was suggested that extracts containing 10% of raw propolis stimulate the production of antibodies (Sforcin et al., 2005). Propolis can modulate the immune system inhibiting *in vitro* and *in vivo* murine peritoneal macrophages. It stimulates the lytic effect on natural killer (NK) cells against tumor cells and inhibits lymphoproliferation induced by inflammation (Sforcin, 2007).

Propolis acts directly on immune cells 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 (Ansorge et al., 2003). The administration of green propolis ethanol extract (200 mg/kg) to mice improves innate immunity through the activation of the initial phases of the immune response via upregulation of toll like receptor 2

(TLR) and TLR-4 and pro-inflammatory cytokines (IL-1 and-6), the production of macrophages and spleen cells contributing to the recognition of foreign microorganisms by activating lymphocytes from the cells presenting the antigen (APC) (Orsatti et al., 2010). The anti-inflammatory property of propolis was also studied in a clinical trial in relation to chronic stomatitis. This pathology is characterized by erythema, edema and mucous ulcers. All patients treated with the Brazilian propolis gel had a complete clinical remission of oral stomatitis (Santos et al., 2008). Green Propolis was evaluated as mouth ulcer treatment also on other studies and the results showed a statistically significant increase in the reduction of oral ulcerations (Samet et al., 2007; Santos et al., 2008).

Antioxidant activity

The antioxidant activity of the propolis is mainly due to polyphenols which are involved in many biological functions such as the protection of plants against ultraviolet ray (UV), pathogenic microorganisms and predators (Petty & Scully, 2009); in humans, polyphenols have multiple effects mainly related to their antioxidant one. This effect of polyphenols depends on the number of phenolic rings, and it vary according to number and position of hydroxyl groups, double bonds in the molecule, the presence of a catechol group, an unsaturation in position 2,3 associated with a 4-carbonyl function in C-ring and functional groups able to chelate metals (Bors et al., 2001; Spencer et al., 2003). In addition to classical antioxidant action it is possible to highlight *in vitro* pro-oxidative actions (Elbling et al., 2005). In fact, on one hand polyphenols act as antioxidants improving cell survival; on the other hand, they can act as pro-oxidant molecules inducing apoptosis, necrosis or proliferation arrest (Lambert et al., 2005).

Phenolic compounds help to maintain a balance between oxidant and antioxidant substances. Flavonoids and phenolic acids are the main classes of phenolic compounds whose activity-structure relationship allows the antioxidant function in hydrophilic or lipophilic systems (Salatino et al., 2011; Banskota et al., 2001; Marcucci et al., 1995).

At mitochondria level, during the oxidative phosphorylation process, the electrons are transferred by NADH (Nicotinamide Adenine Dinucleotide) and $FADH_2$ (Flavin Adenine Dinucleotide) cofactors to oxygen, with subsequent formation of a water molecule. The transport of electrons generates a protonic 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 formed by other mechanisms: 1) the activity of some enzymes such as xanthine oxidase, lipoxygenase and cyclooxygenase (Szocs, 2004); 2) during the biotransformation of foreign compounds, toxins or drugs, through the cytochrome P-450 monooxygenase activity; 3) the exposure to environmental factors such as high concentrations of iron salts or UV ray, leading to lipid peroxidation (Ichihashi et al., 2003); 4) elimination of foreign microorganisms thanks to the action of macrophages, granulocytes and neutrophils.

The toxic effect of ROS is related to the redox equilibrium lost: cellular decreased anti-oxidant capacity and the concomitant ability of these molecules to oxidize biological macromolecules, such as lipids, proteins and DNA, resulting in alterations of cellular membranes, inactivation of enzymes and receptors, modification of cytoskeletal proteins and genome damage. Nevertheless, ROS production is also a physiological event and it has not only a

negative impact for the organism. In fact, ROS are also produced during the elimination of pathogens after the infection (Kumar, 2014). To contrast the oxidative damage of ROS, there are sophisticated cellular antioxidant mechanisms in humans that include both endogenous and exogenous molecules. Among the endogenous antioxidant defenses in animal there are: iron and copper, proteins such as transferrin, ferritin and lactoferrin; the catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzymes. Among the exogenous antioxidant defenses there are: the liposoluble vitamins E and A, and polyphenolic compounds.

The *in vitro* antioxidant activity of propolis extracts is commonly studied using β-carotene bleaching, 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical-scavenging, oxygen radical absorbance capacity (ORAC) and 2,2′-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) and radical cation decolorization assays (Kumazawa et al 2004; Ahn et al., 2007; El Sohaimy et al., 2014).

Propolis extract inhibits lipoxygenase and protects the gastric mucosa from oxidative stress. Brazilian propolis at 50 and 250 mg/kg highlights anti-ulcer action in particular thanks to the activity of cumaric and cinnamic acids (Barros et al., 2008). Moreover, propolis has spasmolytic action in the gastrointestinal tract and protects the stomach from ethanol-induced lesions (Liu et al., 2002). Although green propolis is poor in flavonoids, studies on this propolis showed a 40% or 57% scavenging activity at a concentration of 500 μg/ml (Buratti, 2009).

Antioxidant activity is a very important topic since many syndromes are linked to an imbalance between the antioxidant defense system and the production of free radicals (i.e. amyotrophic lateral sclerosis, accelerated aging, Alzheimer's

disease, cataracts, cardiovascular disease and rheumatism) (Favier 1997). Oxidative stress and inflammation are closely related phenomena. In fact, the oxidative stress causes inflammation, which in turn induces oxidative stress and causes the emergence of a chronic inflammatory state. Recently, it has been shown that chronic inflammation is a predisposing factor for the onset of some diseases such as atherosclerosis, neurodegenerative diseases and cancer (Ishibashi, 2013). The antioxidant activity of flavonoids could be the basis of the anti-inflammatory activity (Robak & Gryglewski, 1996) due to their structure, their ability to penetrate the cell lipid membrane (Saija et al., 1995) and their own ability to modulate the expression of closely related anti-inflammatory genes (Sperandio, 2006).

Activity on glycemic equilibrium

Some studies show that propolis has a hypoglycemic effect in patients with type II diabetes and it contributes to reducing the risk of metabolic syndrome in healthy subjects. Propolis stimulates the activity of damaged pancreatic cells, accelerates tissue regeneration and repair and promotes bone remineralization (Al-Hariri et al., 2011; Zhao et al., 2016).

Activity on cardiovascular diseases

Propolis exerts a cardiovascular protective activity. In fact, it protects the blood vessel wall whose degeneration can cause arteriosclerosis (Fuliang et al., 2005). A constant administration of polyphenols in diet reduces the risk of cardiovascular disorders (Gorinstein et al., 2011; Grassi et al., 2008; Norata et al., 2007). Propolis is able to modulate lipid and lipoprotein metabolisms acting on the hepatic triglyceride synthesis in rats (Fuliang et al., 2005; Li et al.,

2012). Propolis causes the decrease of total cholesterol and the increase of high density lipoprotein (HDL) cholesterol in mice. This mechanism involves the ATP-binding cassette transporter (ABCA) 1 receptor through the upregulation of ABCA1 gene expression, which is associated with increased HDL levels (Daleprane et al., 2012).

Activity on respiratory tract

Bee products have been used empirically for centuries especially for the treatment of respiratory diseases. Propolis can significantly reduce the number and severity of nighttime asthma attacks, improve pulmonary function and reduce inflammation (Khayyal et al., 2003). Subjects with pharyngitis, treated with an extract containing 75 mg of raw propolis, showed a significant positive trend in symptom relief with a reduction in sore throat, fever, adenomegaly, pharyngeal erythema and exudate with the only exception in the nasal secretion that showed no clear signs of improvement (Di Pierro et al., 2016). Propolis can be effective in relieving symptoms of allergic rhinitis by inhibiting the release of histamine (Shinmei et al., 2009). In addition, propolis is able to reduce allergic pulmonary inflammation in murine model through the involvement of lung inflammatory cells and the decrease of polymorphonuclear inflammatory cells (de Farias et al., 2014).

Molecular biology studies of propolis and its components

Studies in the field of molecular biology have shown that both micronutrients and macronutrients represent potent signals to influence gene expression. They play a crucial role in the cell epigenetic. Epigenetic (literally from Greek επί, epì = "above" and γεννετικός, gennetikòs = "Family Heritage") studies the phenotype changes without altering the genotype (Wolffe & Matzke, 1999). The term was coined in 1942 by the biologist of development Conrad H. Waddington. Epigenetic mechanisms include: DNA methylation, histone protein modification and noncoding RNA (ncRNA) (Luo et al., 2015). Among the ncRNA there are the so called microRNA (miRNAs). MiRNAs are small non-coding RNAs, of about 22 nucleotides. They are able to anneal complementary sequences of target messenger RNA (mRNA), interfering with the translation of the corresponding protein and preventing or altering the gene expression (Bartel, 2004). The mature miRNAs negatively regulate gene expression and the strength of this regulation depends on the degree of complementarity between the miRNA and its target sequences. MiRNAs can bind the mRNA 3′ UTR (untranslated region) sequence either with imperfect complementarity blocking the protein translation or with perfect complementarity inducing the cleavage of target mRNA. Moreover, the modulation of gene expression could also depends on the number of annealed bases (Breving & Esquela-Kerscher, 2010). The genome sequences coding for miRNAs are found primarily in the intergene and intronic regions, although a small part of them could also be found in exons. The synthesis of miRNA is a complex process made up of different steps some of which occur in the nucleus and others in the cytoplasm. MiRNAs transcription begins by RNA polymerase

II (Pol II) in the nucleus (Borchert et al., 2006) to form large pri-miRNA transcripts (100-1000 nucleotides long). In some cases, transcripts may be polycystronic, meaning that more mature miRNAs may reside on the same precursor. Pri-miRNAs are processed by the RNase III enzyme, than the activity of Drosha lead to the formation of pre-miRNAs which are transferred from the nucleus to the cytoplasm by RAN-GTP mechanism. Subsequently Dicer generates 18- to 24-nucleotide mature miRNAs (Carthew et al., 2009), which are then incorporated into the RNA-induced silencing complex (RISC). It is thanks to this complex that the miRNAs can exert their function by the annealing with target mRNAs. The consequence is a transcriptional and post-transcriptional alteration within the cell. It is believed that miRNAs control the posttranscriptional regulation of 30% of mammalian genes (Esquela-Kerscher et al., 2006; Miska, 2005). The importance of miRNAs studies is related not only to nutraceutical activity of micro or macronutrients, but also to the modulation of the risk of onset/ severity/protection of a large number of pathologies, such as neurodegenerative diseases, cardiovascular disease and tumors (Bladè et al., 2014). Today in miRBase, the miRNA reference database (http://www.mirbase.org/), over 20.000 miRNAs have been annotated. This database could be used to know which miRNA is involved in specific pathways and in turn in the prevention or pathogenesis of pathologies. The mechanism by which miRNAs work is that a single miRNA may regulate the expression of many target mRNAs and, in turn, a given mRNA may be the target of several miRNAs. This mechanism introduces a degree of redundancy of post-transcriptional gene regulation and makes the data interpretation quite complex, since a single miRNA can be involved in more than one signal transduction path, resulting in different effects depending on the pathway in which it is

acting on. This is one of the reasons why little is still known about miRNAs even if the number of scientific literature is growing day by day. Moreover, these molecules are good biomarkers, since they are ubiquitous, small, easily extractable and quantifiable (Kuzuhara et al., 2006; Xiao et al., 2012). It was demonstrated that polyphenols are able to modulate expression and/or the activity of numerous enzymes involved in different pathways from inflammation to oxidative stress response, cell survivor and differentiation (Naasani et al., 2003; Laughton et al., 1991; O'Leary et al., 2004; Hussain et al., 2005; Schewe et al. 2001; Sadik et al., 2003). There are only few *in vivo* researches. In most cases, studies pointed the polyphenol effects on the expression of miRNAs *in vitro* using polyphenols in their native form at high concentrations and not the physiological metabolites normally present in the blood circulation at low concentration after ingestion of polyphenols-rich food. This represents one of the major bias since the polyphenols metabolism and metabolites are not taken in consideration in these studies (Milenkovic et al., 2013). The way polyphenols interact with miRNAs is still to be clarified. Evidence shows that polyphenols can bind to both RNA sequences and proteins (Kuzuhara et al., 2006; Xiao et al., 2012). This indirectly suggests that polyphenols are also able to bind miRNAs and/or some proteins involved in their biogenesis. In addition, since most of the sequence of miRNAs are located in the intronic regions of the genes, polyphenols may result in modulation of the expression by regulating host gene expression (Bladé et al., 2013). The study of relation between miRNAs, polyphenols and propolis started only recently. In 2014 Kumazaki et al. showed that propolis polyphenols (as cinnamic acid derivatives, baccharin and drupanin) induce apoptosis in drug-resistant colon cancer cells. These polyphenols act by increasing the expression

level of anti-oncogenic miR-143 leading to the down-regulation of Erk5 target gene. Similar findings were provided by the studies of Cuevas et al., (2014; 2015) in which they observed an attenuation in atherosclerotic lesions in low density lipoprotein receptor gene knockout mice, through an overexpression of three miRNAs (miR-181a, miR-106a and miR-20b) involved in the modulation of pro-angiogenic factors (Cuevas et al., 2014). In 2015, a study on Chilean propolis linked the effect of propolis at molecular level by an overexpression of miR-19b that has as targets mRNAs coding for proangiogenic proteins in human endothelial cells (Cuevas et al., 2015).

BIOAVAILABILITY OF PROPOLIS POLYPHENOLS

Bioavailability expresses the amount/proportion of a drug, nutrient or other compounds able to reach, through systemic circulation, its site of action where it exerts its biological effects (Porrini et al., 2008).

Many epidemiological studies have demonstrated the inverse correlation between incidence of chronic degenerative and cardiovascular diseases and intake of food rich in polyphenols (Arts et al., 2005; Hertog et al., 1995; Hirvonen et al.,2001; Lambert et al., 2005; Williamson et al., 2005).

These effects were demonstrated both *in vitro* and *in vivo* experiments by using high concentrations often not comparable to those of a common human intake. Moreover, the compounds used in the *in vitro* experiments were polyphenol aglycones or their sugar conjugates rather than their active metabolites. The dose used should reflect real polyphenol intake: the tested concentrations commonly range from low µmol/l to mmol/l, while the concentrations of plasma metabolites, after a normal dietary intake, rarely exceed nmol/l, in urinary excretion may vary from 0.3% to 43% of the ingested dose, according to the polyphenol considered (Manach et al., 2005; Monteiro et al., 2007; Seeram et al., 2008).

It is difficult to carry out bioavailability studies since there are several disturbing factors such as the environmental one, food matrix, polyphenols interactions with other compounds, chemical structure, concentration in food, intake, intestinal and systemic factors (D'Archivio et al., 2010).

One of the *in vivo* approach generally used to study the bioavailability of the polyphenols is the single-dose design. It is based on the intake of one portion of food containing the studied polyphenols. Than the concentration in blood is measured at different time recording a transitional increase of metabolites. This

increase reflects the ability of the model organism to absorb the polyphenol from the food matrix. However, to obtain a significantly increase and biological effect it is necessary a chronic administration of food rich in polyphenols (Scalbert et al., 2000). Since food matrices contain a mixture of polyphenols, it is difficult to correlate a biological activity to just a single compound (D'Archivio et al., 2010). Other approaches are related to *in vitro* studies, using tissue slices or cultured cells. However, also in these cases, the data analyses and interpretation are critical steps. Moreover, it is very difficult to transfer studies carried out on animals or cells to humans.

As already mentioned, there are many factors influencing the bioavailability. One of the most important is the chemical structure since polyphenols are found as polymers or in glycosylated forms. In these forms polyphenols cannot be absorbed due to their high molecular weight and polarity, in fact once they arrived in the colon, they are hydrolyzed by enzymes or colonic microflora. The compounds not absorbed in the small intestine, directly reach the colon where they undergo substantial structural modifications induced by colonic microflora that hydrolyzes glycosides into aglycones (Aura et al., 2005; Kuhnau et al., 1976). The polyphenols transformed in simple aglycones undergo to other structural modifications due to the conjugation process in the small intestine and liver (figure 4).

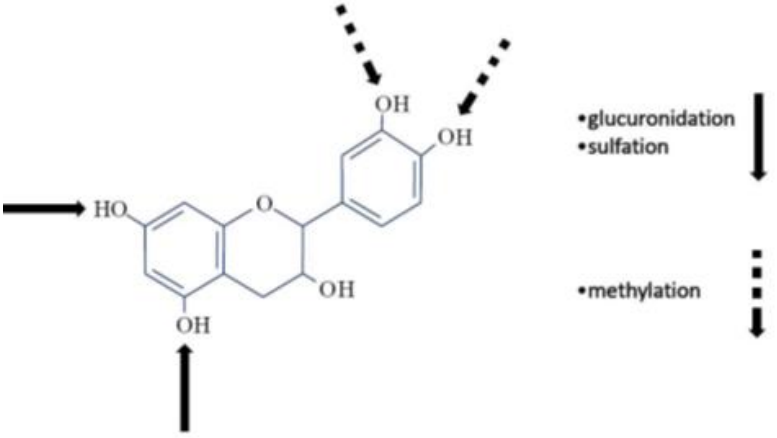

Fig. 4- Potential sites of the conjugation process of the polyphenols. The broken arrows represent the potential methylation sites; the full arrows represent the potential glucuronidation and sulfation sites. (D'archivio et al., 2010)

These structural modifications increase the solubility and the molecular weight. Among them there are methylation, sulfation, and glucuronidation, which represent the mechanism of detoxification also used for xenobiotics to restrict their potential toxic effects and facilitate their elimination from the organism. Although this process of conjugation produces active metabolites from some dietary polyphenols, in the meantime it reduces the total amount of circulating polyphenols in the blood increasing their excretion (D'Archivio et al., 2010; Felgines et al., 2005).

Glucuronidation is particularly important to increase the molecular weight, necessary for the excretion in the bile (Day et al., 2000). Catechol-O-methyltransferase (COMT) catalyzes the transfer of a methyl group from adenosylmethionine to polyphenols that contain a diphenolic moiety, such as

quercetin, catechin, caffeic acid and cyanidin. This enzyme is ubiquitously expressed in many tissues, but the highest activity is registered in the liver and kidneys (Piskula et al., 1998; Tilgmann etal., 1996). Sulfotransferases (SULT) catalyze the transfer of a sulfate moiety from phosphoadenosine-phosphosulfate to a hydroxyl group on various substrates, among which polyphenols. Sulfation occurs mainly in the liver (Falany, 1997; Piskula et al., 1998). Uridine-5'-diphosphate glucuronosyltransferases (UGTs) are membrane-bound enzymes located in the endoplasmic reticulum of cells of many tissues and catalyze the transfer of a glucuronic acid from UDP-glucuronic acid to polyphenols. Glucuronidation of polyphenols first occurs in the enterocytes before further conjugation in the liver (Boersma et al., 2002; Crespy et al., 2001; Spencer et al., 1999). The balance between structural modification could also depends on species and sex as in the case of sulfation and glucuronidation of polyphenols (DuPont et al., 2000). The consequence of all these modifications is that any single polyphenol can generates several metabolites, as many as 20 in the case of quercetin glycosides (Mullen et al., 2006).

The only exception is represented by anthocyanins that can be absorbed also in the glycosylated form and detected directly in the blood (Nurmi et al., 2009). Many studies explained this peculiarity with the instability of the aglycone form and suggested specific mechanisms of absorption/metabolism for anthocyanins as the action of lactase phloridizin hydrolase (LPH) in the small intestine epithelial cells (Passamonti et al., 2002; Wu et al., 2002; Semenza, 1987) or of cytosolic b-glucosidase (CBG) (Gee et al., 2000).

AIM OF THE INVESTIGATION

The first studies on the chemical characterization of propolis date back to the beginning of the 20th century. With the progress being made in analytical methods, more than 300 compounds have been identified in propolis, including flavonoids, terpenoids, phenolic acids, phenolic esters and sugars.

Despite the great number of investigations, the common scientific approaches to study biological activities of propolis show some limits due to the high natural variability of propolis and to the different extracts used in the investigations. Therefore the results obtained are often not comparable each other and poorly reproducible.

For these reasons the first aim of this thesis, carried out in B Natural, was **the improvement of propolis extraction method** to standardize the process and guarantee a high purification and a constant concentration and ratio of active polyphenol compounds in each extract also starting from different propolis raw materials. The obtained standardized extracts were then used for the experiments carried out at the University of Pavia.

As far as biological activities are concerned, there are hundreds of studies present in the scientific literature supporting the healthy properties of propolis, such as gastroprotective, hepatoprotective, immunomodulatory, wound healing, antidiabetic and antineoplastic. These properties are ascribed to the three main activities of propolis, namely antioxidant, anti-inflammatory and antimicrobial.

Although the antioxidant and anti-inflammatory properties of propolis have been extensively studied, the molecular mechanisms of actions are still unknown.

Therefore, the second aim was the investigation of the **mechanisms of action behind the antioxidant and anti-inflammatory activities** through the analyses of the expression levels of miRNAs, mRNAs and proteins associated with these pathways in human keratinocyte (HaCat) cell line after the treatment with two types of standardized propolis extracts, characterized by RP-HPLC-PDA-ESI-MSn.

Since there are few *in vivo* studies on propolis, the third aim was the evaluation of **propolis bioavailability** using galangin as chemical marker after acute and chronic treatments. In addition the ***in vivo*** **antioxidant activity** of propolis extract was then checked after chronic treatment.

The antibacterial activity of propolis is the most known and studied properties, but previous literature data provided non-homogeneous and conflicting information due to the high natural variability of propolis used. Thus the fourth aim was the determination of ***in vitro*** **antibacterial activity of propolis** on different microorganism species and strains (Staphylococci, Streptococci, Fungi, Listeria, Bacilli, Clostridi, Gram negative and vaginal flora Bacteria spp).

Improvement of propolis extraction method

Materials and methods

Selection of raw material

Green and brown raw propolis from different origins such as Asia, Europe and South America were used in this study. In particular, green propolis comes from Brazil while brown propolis comes from Italy, Romania, Poland, Mongolia, China, Kazakistan, Chile, Argentine and Paraguay.

Sample preparation

Raw propolis was submitted to extraction process including several steps for the preparation of polyphenol-rich propolis extracts. These steps consist of an initial aqueous extraction from dewaxed raw propolis, a series of extractions on the residue using an ethanol/water mixture, with each extraction being carried out on the residue from the previous extraction using a higher degree of alcohol.

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

- aqueous extraction, 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 circa 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.

(1) The first extraction step used a water/ethanol mixture with an alcoholic concentration ranging from 35 to 40 alcoholic degrees, at 50 °C;

(2) The second extraction step used a mixture with an alcoholic concentration ranging from 55 to 60 alcoholic degrees, at 70 °C;

(3) The third extraction step used a mixture with an alcoholic concentration ranging from 70 to 80 alcoholic degrees, at 80 °C

- Concentration: the combined extracts were mixed and concentrated to a residual humidity value ranging from 15 to 20% by weight.

Analyses of Propolis Extracts (RP-HPLC-PDA-ESI-MSn)

The chromatographic analyses were performed by means of the RP-HPLC-PAD-ESI-MSn method, set up by Cui-ping et al., with some modifications (Zhang et al., 2014). The chromatographic analyses were performed using a Thermo Finnigan Surveyor Plus HPLC, equipped with a quaternary pump, a Surveyor UV−Vis diode array detector and a LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Xcalibur software (2.0 SR2, Thermo Fisher Scientific, Waltham, MA, USA) was used to control the HPLC instrumentation and to analyze the data. Compound separation was obtained with an analytical Synergi Fusion RP-18 column (150 × 4.6 mm, 5 μm), equipped with a Hypersil Gold C18 precolumn (10 × 2.1 mm, 5 μm), all produced by Phenomenex (Torrance, CA, USA). The mobile phase used was acidified water, with 0.1% formic acid (eluent A) and methanol

(eluent B). The run time was 110 min in total, including the reconditioning of the column. The flow rate was maintained at 1.00 ml/min, and the temperatures of the autosampler and column were kept at 4 and 33 °C. The volume of injection was set to 5 µl. The elution method is described in Table 3. Chromatograms were registered at 5 different wavelengths (λ = 254, 280, 330, 370 and 395 nm). The HPLC-ESI-MSn data were collected using Xcalibur software under a negative ionization mode. For this purpose, the ion trap was set in full scan mode to detect all mass-to-charge ratios (*m/z*) in the selected range, data dependent scan, and MSn mode, in order to obtain further discrimination between compounds.

Time (min)	% Eluent A	% Eluent B
0	85	15
30	60	40
65	45	55
70	38	62
85	0	100
90	0	100
100	85	15
110	85	15

Tab. 3. RP-HPLC-PDA-ESI-MSn analysis elution method.

Determination of polyphenolic content in propolis extract by HPLC-UV

The chromatographic analyses were performed by means of the HPLC-UV method, set up by Sha et al., 2009. A calibration curve using pure galangin (Sigma Aldrich) has been built to determine the total content of polyphenols in

propolis. The chromatographic analyses were performed using Jasco HPLC, equipped with a quaternary pump. Jasco-Borwin software (relase 1.5, Jasco-Browin software, Easton, MD, USA) was used to control the HPLC instrumentation and to analyze the data. Compound separation was obtained with an analytical Discovery® C18 HPLC column (250 × 4.6 mm, 5 μm) (Sigma Aldrich). The mobile phase used was acidified water, with 0.1% acetic acid (eluent A) and acetonitrile (eluent B). The run time was 102 min in total, including the reconditioning of the column (10 min). The flow rate was maintained at 1.00 ml/min at RT and the volume of injection was set to 20 μl. The chromatograms were registered at 260 nm. The elution method is described in Table 4.

Time (min)	% Eluent A	% Eluent B
0	75	25
3	75	25
10	70	30
40	60	40
60	40	60
80	10	90
92	10	90
102	10	90

Tab. 4. HPLC-UV analysis elution method.

To perform the calibration curve, 5 standard solutions with a range of concentration from 5 to 50 μg of pure galangin were prepared and analyzed by HPLC using the analytical method just described. This calibration curve covered an area under the curve (AUC) range from 29.76 to 59.81. The

coefficient of correlation obtained was 0.9567, it was calculated using the regression software FigSys release 2.4.3 from Biosoft (figure 5).

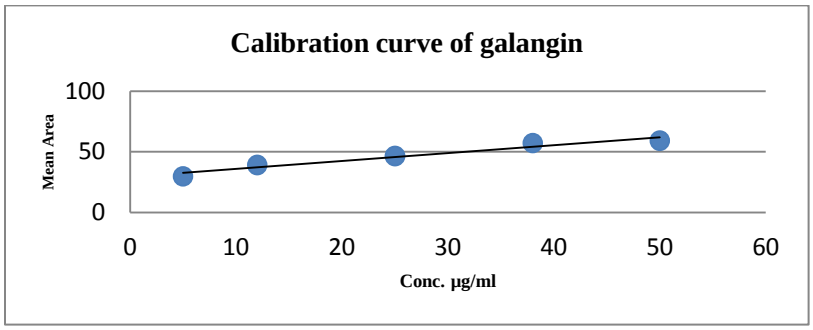

Fig. 5. Galangin Calibration curve in HPLC-UV.

Preparation of formulated extracts

Hydrogliceric extract:

the concentrated extracts were evaporated to remove ethanol. The dough (10%) was mixed with hot glycerin (95%) and water (5%) for 2 h within a mixer, and then cooled at 10 °C to give a non-alcoholic liquid. After precipitation the solution was filtered twice, using 30 µm and 10 µm filters.

Dry extracts:

the concentrated extracts (20%) was mixed with 68% of arabic gum, water, 10% of sucrose, 2% of burnt sugar and it was microencapsulated by spray dry at following conditions: 30% of dry residue, input temperature 185 °C, output temperature 85 °C.

Syrupy extract:

the concentrated extracts (25%) was mixed with arabic gum (6%), sugar (35%), maltodextrin (9%) and water (25%) to make the syrupy extracts.

Results

The research carried out in B Natural contributed to a patent deposition on February 2017 (patent n. 0001425516) concerning a new propolis extraction method which was called Multi Dynamic Extraction (M.E.D.®) (figure 6).

Ministero dello Sviluppo Economico
Direzione generale per la lotta alla contraffazione
Ufficio Italiano Brevetti e Marchi

ATTESTATO DI BREVETTO PER INVENZIONE INDUSTRIALE

N. 0001425516

Il presente brevetto viene concesso per l'invenzione della domanda sotto specificata:

num. domanda	anno	C.C.I.A.A.	data pres. domanda	classifica
001434	2014	MILANO	05/08/2014	A61K35 644

TITOLARE/I B NATURAL SRL
CORBETTA (MI)

MANDATARIO MINOJA FABRIZIO

INDIRIZZO BIANCHETTI BRACCO MINOJA S.R.L.
VIA PLINIO, 63
20129 MILANO

TITOLO PROCEDIMENTO PER L'OTTENIMENTO DI ESTRATTI INTEGRALI DI PROPOLI RICCHI IN POLIFENOLI E DOTATI DI ATTIVITA' ANTIBATTERICA E SUA APPLICAZIONE NELLA PREVENZIONE E TRATTAMENTO DI PROCESSI INFETTIVI DI ORIGINE BATTERICA

INVENTORE/I VOLPI NICOLA
FACHINI ALFREDO

CAMERA DI COMMERCIO MILANO
02 FEB. 2017
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Roma, 03/11/2016

IL DIRIGENTE
Dr.ssa Loredana Guglielmetti

Fig.6. Patent registration.

It implies that the concentrated extract obtained contains about 5-25% of phenolic acids and 75-95% of flavonoids of which flavons and flavonons from 10% to 40%, flavonones and diidroflavonones from 10% to 40% and glicosilated flavonoids from 20% to 80%. 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).

The chemical characterization and the polyphenolic quantification of formulated extracts prepared for *in vivo* and *in vitro* experiments were reported into each result chapter.

In this section an example of propolis M.E.D.® concentrated extract was reported. In particular metabolic profile (table 4), HPLC-ESI-MS and HPLC-UV chromatograms (figures 7 and 8) are shown:

Polyphenols	% (w/w)
Phenolic acids	17.1
Flavonoids	82.9
of which flavons and flavonols	18.9
flavonones and diidroflavonones	14.6

Tab.4. Metabolic profile of concentrated propolis extract.

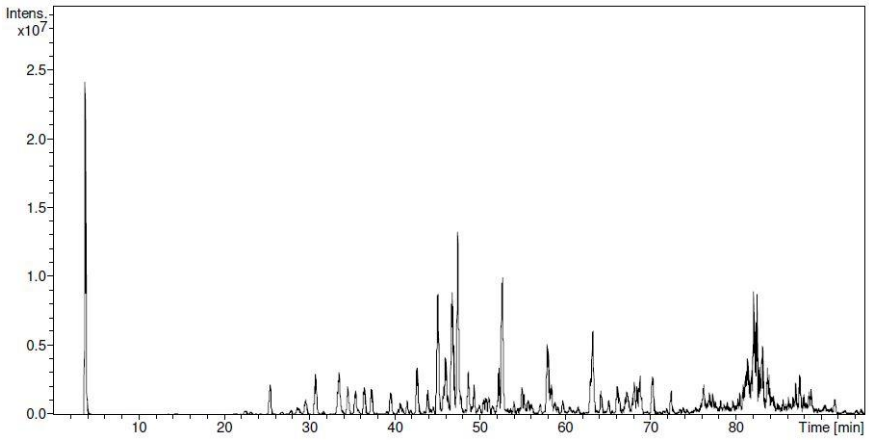

Fig.7. MS spectrum of M.E.D.® concentrated extract

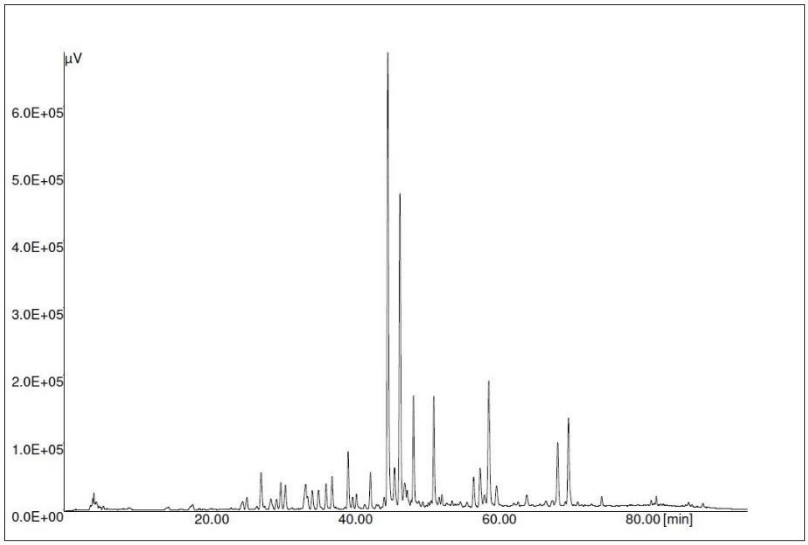

Fig.8. UV spectrum of M.E.D.® concentrated extract

Discussion

The propolis, its extracts and its molecular components possess many different biological activities. However, due to the well-known high chemical heterogeneity of propolis, to the different purification and preparation methods, to the several solvents used to extract selectively some compounds, the literature data are not comparable. In fact, it is not possible to correlate the molecular composition of a given extract with a specific biological activity.

In literature, several solvents to extract propolis compounds are reported: water, absolute ethanol, ethanol-water mixtures (80, 90, and 96%), glycerol, methanol, hexane, acetone, DMSO and chloroform (Gómez-Caravaca et al., 2006; Miguel et al., 2010; Netı´kova' et al., 2013; Park et al., 1998; Sforcin & Bankova, 2011). However, none of them is able alone to obtain an extract with all the most important active compounds.

The improvement of extraction process of propolis, conducted during these years in B Natural, led to the registration of a new patent for the production of integral extract of propolis rich in polyphenols in the beginning of 2017.

M.E.D.® method is characterized by a variable alcoholic grade to extract the most of the polyphenolic compounds from propolis raw material with different solubility. The extract is pure from inactive resins and is rich in polyphenols; in particular the obtained propolis extract is characterized by the presence of a biologically active polyphenol complex, identified in six major polyphenols, galangin, chrysin, pinocembrin, apigenin, pinobanksin, quercetin, having a relative concentration in the extract always greater than 25% (w/w). This method uses a mixture of raw materials, previously selected, coming from

different geographical origin such as Asia (China, Mongolia, Iran, Turkey), Europe (Italy, Spain, Poland, Russia) South America (Brazil, Argentine, Chile) to ensure a continuous supplying to produce extracts with rich and similar chemical profile. M.E.D.® is flexible and it can be adapted to raw materials with different chemical composition. Moreover, M.E.D.® extracts is free from organic contaminants and soluble in aqueous solvents and/or organic mixtures.

Conclusions

Using M.E.D.® it is possible to obtain low alcoholic grade, glycerin and water-soluble products with high content of bioactive molecules. M.E.D.® method allowed the preparation of standardized extracts with constant composition that were used for the subsequent studies.

Mechanisms of action at the basis of the antioxidant and the anti-inflammatory activities of propolis

nutrients

MDPI

Effect of Green and Brown Propolis Extracts on the Expression Levels of microRNAs, mRNAs and Proteins, Related to Oxidative Stress and Inflammation

Materials and Methods

Sample Preparation

To evaluate the activity of green and brown propolis extracts, hydroglyceric samples were prepared and analyzed as previously reported in "improvement of propolis extraction method" section.

Cell culture

Human keratinocyte cell lines (HaCaT, code BS CL 168) from the IZSLER Institute (Instituto Zooprofilattico of Lombardy and Emilia Romagna) were selected for this study. Dulbecco's Modified Eagle Medium (D-MEM) High-Glucose was used as the culture medium (complete medium), supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (2 mM) and antibiotics (penicillin, 100 IU/ml and streptomycin, 100 μg/ml). Cells were grown in sterile conditions and kept at 37 °C in an atmosphere with 5% carbon dioxide.

Cell viability test

Preliminary experiments, using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, have allowed us to identify non-cytotoxic concentrations of propolis. HaCat cells were seeded in 96-well plates, at a density of 1.5 × 104 cells per well and incubated at 37 °C with 5% CO2. The HaCat cells were treated with propolis extracts 24 h after seeding. The extract was weighed and dissolved in complete culture medium, at a concentration of 25 mg/ml. Subsequent serial dilutions (1:2) were prepared to reach the final concentration of 0.019 mg/ml. The control solution was prepared using 90% glycerol (from B Natural S.r.l.) and 10% H_2O, and was tested under the same conditions, in order to exclude its direct cytotoxicity. The treatments were performed for 24 h. At the end of this period, and after morphological observation under a microscope, 10 µl of the stock 5 mg/ml of MTT in phosphate-buffered saline (PBS) were added to the HaCat cells, for 2 h, at 37 °C. At the end of the incubation period, after removal of the culture medium and washing with PBS, cells were added to 100 µl of DMSO to solubilize the formazan crystals. Spectrophotometric readings were then carried out at a wavelength of 570 nm. Cell viability was calculated by measuring the optical densities of treated samples compared to control samples (cells plus glycerol). Each value given in the results represents the mean ± standard deviation of three independent experiments, each consisting of three replicates (Curti et al., 2017).

Cell treatment with green and brown propolis

HaCat cells were seeded in Petri dishes at a density of 1.5 × 106, for 24 h. Cells were treated for a further 24 h with the first three non-cytotoxic concentrations

of propolis extracts: 3.125 mg/ml, 1.56 mg/ml, 0.78 mg/ml. Untreated cells were used as controls. Cells were collected and counted at the end of the incubation period, according to the standard protocol, which includes a brief wash in PBS to eliminate the supernatant. The resulting pellets were stored at −80 °C.

RNA extraction and miRNA Real-Time PCR

Total RNA was extracted from the cell pellets using the miRNAeasy Mini Kit Qiagen, according to manufacturer's instructions (Curti et al., 2014).

Quantitative RNA analyses were performed using a fluorometric method with a Qubit tool (Invitrogen, CA, Grand Island, NY, USA), using the Quant-iT RNA Assay Kit (sensitivity from 5 to 100 ng) with the following protocol: 2 µl of RNA were added to 200 µl of a "working solution", obtained by mixing 1 µl of Qubit RNA reagent with 199 µl of Qubit RNA buffer. The quantification was performed following calibration of the instrument using appropriate standards (0 and 10 ng/ml).

The total RNA was retro transcribed to a DNA copy (cDNA) using the miRCURY LNATM Universal RT microRNA PCR Kit. This reaction only targets mature miRNA from the total RNA pool. The retro transcription protocol is as follows: 4 µl of total RNA (5 ng/l) was added to 4 µl of 5 × reaction buffer, 2 µl of enzyme mix, 1 µl of synthetic spike-in and 9 µl of nuclease free water. The mixture was then incubated in a thermocycler (SureCycler 8800-Agilent Technologies, Cernusco sul Naviglio, Milano, Italy) at 42 °C for 1 h, then 95 °C for 5 min and then immediately cooled to 4 °C.

In order to evaluate the expression of miRNAs, RT-PCR reactions were set up using the EcoTM Real-Time PCR System (Illumina, Milano, Italy) instrument and the Universal cDNA Synthesis Kit and SYBRR Green Master Mix. The PCR reaction was performed with a volume of 10 µl, containing 4 µl of cDNA, diluted 1:80, 5 µl of SYBRR Green Master Mix, and 1 µl of miR-19a-3p, 17-3p, 27a-3p, 203a-3p probes, provided by Euroclone (Pero, Milano, Italy). The reaction conditions were as follows: a first step at 95 °C for 10 min, 45 amplification cycles at 95 °C for 10 seconds, followed by a step at 60 °C for 1 min. The U6 small nuclear RNA (snU6) was used to normalize the expression data of miRNAs and each assay was performed in triplicate. To evaluate the levels of mRNA, coding for Tumor necrosis factor alpha (TNF-α), nuclear factor erythroid 2 like 2 (NFE2L2), manganese SOD (MnSOD), glutathione peroxidase 2 (GPX2) and Thioredoxin Reductase 2 (TRXR2), RT-PCR reactions were performed with the AriaMX Real Time PCR System, using Brilliant III Ultra-Fast SYBR® Green RT-PCR Master Mix (Agilent, Cernusco sul Naviglio, Milano, Italy), according to the manufacturer's protocol. Primers were designed using Primer-BLAST software (available online on 10 July 2017 at http://www.ncbi.nlm.nih.gov/tools/primer-blast). The sequences for the used primers were:

TNF-α forward: 5′-CATCCAACCTTCCCAAACGC-3′

TNF-α reverse: 5′-CTGTAGGCCCCAGTGAGTTC-3′

NFE2L2 forward: 5′-CAGTCAGCGACGGAAAGAGT-3′

NFE2L2 reverse: 5′-ACGTAGCCGAAGAAACCTCA-3′

MnSOD forward: 5′-AAACCTCAGCCCTAACGGTG-3′

MnSOD reverse: 5′-CCAGGCTTGATGCACATCTTA-3′

GPx2 forward: 5′-GAGGTGAATGGGCAGAACGA-3′

GPx2 reverse: 5′-CTCTGCAGTGAAGGGGACTG-3′

TNF-α forward: 5′-CCTCTCTGCCATCAAGAGCC-3′

TNF-α reverse: 5′-TTGAGTAACTTCGCCTGCGT-3′

TRXR2 forward: 5′-CCCTATCCCAGTGTTCCACC-3′

TRXR2 reverse: 5′-AAGGTTCCACGTAGTCCACC-3′

Protein analyses

The analyses of protein expression levels were performed using an Enzyme-Linked Immunosorbent Assay (ELISA) with the microplate reader FLUOstar® Omega by BMG Labtech (BMG Labtech, Ortenberg, Germany). TNF-α, NFE2L2, GPX2, MnSOD and TRXR2 were quantified in the culture supernatants using a Cloud-Clone Corp Kit (respectively SEA133Hu, SEL947Hu, SEC993Hu, SES134Hu and SED376Hu kits) in 96-well plates (Cloud-Clone Corp, Houston, TX, USA)

In brief, the culture supernatant was added to 100 µl of standard diluent, and then incubated for 1 h at 37 °C. After the removal of liquid, 100 µl of detection reagent A was added and incubated for 1 h at 37 °C. Plates were then washed with 350 µl of wash solution 3 times. Next, 100 µl of detection reagent B was

added and incubated for 30 min at 37 °C. After adding horseradish peroxidase-conjugated monoclonal antibodies, with the detection reagents A and B, and following a repeat wash process for a total of 5 times, samples were incubated with 90 µl of colorimetric substrate solution (3,3′,5,5′-tetrametylbenzidine) for 20 min at 37 °C, and then 50 µl of stop solution was added. Finally, a colorimetric measurement was conducted at 450 nm.

Statistical analyses

The cellular effects of the treatments were tested using mixed models, in which treatments with different concentrations of a substance were considered to be a fixed effect, and the sending cell culture was considered to be a random effect. Significant values were taken to be $p < 0.05$.

Statistical analyses of quantification cycle (Cq) values were carried out using software R (ver. 3.0.3, R e2sCore Team, 2014) (Vienna, Austria). Differences between group means were estimated using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, with measurements of $p < 0.05$ being taken as significant.

Results

To evaluate the potential effects of propolis on the expression of miRNAs associated with oxidative stress and inflammatory processes, the human keratinocyte cell line HaCat was treated with chemically characterized green and brown hydroglyceric propolis extracts, obtained as reported in the Materials and Methods section.

Propolis Extracts RP-HPLC-PDA-ESI-MSn Analyses

RP-HPLC-PAD-ESI-MSn analyses of the propolis extracts led to the identification of 16 compounds in each propolis sample, as shown in tables 5 and 6.

Peak *n*	RT* (min)	*m/z*	MS^2 *m/z*	Proposed Structure	Area %
1	12.39	179	135	caffeic acid	2.4
2	17.36	163	119	p-coumaric acid	10.5
3	20.12	193	149; 134	ferulic acid	0.2
4	25.67	287	269	dihydrokaempferol	0.4
5	28.75	515	353	dicaffeoylquinic acid	2.6
6	37.21	271	253; 165; 151; 243	pinobanksin	3.4
7	39.48	301	283; 255; 215; 187	not identified	
8	53.79	247	203; 204	not identified	
9	57.02	255	213; 151	pinocembrin	0.2
10	58.55	285	257	kaempferol	2.4
11	59.57	231	187	drupanin	7.2
12	63.01	313	253; 271	pinobanksin-3-*O*-acetate	0.4
13	65.96	253	209	Chrysin	0.2
14	69.83	315	300; 271	quercetin-3-methyl-ether or quercetin-4-methyl-ether	2.8
15	71.45	299	284	luteolin-methyl-ether	3.4
16	76.72	315	300; 271	quercetin-3-methyl-ether or quercetin-4-methyl-ether	3.6
17	77.73	329	314; 299	quercetin-dimethyl-ether	4.0
18	80.13	299	200; 255	artepillin C	7.4
Cinnamic Acid Derivatives					**30.5**
Flavonoids (Area % 20.8)				**Flavonols and Dihydroflavonols**	**17.0**
				Flavones and Flavanone	**3.8**

Tab.5. Identified compounds in green propolis by RP-HPLC-PDA-ESI-MSn analyses, registered at 330 nm. (* RT = retention time).

Peak *n*	RT * (min)	*m/z*	MS^2 *m/z*	Proposed Structure	Area %
1	2.10	225 (179 and 46)	179	caffeic acid	1.1
2	23.00	193	193; 149; 134	ferulic acid	6.3
3	32.46	137	93	p-hydroxybenzoic acid	8.5
4	33.76	285	241; 257	kaempferol	1.9
5	39.26	271	253; 243; 151; 165; 107; 225	pinobanksin	3.7
6	54.92	269	117; 149; 225	apigenin	1.8
7	56.04	315	300; 228	quercetin-3-methyl-ether	0.7
8	59.98	329	314; 299; 285	quercetin-dimethyl-ether	0.6
9	61.19	255	213; 187; 151	pinocembrin	5.1
10	68.72	313	253; 271; 299	pinobanksin-3-*O*-acetate	1.0
11	71.35	253	209	chrisin	7.8
12	73.06	269 (538)	227	galangin	7.1
13	75.20	283	239; 268	galangin-5-methyl-ether	1.1
14	76.48	327	253; 271	pinobanksin-3-*O*-propionate	0.9
15	78.83	373	279; 161; 277; 256; 305; 258	not identified	
16	79.71	341	271; 253	pinobanksin-3-*O*-butyrate	0.5
17	80.33	389	295	not identified	
18	81.58	355	253; 271; 225	pinobanksin-3-*O*-pentanoate	0.2
Hydroxycinnamic Acids					**6.4**
Flavonoids (Area 32.4%)			**Flavonoids and Dihydroflavonoids**		**22.8**
			Flavones		**9.6**
Phenolic Acid					**8.5**

Tab. 5. Identified compounds in brown propolis registered at 330 nm.

Identification was performed through the comparison of experimental data (chromatographic behavior, UV-Vis, MS and MSn spectra) with the literature, and with commercially available standard compounds, where possible. Figures 9 and 10 show green and brown propolis extract chromatograms, acquired at 330 nm.

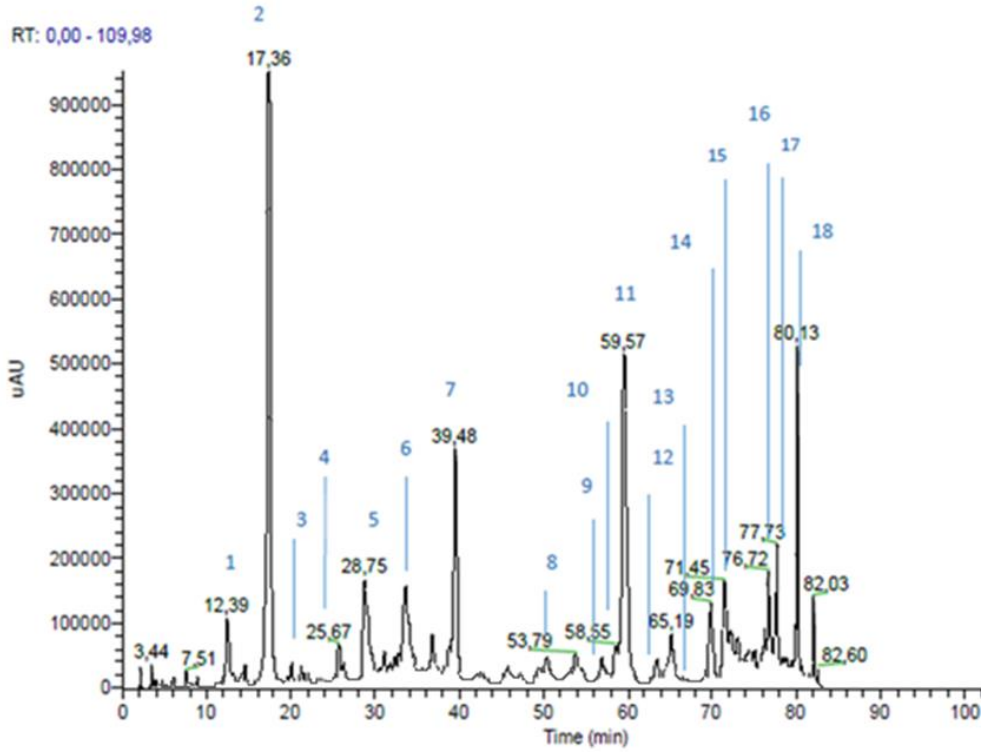

Fig. 9. Green propolis extract chromatogram, registered at 330 nm.

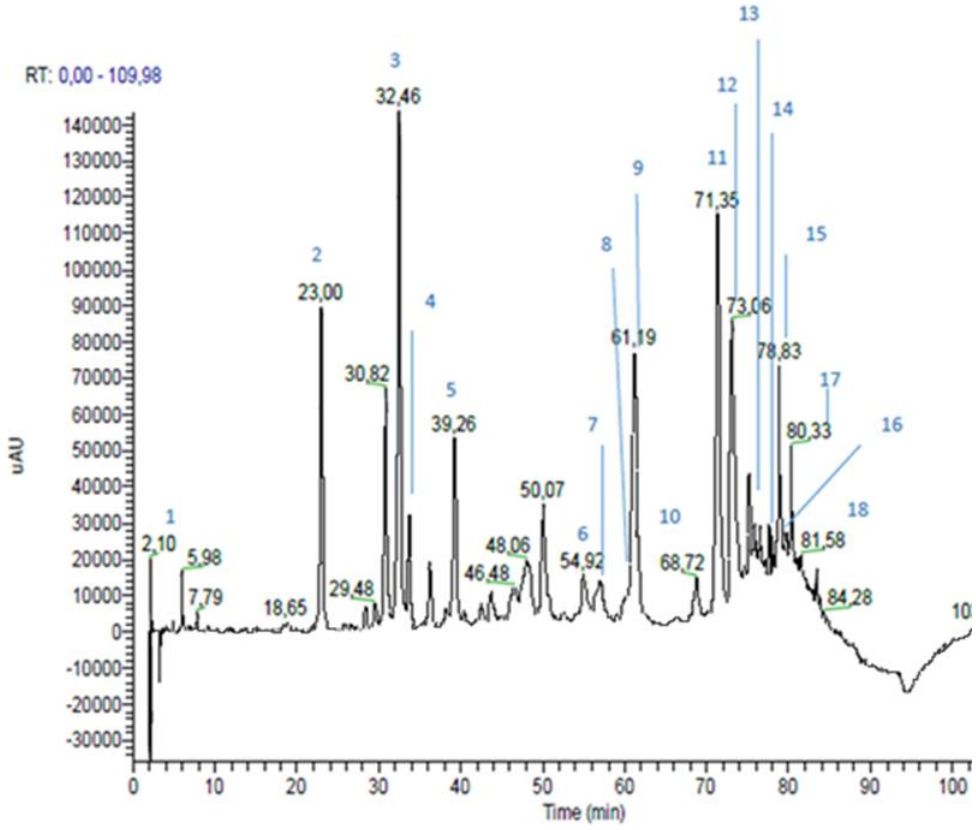

Fig. 10. Brown propolis extract chromatogram, registered at 330 nm.

In the green propolis, six hydroxycinnamic and cinnamic acid derivatives and 11 flavonoids were identified; the percentages of the sum of their peak areas were 30.5% and 20.8%, respectively. In brown propolis, two hydroxycinnamic acids, 13 flavonoids, and one phenolic acid were identified. In contrast to green propolis, in brown propolis the major components were found to be flavonoids (sum of peak area 32.4%), followed by phenolic acids (peak area 8.5%) and hydroxycinnamic acid derivatives (sum of peak area 6.4%). Among the flavonoids, brown propolis showed a higher content of flavonols and

dyhydroflavonols and a content of flavones greater than double those determined in green propolis. These results are in agreement with the results reported by similar studies. In fact, in green propolis, p-coumaric acid and artepillin C are reported to be typical components of green Brazilian propolis (Machado et al., 2016). As far as European brown propolis is concerned, flavonoids are the major components, with flavones (i.e., chrysin and apigenin), flavanones (pinocembrin) and flavonols (galangin) being the most common brown propolis components (Volpi & Bergonzini, 2006)

Cell viability test

For the determination of propolis non-cytotoxic concentrations, MTT 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.

miRNA

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. In particular, 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 (green propolis: $\chi 2 = 17.56$, $df = 3$, $p < 0.001$; brown propolis: $\chi 2 = 13.27$, $df = 3$, $p = 0.004$), when compared to the control sample (figure 11).

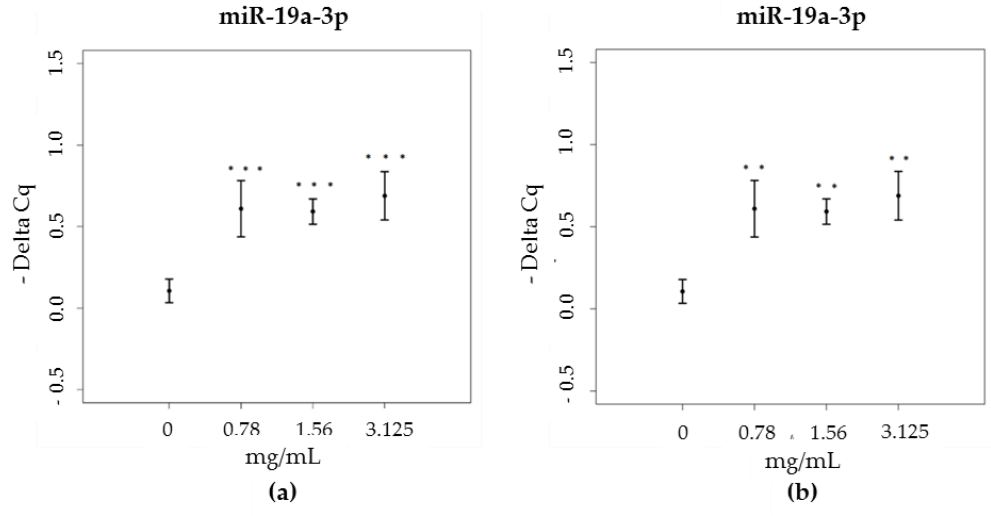

Fig.11. miR-19a-3p expression levels (expressed as difference of Cq – Delta-Cq) in HaCat cells, treated with increasing concentrations of (a) green propolis extract (0.78–3.125 mg/ml) and (b) brown propolis extract (0.78–3.125 mg/ml).

On the other hand, the levels of miR-203a-3p only increased in cell cultures treated with brown propolis, at all tested concentrations ($\chi 2 = 41.92$, df = 3, $p < 0.001$), when compared to the control sample (figure 12). Green propolis did not induce any significant changes in the expression level of miR-203a-3p (data not shown).

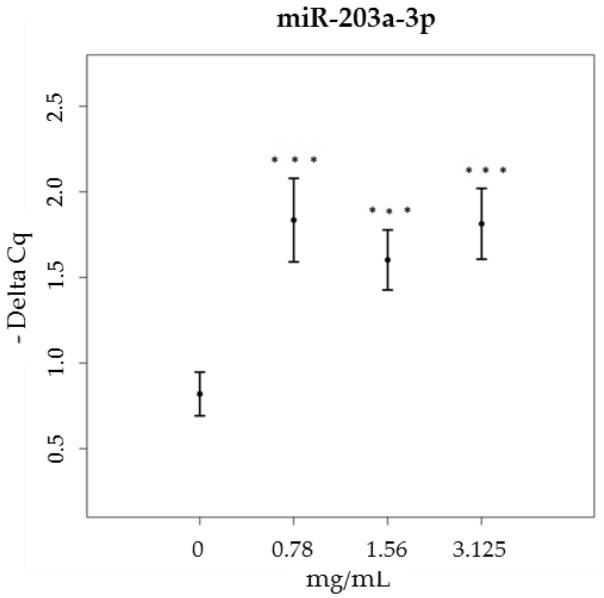

Fig.12. miR-203a-3p expression levels (-Delta Cq) in HaCat cells, treated with increasing concentrations of brown propolis extract (0.78–3.125 mg/ml).

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 (green propolis: $\chi 2 = 11.28$, df = 3, p = 0.01; brown propolis: $\chi 2 = 12.90$, df = 3, p = 0.004), compared to the control sample (figure 13).

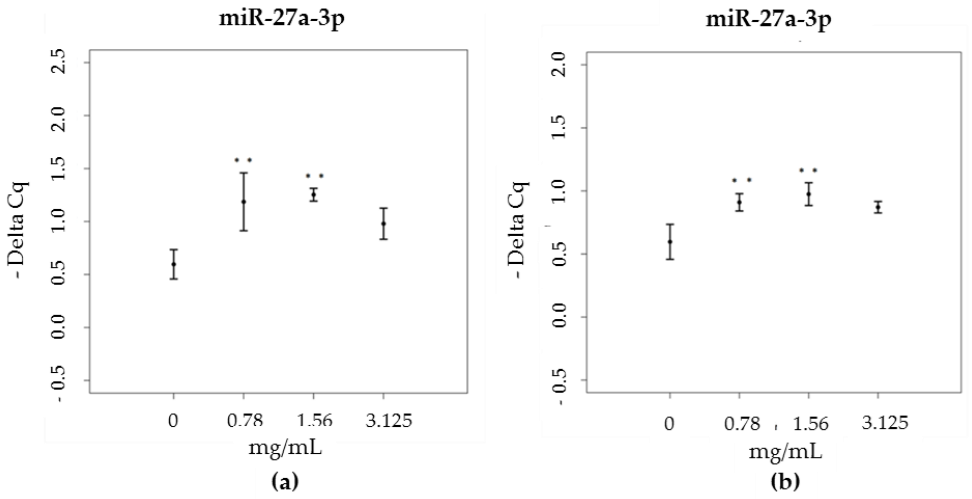

Fig.13. miR-27a-3p expression levels (-Delta Cq) in HaCat cells, treated with increasing concentrations of (a) green propolis extract (0.78–3.125 mg/ml) and (b) brown propolis extract (0.78–3.125 mg/ml).

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 ($\chi 2 = 25.63$, df = 3, $p < 0.001$), compared to the control sample (figure 14, data not shown for miR-17-3p expression levels of green propolis treated cells).

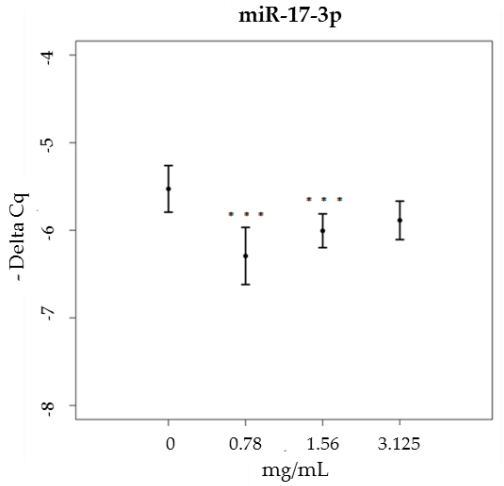

Fig.14. miR-17-3p expression levels (-Delta Cq) in HaCat cells, treated with increasing concentrations of brown propolis extract (0.78–3.125 mg/ml).

mRNA and Proteins

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, we 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 ($F = 16.181$, $p < 0.001$; Tukey, $p < 0.05$) (figure 15a). Conversely, green propolis did not induce any significant changes in mRNAs coding for TNF-α (data not shown). 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.

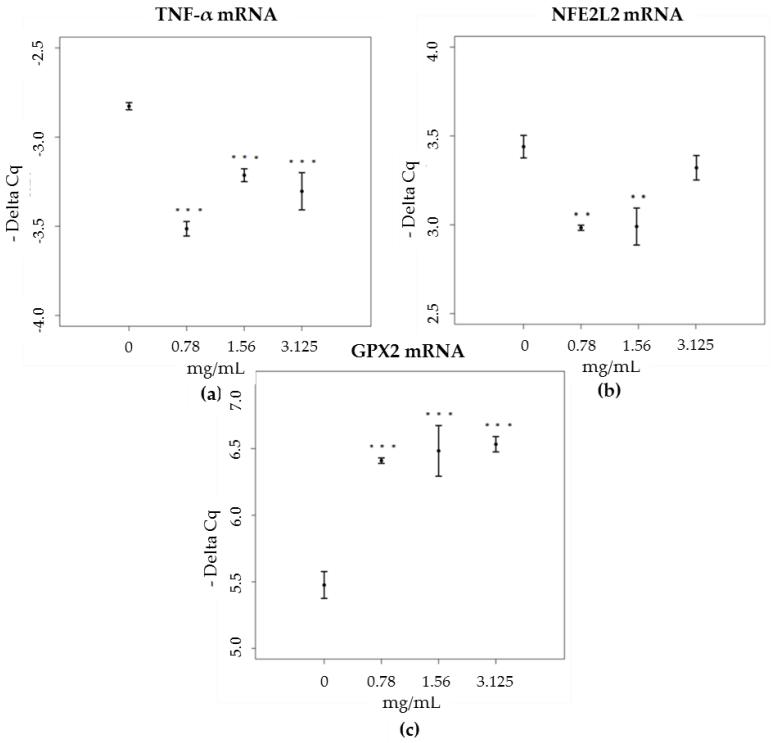

Fig.15. Expression levels (-Delta Cq) of mRNA coding for (a) TNF-α, in HaCat cells, treated with increasing concentrations of brown propolis extract (0.78–3.125 mg/ml), (b) NFE2L2, in HaCat cells, treated with increasing concentrations of brown propolis extract (0.78–3.125 mg/ml), (c) GPX2, in HaCat cells, treated with increasing concentrations of brown propolis extract (0.78–3.125 mg/ml).

TNF-α protein concentrations confirmed the expression levels of mRNAs. Significant decrease in expression levels were measured at all tested concentrations for brown propolis ($F = 6.7292$, $p < 0.05$), compared to the control sample (figure 16). For the green propolis treatments, TNF-α

concentrations did not change significantly, which also correlates with the mRNA expression levels registered (figure 16).

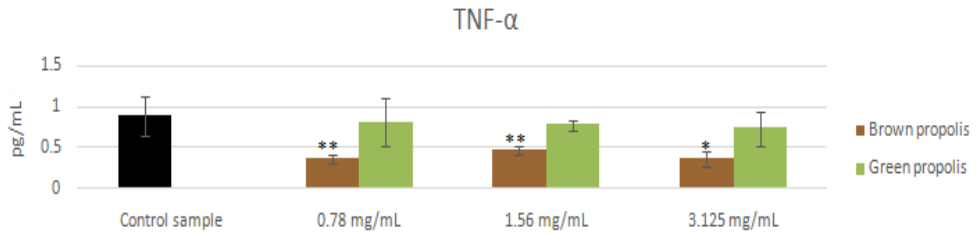

Fig. 16. TNF-α levels in HaCat cells treated with increasing concentrations of brown propolis extract (0.78–3.125 mg/ml). * Indicates statistically significant differences ($p < 0.05$) between treated and untreated cell cultures as reported in the text. ** Indicates statistically significant differences ($p < 0.01$) between treated and untreated cell cultures as reported in the text.

For miR-27a-3p, we studied changes in the expression levels of mRNA coding for NFE2L2. As expected, we 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 ($F = 4.406$, $p < 0.05$) (figure 15b). Green propolis did not induce any significant changes in mRNAs coding for NFE2L2 (data not shown).

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 ($F = 9.4892$, $p < 0.05$). In agreement with the mRNA expression levels (figure 17), the green propolis treatment did not generate any significant changes in the concentration level of the protein, compared to the control sample (figure 17).

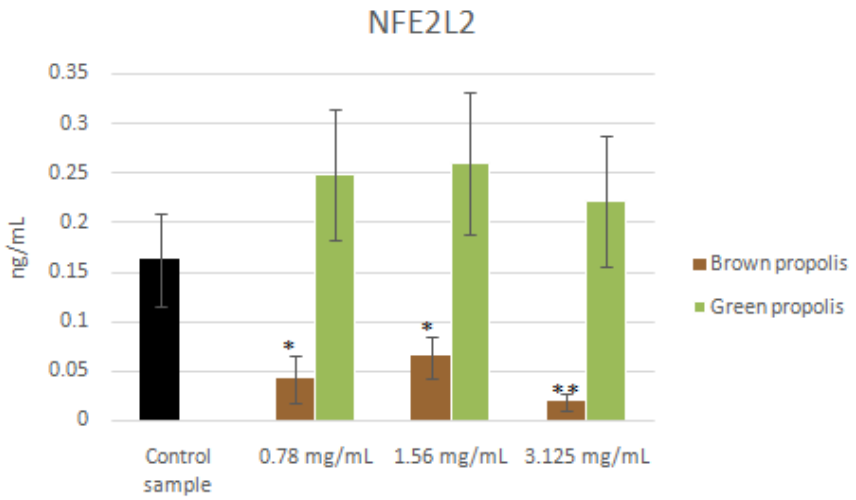

Fig. 17. NFE2L2 levels in HaCat cells treated with increasing concentrations of brown propolis extract (0.78–3.125 mg/ml). * Indicates statistically significant differences ($p < 0.05$) between treated and untreated cell cultures as reported in the text. ** Indicates statistically significant differences ($p < 0.01$) between treated and untreated cell cultures as reported in the text.

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 ($F = 20.228$, $p < 0.001$; Tukey, $p \leq 0.001$), which agrees with the expression trends of the corresponding miRNA (figure 15c).

Brown propolis treatment did not induce any significant changes in GPX2 concentrations compared to the control sample. These results suggest that GPX2 synthesis is regulated by molecular mechanisms which have not been taken into account in this study.

Discussion

Anti-inflammatory activity is one of the most studied properties of propolis. Many investigations have been performed in recent years on the effects of propolis on inflammation, in both *in vitro* and *in vivo* conditions, though the mechanism is still unclear at the molecular level.

In a model system mimicking physiological conditions, this investigation has found that brown propolis exerts anti-inflammatory activity through an epigenetic mechanism of action, being able to increase the expression levels of miR-19a-3p and miR-203a-3p, downregulate mRNA coding for TNF-α and downregulate TNF-α itself—a well-known proinflammatory cytokine involved in the initiation and propagation phases of inflammatory response—by the induction of NF-kB, which is in turn involved in many biological processes, such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis (Chang et al., 2017). To the best of our knowledge, no studies have previously been performed on the anti-inflammatory activity of brown propolis on cell cultures under physiological conditions. In fact, studies performed thus far on the anti-inflammatory activity of brown propolis were carried out in model systems, in which inflammation was induced by pro-inflammatory agents (Bufalo et al., 2014; Erturkuner et al., 2016; Khayyal et al., 2015; Wang et al., 2014). Our results, therefore, are the first to show brown propolis exerting anti-inflammatory activity in physiological conditions and decreasing the expression of a pro-inflammatory cytokine through an epigenetic mechanism of action. This result suggests that brown propolis exerts a protective effect in healthy subjects, avoiding the development of chronic

inflammation, which is a common pathological basis for many diseases, including cardiovascular disease, diabetes, and cancer.

Moreover, our investigation showed that green propolis increases the expression levels of miR-19a-3p, but does not significantly modify mRNA and TNF-α expression levels. These results are in agreement with those obtained by Kathleen et al. in 2014, who investigated TNF-α in both inflamed and non-inflamed cell cultures (mouse odontoblast-like cells, MDPC-23; macrophages, RAW264.7 and osteoclasts). They confirmed that green propolis was able to reduce TNF-α in inflamed cell cultures, as shown by other previous investigations (Neiva et al., 2014; Takeshita et al.,2013; Tiveron et al., 2016; Wu et al., 2013; Zhao et al 2016), but was unable to influence TNF-α levels of cells grown in physiological conditions.

To investigate the influence of propolis on oxidative stress, we first studied the NFE2L2 transcription factor, which is encoded by a mRNA, including miR-27a-3p as a validated target. NFE2L2 is a member of the "basic leucine zipper protein" family, which regulates the transcription of genes that contain the antioxidant response element (ARE) as part of their promoter sequence; many of these genes code for proteins involved in the response to damage induced by oxidative stress and inflammation. Under physiological conditions, NFE2L2 is localized in cell cytoplasm, where the Keap1 protein mediates its transfer and degradation (Chen et al., 2015). Oxidative stress promotes the dissociation of Keap1 from NFE2L2, which translocates into the nucleus when freed, there activating the transcription of antioxidant genes. Thus, an increase in NFE2L2 levels is associated with oxidative stress. In our experimental conditions, brown propolis was found to increase the expression levels of miR-27a-3p, confirming

that brown propolis exerts an epigenetic effect. As expected, brown propolis decreased the expression levels of mRNAs coding for NFE2L2 and NFE2L2. Therefore, brown propolis acts by attenuating an oxidative stress marker in the physiological conditions applied.

This result was consistent with that obtained for the expression levels of miR-17-3p, which is involved in the regulation of mitochondrial antioxidant enzymes, namely MnSOD, GPX2 and TRXR2. Mitochondrial antioxidant defenses are responsible for the prevention of damage to cells, caused by free radicals produced by mitochondrial metabolism. The decrease of miR-17-3p after treatment with brown propolis confirms that brown propolis has the capacity to modulate miRNAs involved in protection against oxidative stress. Nevertheless, brown propolis increased the expression levels of mRNAs coding for GPX2, but did not modify the expression levels of this antioxidant enzyme itself, suggesting that the process of GPX2 synthesis is regulated by other molecular mechanisms and that no GPX2 level increase is induced in physiological conditions. In addition, brown propolis did not show any influence on mRNAs coding for the other mitochondrial enzymes, MnSOD and TRXR2.

On the whole, these results are consistent with those obtained in different conditions by Zhang et al. which showed that brown propolis exerts radical scavenging and reducing activities, and is able to induce the nuclear translocation of NFE2L2, which, in turn, can activate the translation of antioxidant genes and phase II detoxication genes, such as HO-1 and GCLM (Zhang et al., 2016).

Green propolis only increased miR-27-3p expression levels and did not induce any modification in miR-17-3p or their mRNAs and related proteins.

The different capacities to modulate the expression levels of miRNAs, mRNAs and proteins involved in the anti-inflammatory response and antioxidant activity, shown by brown and green propolis, can be ascribed to the different polyphenolic profiles of these types of propolis. The most notable difference in the chemical compositions of brown and green propolis is the higher content of flavonoids found in brown propolis, relative to hydroxycinnamic acid derivatives. This difference could be at the basis of the different behaviors. In particular, brown propolis showed higher levels of chrysin and apingenin. A large body of evidence suggests that flavones exert anti-inflammatory and antioxidant activities (Nabavi et al., 2015). A recent study showed that chrysin reduced the levels of TNF-α and other pro-inflammatory cytokines and increased the activity of antioxidant enzymes in *in vivo* conditions (Sprague–Dawley type male rats) (Eldutar et al., 2017). Similar results were achieved in a recent investigation, where, in *in vitro* conditions (RAW-264.7 cell line), it was shown that apigenin reduces TNF-α expression and secretion (Palacz-Wrobel et al., 2017). In addition, apigenin was found to improve the loss of antioxidant enzymes *in vitro*, exerting its activity at gene transcription, protein expression, and enzyme activity levels. Another compound which is abundant in brown propolis, in comparison with green propolis, is the flavanone, pinocembrin. In *in vitro* conditions (hBMEC cell line), pinocembrin regulated the NF-κB signal pathway and inhibited the release of pro-inflammatory cytokines, although it was not able to ameliorate the oxidative stress induced by cell treatment with toxic molecules, such as amyloid-β peptides (Rui et al., 2014). On the other

hand, considering the complexity of propolis, it must be highlighted that probably all these substances as a whole are responsible for the higher activity of the brown propolis.

An *in vivo* investigation on experimental animals is currently ongoing to verify the effects of brown propolis, which showed more promising results than green propolis, against oxidative stress and inflammation.

Conclusions

Based on the results of this study, the antioxidant and anti-inflammatory effects attributed to green and brown propolis could be due 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 and TNF-α and NFE2L2 protein levels.

Propolis bioavailability and *in vivo* antioxidant activity

Materials and method

Sample preparation

For *in vivo* tests, in order to increase the compliance of the animals, a brown propolis M.E.D.® dry extract was prepared and analyzed as previously reported in "improvement of propolis extraction method" section (pages 49-53). 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) (Regan-Shaw et al., 2008).

Experimental design

Adult male mice (c57bl6, 8 weeks old) were subdivided into 4 groups, one was used as control and the other three were subjected to acute bolus with a dosage of 500 mg/kg containing 14 mg of propolis, corresponding to 1 mg of total polyphenols, under anesthesia (intraperitoneal administration of Avertin, 0.024 ml/g) to minimize the suffering of the animals. Following the bolus, blood drawings were performed at different times (30 ", 2, 5, 10, 15, 20, 25, 30, 45, 60 and 120 min) (table 7). Then, the plasma was separated from the corpuscular part and processed for the subsequent analysis of specific components of propolis by HPLC-PAD-ESI-MSn.

Mouse n.	Time 1	Time 2
1	5’	20’
2	10’	25’
3	30’	
4	5’	20’
5	10’	25’
6	15’	30’
7	5’	20’
8	10’	25’
9	15’	30’
10	5’	20’
11	10’	25’
12	15’	30’
13	5’	
14	10’	25’
15	15’	25’

Mouse n.	Time 1	Time 2
16		
17	10’	25’
18	15’	30’
19	30”	45’
20	60’	120’
21	30”	45’
22	60’	120’
23	30”	45’
24	60’	120’
25	30”	45’
26	60’	120’
27	30”	45’
28	60’	120’
29	30”	45’
30	60’	45’

Tab.7. Sampling at different time for acute treatment

At the end of acute tests, mice underwent to chronic treatment for almost one month: three doses of propolis extracts were prepared and incorporated in the experimental animal pellet at three concentrations (500 mg/kg/day, 250 mg/kg/day, 100 mg/kg/day) (Charles River). Each group was treated with one of the obtained pellet ad libitum for 30 days.

During the chronic treatment blood samplings at 10, 20 and 30 days were performed for the evaluation of the circulating levels (HPLC-PAD-ESI-MSn assay) of specific propolis components. At the end of the treatment the mice were sacrificed with cervical dislocation and their liver was used to evaluate the

differences in the expression of proteins involved in antioxidant pathway (ELISA assay) and the presence of propolis metabolites.

Bioavailability: plasma sample preparation for chromatographic analyses

100 µl of mouse plasma were added to 300µl of methanol (Sigma Aldrich) and centrifuged at 13000 rpm (4 °C) to precipitate the proteins. The surnatant was separated and the methanol was evaporated with nitrogen at low temperature. Then the samples were reconstituted with 100 µl of methanol. The samples were centrifuged at 13000 rpm at 4 °C and the surnatants were used for HPLC-UV-DAD-MS analysis.

RP-HPLC-PAD-ESI-MSn analyses

RP-HPLC-PAD-ESI-MSn analyses were carried out using a Thermo Finningan Surveyor plus HPLC system consisting of a quaternary pump, thermostat self-sampler and equipped with a 5 ul injection loop, thermostat per column, UV-DAD detector and ion-trap mass spectrometer with ESI ionization (Thermo Fisher). Excalibur software was used for system control and data analysis. Separation of the propolis components was obtained using a SINERGY^{TM} 4µm Fusion-RP 80 Å (150 x 4.6 mm) column equipped with pre-column (Agilent Technologies, Waldbronn, Germany). The mobile phase employed consists of acidified water with 0.1% formic acid (eluent A) and methanol (eluent B), eluted in gradient as in table 8.

Time (min)	%Eluent A	%Eluent B
0	70	30
10	60	40
30	45	55
50	30	70
55	0	100
60	0	100
65	70	30
70	70	30

Tab.8. RP-HPLC-PDA-ESI-MSn analysis elution method.

The flow rate was set at 0.5 ml/min, the thermostated column at 30 °C while the autosampler at 4 °C. The chromatograms were obtained at wavelengths of 395, 266 and 207 nm. UV-Vis spectra were registered in the range of 200-800 nm.

Calibration curve

To identify and quantify the compounds of interest a calibration curve was prepared using solutions obtained from the dilution of galangin standard solutions in the plasma of untreated mice at concentrations of 1, 2, 4, 8, 12 and 15 ng/ml. The six solutions obtained were analyzed by of HPLC using the analytical method above described.

Extraction of galangin and its metabolites from the liver

At the end of chronic treatment, 500 mg of mice liver were frozen at -80 °C and then ground with 3 ml of acidified (0.1% formic acid) methanol.
The samples were submitted to sonication for 30 minutes and centrifuged at 13000 rpm at 4 °C for 10 minutes.

The surnatant was removed and the methanol was evaporated with nitrogen at low temperature and protected from light. Then the samples were reconstituted with 100 µl of methanol and centrifuged again at13000 rpm at 4 °C for 10 minutes; the surnatants were used for HPLC-UV-DAD-MSn analysis.

Antioxidant enzyme quantification

Liver sample preparation

Frozen liver samples were defrosted and washed with phosphate buffer saline solutions (PBS; Dulbecco' s PBS without Calcium and Magnesium 10 X EuroClone S.P.A). 25 mg of each sample were homogenized with 5 ml of PBS and 1% of Halt protease inhibitor (Thermo Fisher Scientific) in ice (Dounce homogenator). Each homogenized sample was subdivided into five aliquots of 1 ml each and centrifuged at 13000 rpm for 10 minutes at 4 °C. Then the surnatant was used for the quantification of total proteins (BCA tests) and for quantification of antioxidant enzymes (ELISA tests).

Quantification of total proteins content: Bicinchoninic acid assay (BCA)

Bovine Serum Albumin (BSA) was used at different concentrations (750 µg/ ml, 1000 µg/ml, 1500 µg/ ml, 2000 µg/ ml) as standard solution to build a calibration curve.

25 µl of each surnatant sample/standard were loaded into a well plate, then 200 µl of working reagent were added to each well. The microplate was placed carefully on a stirrer for 30 seconds and then incubated at 37 °C for 30 minutes. After some minutes at RT, the absorbance at 562 nm was read. The experiment was performed on 16 animals (4 per treatment and 4 controls).

ELISA test

ELISA tests (Cloud-Clone Corp) were carried out for three antioxidant enzymes using three specific kits for which the procedures were the same while each microtiter plate was pre-coated with the specific antibody (SOD, CAT and glutathione synthase (GSS)). A calibration curve was prepared according to kit instructions, in which the standard was prepared at seven different final concentrations (10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312ng/ml, 0.156 ng/ml) for SOD and GSS, while for the CAT (4,000 pg/ml, 2,000pg/ml, 1,000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml) together with a negative control. 100 µl of each surnatant sample/standard were loaded into a well plate.

The ELISA plate was covered with a plate sealer and incubated for 1h at 37 °C. Subsequently the exceeding samples/standard were aspirated and 100 µl of Detection Reagent A was added. The plate was further incubated for 1h at 37 °C. Each well was washed three times with a wash buffer, then 100 µl of Detection Reagent B was added. The plate was incubated for 1h at 37 °C. Each well was washed five times with a wash buffer, then 90 µl of substrate solution was added and the plate was incubated for 10-20 minutes at 37 °C; at the end of this step, 50 µl of stop solution was added and the plate was immediately read at 450 nm.

Statistical analyses

For statistical analysis, ANOVA variance analysis and Tukey test were selected. The values reported represent the mean ± DS of the technical quadruplicate and triplicate.

Results

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 amount of total polyphenols.

In this sample the amount of total polyphenols was 7.21 mg/g (HPLC-UV-DAD-MS vs Gal) as reported in table 9 and figures 18 and 19.

	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.9. Amount of total and relative polyphenols (w/w) in dry extract of brown propolis identified through MS spectrum and quantify using HPLC-UV vs GAL.

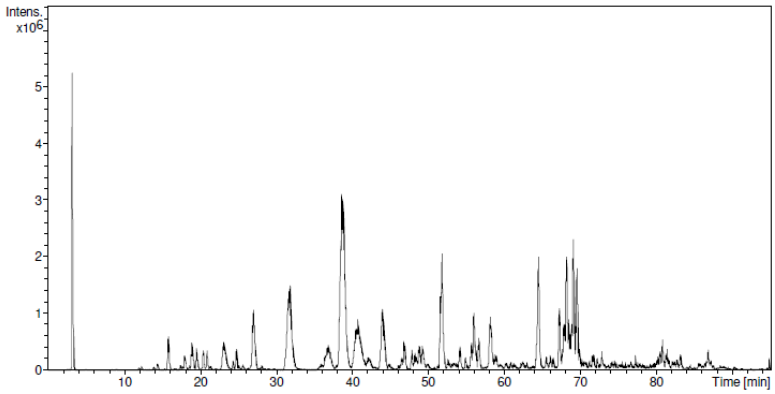

Fig.18. Brown propolis dry extract MS chromatogram.

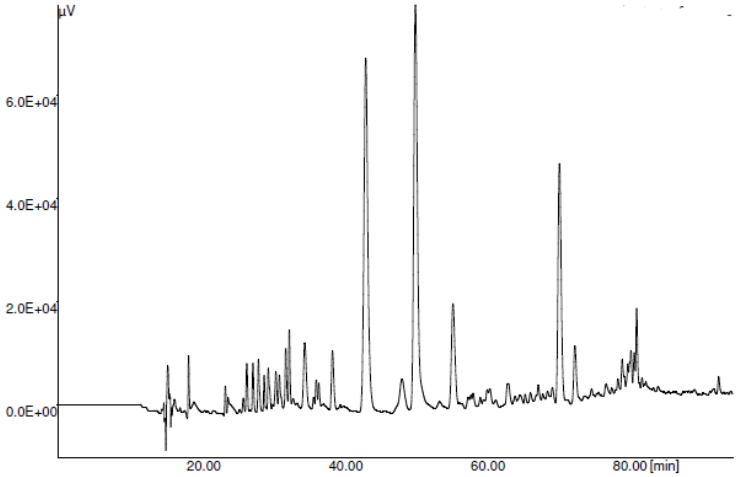

Fig.19. Brown propolis dry extract UV chromatogram.

To study the bioavailability of brown propolis, the galangin was chosen as marker since it is the principal polyphenol occurring in the sample. 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 as showed in table 10 and figure 20.

Conc. (µg/ml)	**Area1**	**Area2**	**Area3**	**Average**	**STD**	**RSD**
1	52544	56438	50604	53195.333	2971.037	5.585
2	114394	133485	117431	121770	10258.497	8.424
4	259185	261624	245570	255459.666	8651.087	3.386
8	493650	495379	538513	509180.666	25417.251	4.991
12	868491	807788	851709	842662.666	31346.302	3.719
15	966338	1099858	1018883	1028359.667	67262.567	6.540

Tab.10. AUC per each known concentration, average and STD values.

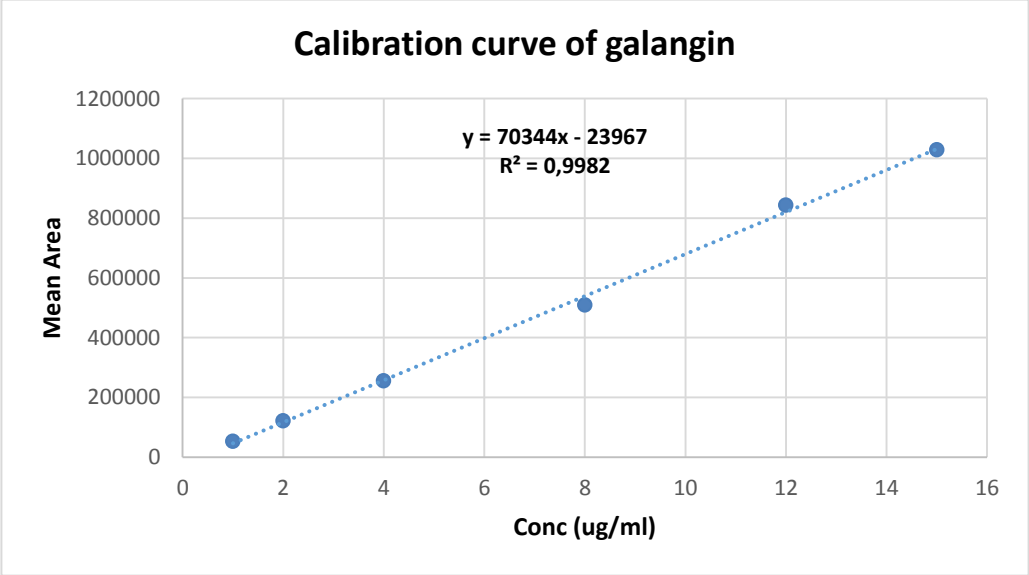

Fig.20 Calibration curve of galangin in plasma.

HPLC-UV-DAD-MS analysis showed that galangin was not found in plasma of treated mice. Galangin-glucuronide was identified (RT 53.34 min; *m/z* 445) using HPL-ESI-MS as reported in figure 21. The fragmentation of the parent ion, reported in figure 23 showed *m/z* value equal to 269, 157, 305, 361 as also reported in literature (Chen et al., 2015).

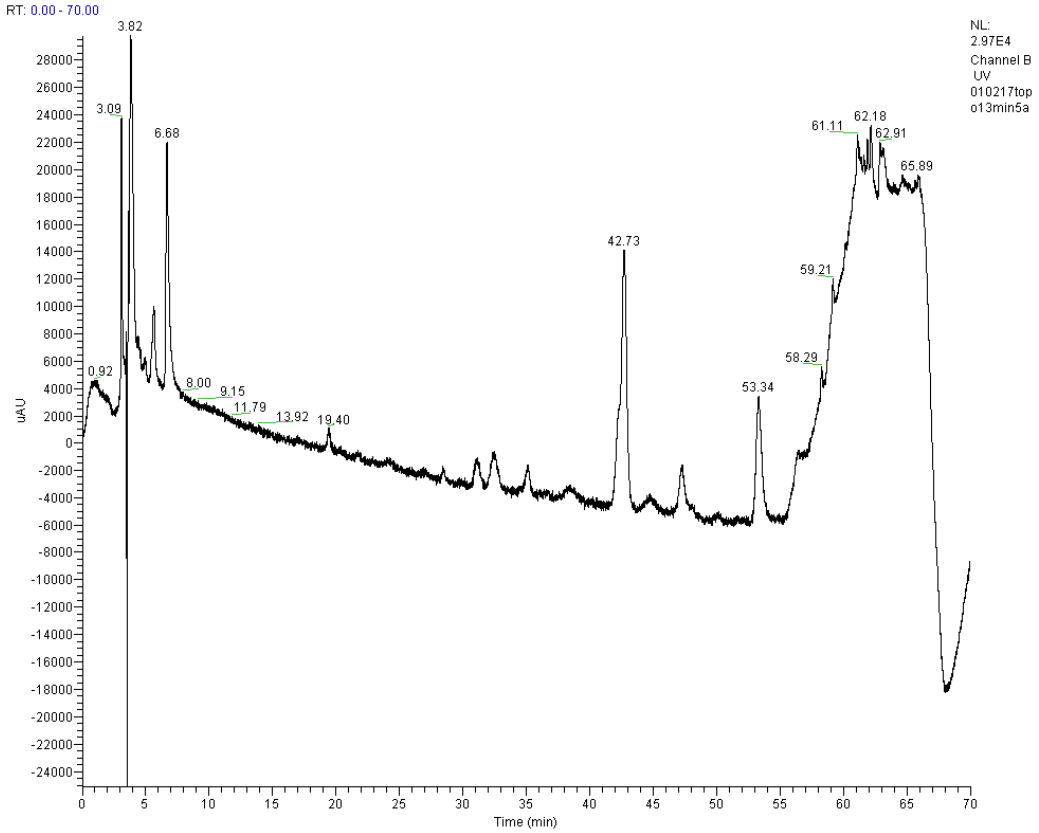

Fig. 21. chromatogram at 5 min after the acute treatment. Plasma sample of mouse n.13

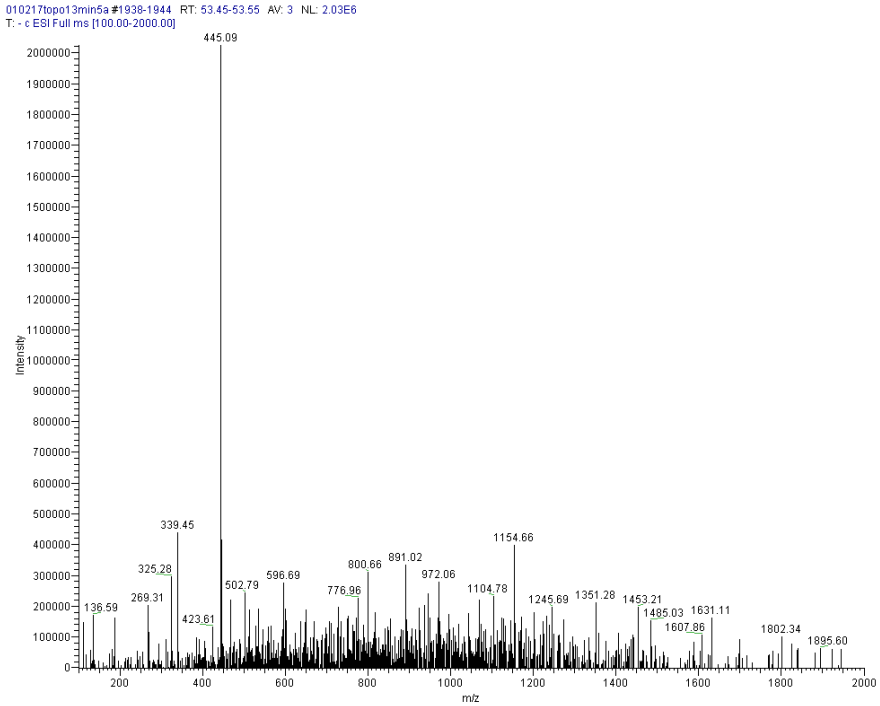

Fig.22. MS spectrum of peak with RT 53,34.

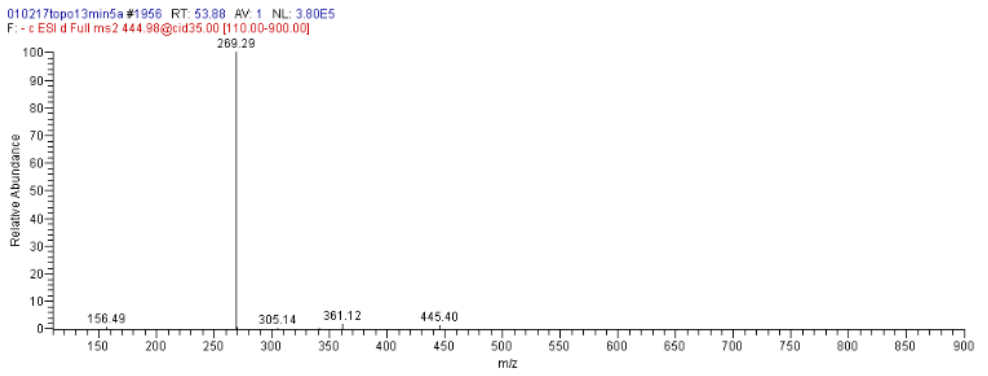

Fig.23. MS/MS spectrum from the fragmentation of the parent ion with *m/z* 445.

To quantify the galangin glucuronide the calibration curve of galangin previously reported was used (figure 20). Neither galangin nor its metabolite (galangin glucuronide) were found in the plasma samples after sampling at 30 sec and 2 min from the propolis administration.

The concentrations of galangin glucuronide, expressed as galangin, calculated were reported in table 11.

Considering these results, 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 (figure 24).

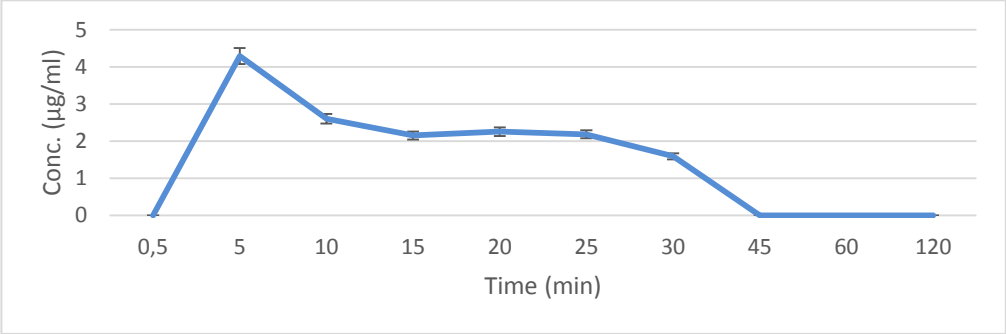

Fig.24. Bioavailability of galangin glucuronide

	Time (min.)									
Mouse	**0.5**	**5**	**10**	**15**	**20**	**25**	**30**	**45**	**60**	**120**
1		3,25			1,93					
2			3,73			2,09				
3							1,81			
4		2,45 2.24 2.48			2.60 2.35 2.34					
5			3.23 3.28 2.93			2.42 2.20 2.08				
6				2.59 2.75 2.49			1.60 1.46 1.59			
7		4.76 4.78 1.97			2.23 2.25 2.11					
8			2.5 2.01 1.94			1.72 1.75 1.62				
9				1.73 1.73 1.60			1.29 1.31 1.29			
10		5.05 5.38 4.88			2.44 2.56 2.66					
11			2.46 2.35 2.33			2.12 1.85 2.13				
12				1.73 1.89 1.68			2.12 1.85 2.13			
13		4.70 4.51 4.36								
14			2.20 2.45 2.40							
15										
16		6.11			1.98					

		6.12 5.53			1.96 1.86					
17			2.84 2.70 2.62			2.37 2.34 2.40				
18				2.11 2.11 2.14			1.43 1.36 1.40			
19	0 0 0									
20									0 0 0	0 0 0
21	0 0 0							0 0 0		
22									0 0 0	0 0 0
23	0 0 0									
24									0 0 0	0 0 0
25	0 0 0							0 0 0		
26									0 0 0	0 0 0
27	0 0 0							0 0 0		
28									0 0 0	0 0 0
29	0 0 0									
Average	0	4.28	2.60	2.15	1.89	2.18	1.59	0	0	0
STD	0	1.37	0.50	0.40	0.80	0.32	0.28	0	0	0

Tab.11. Concentration of galangin glucuronide in plasma of mice treated after different timing**.**

Chronic administration of propolis:

Chromatographic analysis of plasma samples after 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 samples HPLC-PAD-ESI-MS was performed under the same conditions used for plasma samples. None of these molecules were detected as reported in figure 25.

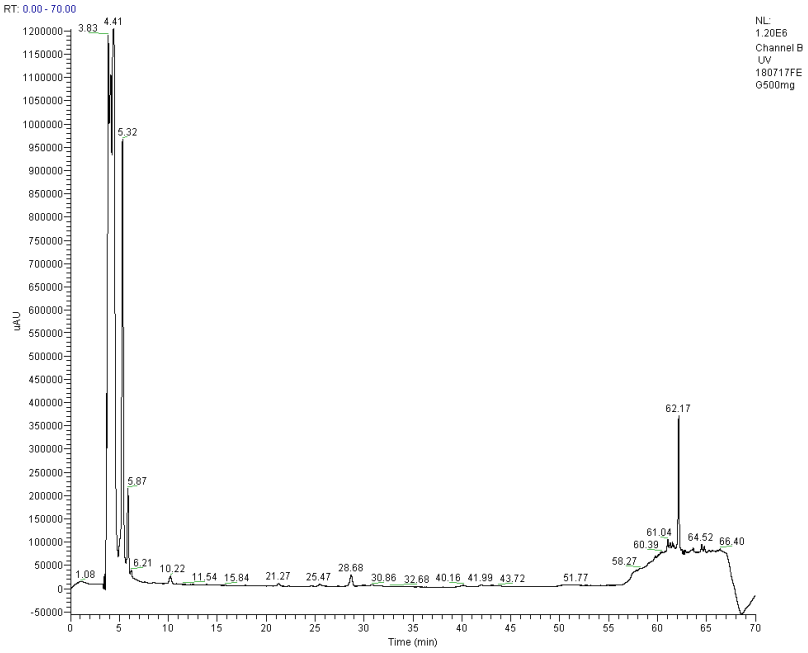

Fig.**25.** Chromatogram with no peak at 47,9 (galangin) or 53.34 (galangin glucuronide) at 266 nm.

Evaluation of antioxidant enzymes in liver samples

To quantify the total protein in the liver samples it was necessary to prepared protein calibration curve. BSA was used as standard with known concentrations (table 12, figure 26).

Samples	**[BSA] µg/ml**	**Absorbance std 1 (nm)**	**Absorbance std 2 (nm)**	**Mean (nm)**	**Normalized mean (nm)**
1	2000	1,366	1,357	1,361	1,25
2	1500	1,084	1,069	1,076	0,96
3	1000	0,784	0,78	0,782	0,67
4	750	0,622	0,637	0,629	0,52
5	500	0,459	0,457	0,458	0,35
6	250	0,279	0,285	0,282	0,17
7	125	0,191	0,193	0,192	0,081
blank	0	0,111	0,111	0,111	0

Tab.12 Absorbance value of BSA at known concentrations

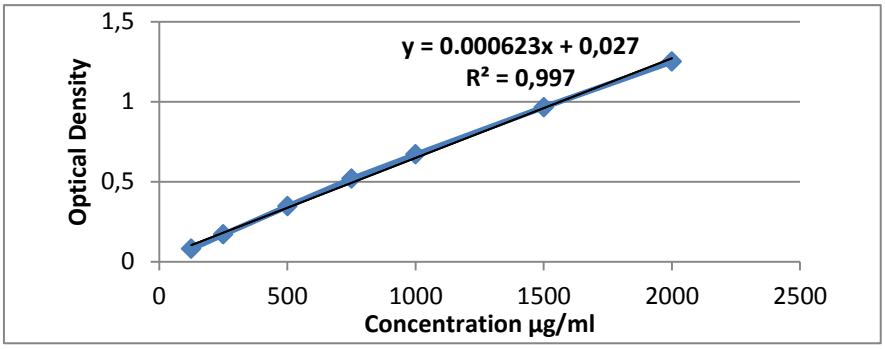

Fig.26 Calibration curve of BSA

Using this calibration curve, the quantification of total proteins in liver samples was calculated. The analyses were performed four times; average values of total protein concentrations and standard deviations are reported in the following tables 13.

Samples	Chronic treatment (mg/kg)	Average concentrations (µg/ml)	STD
17	100	680,1	± 85,5
18	100	648,4	± 82,6
19	100	634,4	± 36,7
23	100	543,3	± 43,6
15	250	408,9	± 46,2
16	250	599,9	± 87,9
20	250	637,6	± 22,1
21	250	555,3	± 24,1

5	500	445,4	± 19,1
6	500	636,8	± 75,8
10	500	574,6	± 37,7
12	500	594,3	± 28,9
8	CTRL	602,7	± 22,1
9	CTRL	526,0	± 10,6
11	CTRL	600,3	± 28,2
22	CTRL	630,8	± 20,1

Tab.13. Statistical analysis of BCA assay.

Superoxide Dismutase (SOD)

The ELISA analyses allowed the quantification of SOD starting from a calibration curve reported below (figure 27).

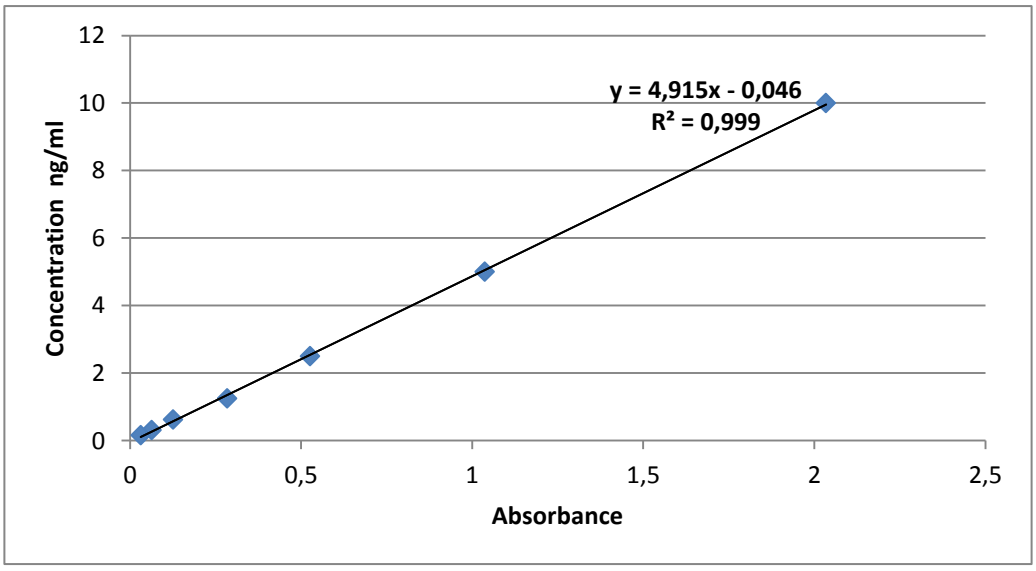

Fig.27 SOD calibration curve.r^2= 0.999

Each sample was analyzed four times. The average of SOD concentration and the standard deviation were calculated per animal and then per group of treated animals with the same concentration of propolis. These results were normalized on total proteins previously calculated through the BCA method. This normalization allowed to express the finally results in µg/mg of total proteins as reported in the table 14.

Treatment	Average concentration (ng/mg)	STD (ng/mg)
Control	7,133	± 1,096
Group 100 mg/kg	7,852	± 1,084
Group 250 mg/kg	8,772	± 0,456
Group 500 mg/kg	7,234	± 0,893

Tab.14. Mean concentration of SOD per each group normalized on total proteins previously calculated through the BCA method and their standard deviation.

Statistical analyses highlighted statistically significant differences between the control group and mice treated with 250 mg/kg of propolis (Anova : F = 4.0738, P = 0.0106, Tukey = 0.014988* P < 0,05) and between the group of mice treated with 250 mg/kg and 500 mg/kg (Anova: F = 4.0738, P = 0.0106, Tukey = 0,0250845* P < 0,05) (figure 28).

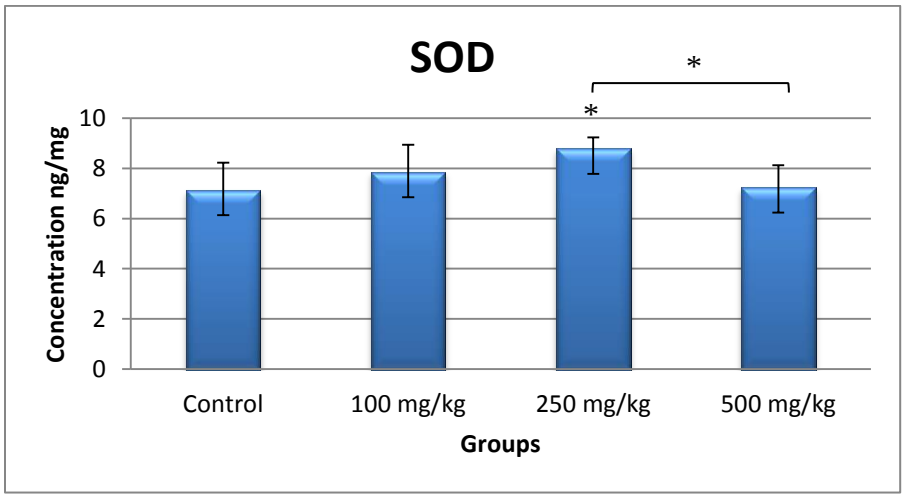

Fig.28. SOD concentration per each treated group

Catalase (CAT)

The ELISA analyses allowed the quantification of CAT starting from a calibration curve reported below (figure 29).

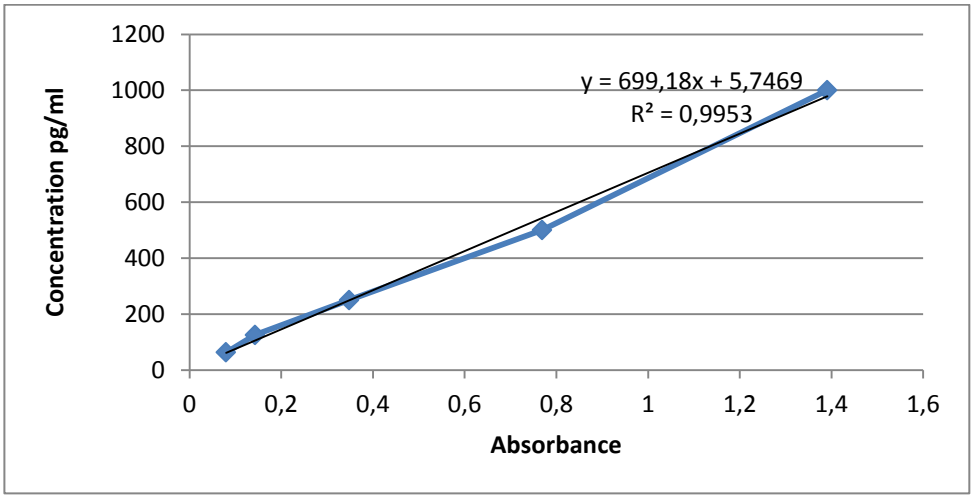

Fig. 29. CAT calibration curve. r^2= 0.995

Each sample was analyzed four times. The average of CAT concentration and the standard deviation were calculated per animal and then per group of treated animals with the same concentration of propolis. These results were normalized on total proteins previously calculated through the BCA method. This normalization allowed to express the finally results in pg/mg of total proteins as reported in the table 15.

Treatment	Average pg/mg	STD pg/mg
Control	538,426	± 226,483
Group 100 mg/kg	745,557	± 309,586
Group 250 mg/kg	751,789	± 170,694
Group 500 mg/kg	581,225	± 328,414

Tab.15. Mean concentration of CAT per each group normalized on total proteins previously calculated through the BCA method and their standard deviation.

Statistical analyses did not highlight any statistically significant differences among all groups using Anova test (Anova F = 2.0612, P = 0.119) (figure 30).

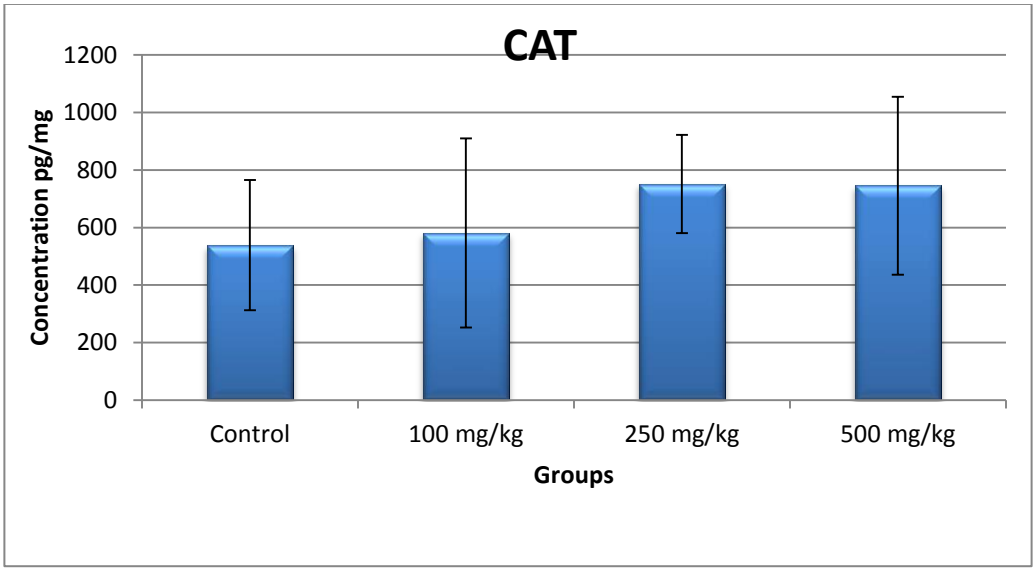

Fig. 30: CAT concentration per each treated group

Glutathione synthetase (GSS)

The ELISA analyses allowed the quantification of GSS starting from a calibration curve reported below (figure 31).

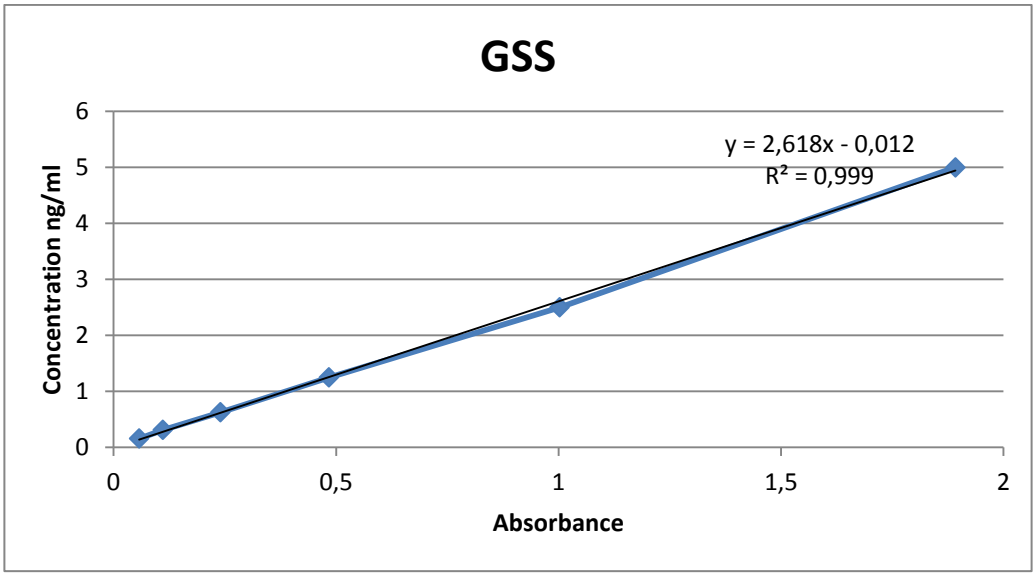

Fig.31. GSS calibration curve. r^2= 0.999

Each sample was analyzed four times. The average of GSS concentration and the standard deviation were calculated per animal and then per group of treated animals with the same concentration of propolis. These results were normalized on total proteins previously calculated through the BCA method. This normalization allowed to express the finally results in ng/mg of total proteins as reported in the table 16.

Treatment	Average ng/mg	STD ng/mg
Control	3,969	± 1,209
Group 100 mg/kg	3,682	± 0,741
Group 250 mg/kg	3,418	± 0,600
Group 500 mg/kg	3,188	± 0,608

Tab.16. Mean concentration of GSS per each group normalized on total proteins previously calculated through the BCA method and their standard deviation.

Statistical analyses did not highlight any statistically significant differences among all groups using Anova test (Anova : F = 1.9872, P = 0.1298) (figure 32).

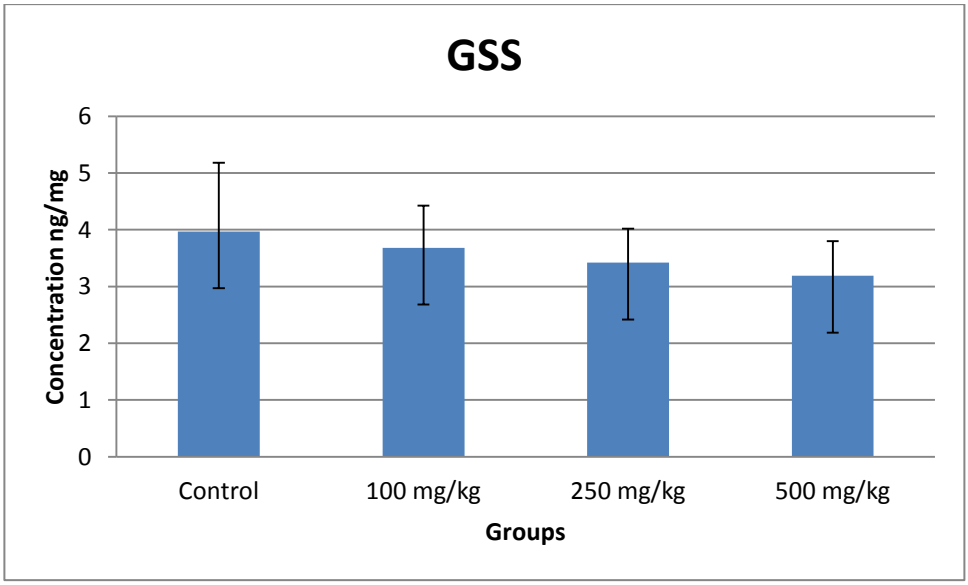

Fig. 32. GSS concentration per each treated group.

Discussion

In poplar brown propolis, the main pharmacologically active constituents are the flavonoids. In raw propolis the most common and abundant flavonoid is galangin (Volpi et al., 2006, Maciejewicz et al., 2001) as well as in the extract used for this *in vivo* study. In light of this, the first aim of this investigation was to evaluate the bioavailability of propolis using galangin as marker in mice (*Mus musculus*) after acute and chronic treatments.

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 (Barrington et al., 2009; Crespy et al., 2003), the quantification of galangin glucuronide was carried out. In particular, 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.

Conclusions

Only few information are present in literature on propolis bioavailability and activity of its metabolites. The study of systemic effects of propolis became important to determine the metabolism of these extracts which are often used in oral pills, capsules, tablets and so on. Since flavonoids are the most active compounds characterizing brown propolis and the galangin is the most abundant, it was used as chemical marker to carry on the bioavailability study. Galangin was not found in plasma samples, while after few minutes from acute propolis administration, the galangin glucuronide, its metabolite, was detectable; this suggests that propolis was absorbed and immediately metabolized.

Chronic administration of propolis was able to increase the endogenous antioxidant defenses in mice. This result was obtained with healthy animals and in physiological conditions, but other studies are needed to evaluate possible changes in pathological conditions.

Antimicrobial activity of propolis extracts

Materials and methods

Samples preparation

To test the antibacterial activity, three brown propolis syrupy extracts have been prepared, starting from raw materials with different geographical origins. In particular, nine raw brown propolis were selected: three of them come from three different European regions (E1, E2, E3), three from different regions of southern America (sA1, sA2, sA3) and three from different regions of Asia (A1, A2, A3). Each sample was made as follow: A+sA+E.

These three raw mixtures (mix A, mix B, mix C) were extracted using M.E.D.® and the samples (Extract A, Extract B, Extract C) were prepared and analyzed as previously reported in "improvement of propolis extraction method" section (pages 49-53).

Antimicrobial assays

Extract A, Extract B, Extract C and blank sample were weighted, resuspended in 50% (v/v) EtOH/water at the concentration of 50 mg/ml and stored at RT. These stock suspension samples were mixed by vortexing and serial 1:2 dilutions were performed in 50% EtOH. Each dilution was then used to prepare the medium to culture the microorganisms, in particular 0.8 ml of each dilution were added to 7,2 ml agar medium previously equilibrated at 70°C. This system covered a range of concentration from 0.007 mg/ml to 0.872 mg/ml of polyphenols.

After mixing, the agar was poured into 6 mm Petri plates. Positive controls plates contained 0.8 ml 50% EtOH of blank stock suspension were prepared as well.

A cell suspension from a frozen vial was spotted on each plate at about 5x103 CFU/spot.

The media and the growing conditions (reported below) were chosen according to microorganism species: Mueller Hinton Agar (MHA), Todd Hewitt Agar (THA), Brucella Agar supplemented with 5% laked horse blood and 1% hemin and vitamin K (BBA), Sabouraud Dextrose Agar (SAB). Antimicrobial tests were determined according to Clinical and Laboratory Standars institute (CLSI) procedures by the broth dilution method (Performance standards for antimicrobial susceptibility testing, 2017; Methods for antimicrobial susceptibility testing of anaerobic bacteria, 2012).

Antimicrobial activities of brown propolis syrups were tested against an enlarged panel of bacterial species from American Type Culture Collection (ATCC) and clinical isolates (Code L) (IRCCS Policlinico San Donato, San Donato M.se, Italy; Centre Hospitalier Universitaire de Limoges, Limoges, France; International Health Management Associates, Inc., Shaumburg, IL, USA; Micromyx, LLC, Kalamazoo, USA; Ospedale Busto Arsizio, VA, Italy; MM: IRCCS MultiMedica, Milan, Italy;, Rockville, USA; S. Raffaele Hospital, Milan, Italy) provided by Naicons srl, Milan, Italy:

a. Staphylococci:, Methicillin-Sensitive *S. aureus* (MSSA): ATCC25923, Glycopeptide-Intermediate Resistant (GISA) and (MSSA) L3797 *S. aureus* (MRSA-GISA) L3798; *S. hominis* ATCC27844; *S. epidermidis* L147; S. haemolyticus L1730; *S. capitis* Meticillin Resistant (Met-R)

ND021008, *S. xylosus* Met-R ND026108, *S. simulans* ND029808, *S. haemolyticus* Met-R ND040809, *S. haemolyticus* Met-R L1729, *S. aureus* Clinda-inducible erm(A)+ ND053410 ATCC *S. aureus* Community Acquired USA300 Met-R ND054910, *S. aureus* Met-R Macrolides-R ND060411, *S. aureus* Met-R L4064, *S. epidermidis* Teicoplanin-R ND042409, *S. epidermidis* ND052110, *S. epidermidis* ND051710. Medium: MHA. Incubation: aerobic, 24 h, 37°C.

b. Streptococci: *S. pneumoniae* Penicillin Susceptible (Pen-S) and L44; *S. pneumoniae* Penicillin Resistant (Pen-R) L3917 *S. pneumoniae* Clindamycin and Erithromycin Resistant L1542; and *S. pneumoniae* Macrolide and Erythromycin Resistant L1402. Medium: THA. Incubation: aerobic, 24 h, 37°C.

c. Fungi: *Candida parapsilosis* L4119 and L3022; *C. albicans* L4120 and L3023; *C. guillermondii* L2065; *C. kruzei* L2880; and *Aspergillus niger* L53 and L54. Medium: SAB. Incubation: aerobic, 48 h, 37°C.

d. Gram negative bacteria: *Escherichia coli* G1640; L1281; L4242; L47, *Pseudomonas aeruginosa* L1367; *Moraxella catarrhalis* L3292; *Acinetobacter baumannii* L3030. Medium: MHA. Incubation: aerobic, 24 h, 37°C.

e. Bacteria present in normal and pathological vaginal flora: *Gardnerella vaginalis* (L1629, L1622, L1630), *Atopobium vaginae* (ND736, ND737), *Neisseria gonorrhoeae* (L1600, L1601, L1599), Bacteroides fragilis (L1011) and Lactobacillus spp (*L. paracasei* L1693, *L. plantarum* L19, *L.gasseri* ND787, *L. acidophylus* ND786). Two strains of *Clostridium difficile* (L1365; L4013) were added to the anaerobe panel. Medium: BBA. 1% Isovitalex supplement was added for

Neisseria strains. Incubation: anaerobic conditions or CO2 atmosphere (GasPak EZ CO2 Container System, BD), 72 h, 37°C.

f. *Listeria monocytogenes* L1450; *Bacillus cereus* L85, Medium: MHA 37ºC for 24 h. *C. perfringens* L3697; L4053. *Propionebacterium acnes* l1016. Medium: BBA, incubated under anaerobic conditions (GasPackEZ anaerobe Container System, BD) 37ºC for 48 h.

Results

The concentrations of total polyphenols found were very close among all extracts. In particular extract A contains 7.59 % (w/w) of total polyphenols, extract B contains 6.85 % (w/w) of total polyphenols and extract C contains 7.2 % (w/w) of total polyphenols (table 17, figures: 33, 34, 35, 36, 37, 38).

UV	*M/Z*	RT MS	Extract A	Extract B	Extract C
Quercetin	301	12	0,13	1,25	0,94
Apigenin	269	17.1	0,17	0,96	1,45
Pinobanksin	271	20.4	1,16	0,83	0,59
Chrysin	253	40.2	13,25	22	22
Pinocembrin	255	47	1,07	1,4	2,43
Galangin	269	47.9	11,45	14,7	15,31
Total polyphenols % (w/w)			**7,59**	**6.85**	**7.25**

Tab.17. Total polyphenols for extract A (7.59%), extract B (6.85%) extract C (7.25%).

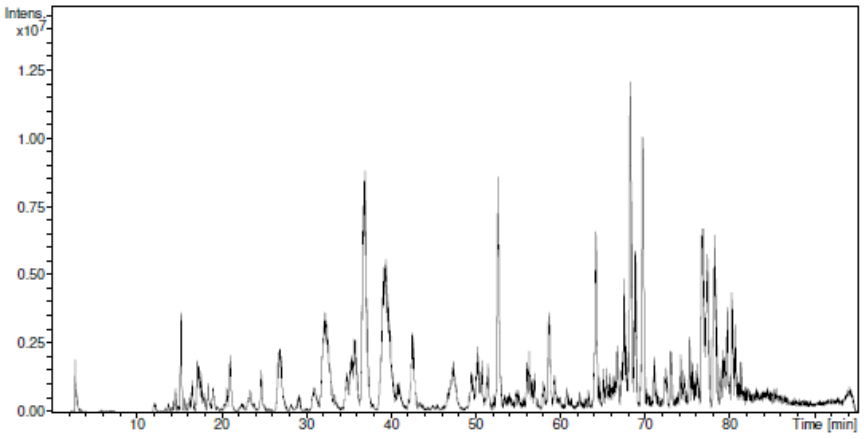

Fig. 33. Brown propolis syrupy extract MS chromatogram of extract A.

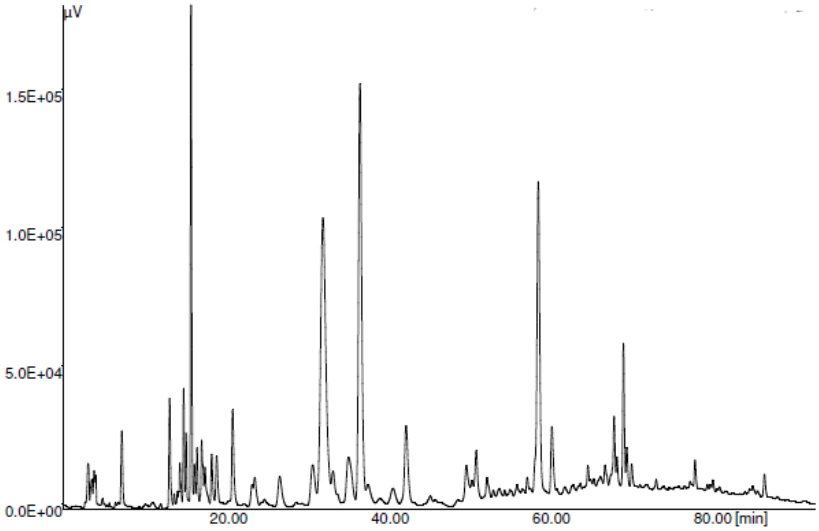

Fig. 34. Brown propolis syrupy extract UV chromatogram of extract A.

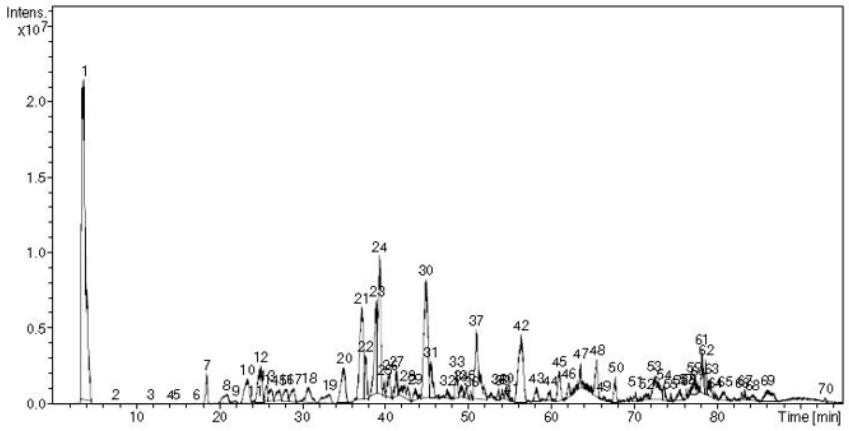

Fig. 35. Brown propolis syrupy extract MS chromatogram of extract B.

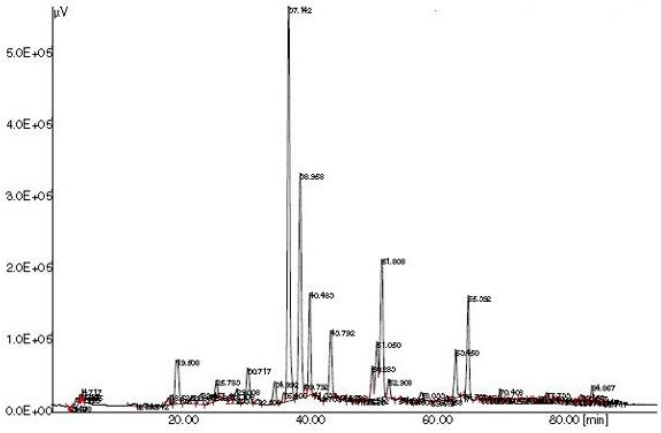

Fig. 36. Brown propolis syrupy extract UV chromatogram of extract B.

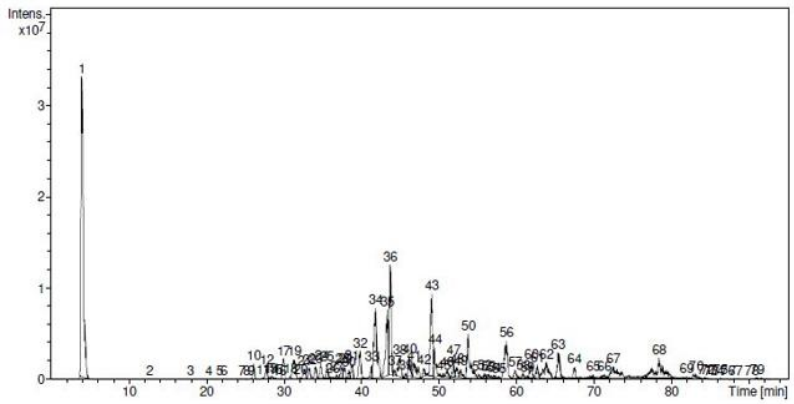

Fig. 37. Brown propolis syrupy extract MS chromatogram of extract C.

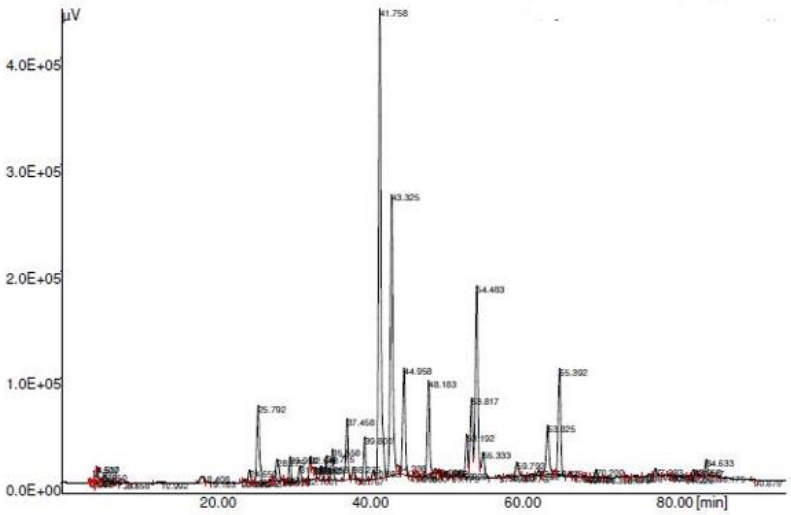

Fig. 38. Brown propolis syrupy extract UV chromatogram of extract C.

The antibacterial activity of these three extracts was tested using a panel of microorganisms containing strains representative of the major families of gram positive, negative and fungi. All extracts showed good activity and comparable Minimum Inhibitory Concentration (MIC) against the same microorganisms. In particular very low MIC value (from 22 to 156 µg/ml) were found in *A. niger*, *S. pneumoniae* Pen-S, *M. catarrhalis, A. vaginae, N. gonorrhoeae*, good activity against Staphylococcus spp and *G. vaginalis* (MIC values some 312 µg/ml). Moderate activity against Candida spp, Clostridium spp and no activity against *B. fragilis, P. acnes* and Lactobacillus spp were found (Table 18).

		MIC (µg/ml)		
STRAIN	**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 Pen-S	L44	22	40	40
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	79	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.18. MIC values of extract A, extract B and extract C.

Since the chemical composition of three tested extracts and the activity showed in the first panel were similar and comparable, the extract A was tested against a wider range of microorganism strains. This second analyses confirmed the previous results (Table 19).

STRAIN	**CODE**	**MIC (µg/ml) Extract A**
Staphylococcus aureus ATCC 6538P		156
Escherichia coli	L4242	312
Staphylococcus aureus GISA MSSA	L3797	625
Staphylococcus aureus GISA MRSA	L3798	312
Staphylococcus haemolyticus MRSH	L1730	312
Staphylococcus hominis ATCC 27844	L323	625
Staphylococcus epidermidis	L147	312
Staphylococcus capitis Met-R	ND021008	156
Staphylococcus xylosus Met-R	ND026108	625
Staphylococcus simulans	ND029808	1250
Staphylococcus haemolyticus Met-R	ND040809	625
Staphylococcus haemolyticus Met-R	L1729	1250
Staphylococcus aureus Clinda-inducible erm(A)+	ND053410	625
Staphylococcus aureus Community Acquired USA300 Met-R	ND054910	625
Staphylococcus aureus Met-R Macrolides-R	ND060411	625
Staphylococcus aureus Met-R	L4064	625
Staphylococcus epidermidis Teicoplanin-R	ND042409	625
Staphylococcus epidermidis	*ND052110*	312
Staphylococcus epidermidis	*ND051710*	625
Streptococcus pneumoniae Cli-Eri-R	L1542	39
Streptococcus pneumoniae Mef (E)+	L1402	39
Streptococcus pneumoniae Pen-S		20
Streptococcus pneumoniae Pen-R	L3917	20

Candida guillermondii ATCC6260	L2065	2500
Candida parapsilosis ATCC22019	L4119	1250
Escherichia coli ATCC 25922	L1281	5000
Escherichia coli	L47	5000
Pseudomonas aeruginosa ATCC 27853	L1367	5000
Acinetobacter baumannii	L3030	5000
Clostridium difficile	L1366	5000
Atopobium vaginae	ND737	156
Lactobacillus paracasei	L1693	5000
Lactobacillus plantarum	L19	5000
Neisseria gonorrhoeae	L1599	156
Gardnerella vaginalis	L1622	312
Listeria monocytogenes ATCC13932	L1450	1250
Bacillus cereus ATCC 10702	L85	312
Clostridium perfringens HSR	L4053	5000
Clostridium perfringens ATCC13124	L3697	2500

Tab.19. MIC values of extract A.

Discussion

Since ancient times, propolis has been used for its antimicrobial properties in folk medicine. Nowadays, due to the increase of antibiotic resistance and thanks to the increased attention of scientific community and consumers on natural products, propolis is considered for new applications and the need of new studies is required to demonstrate the activity of propolis against microbes.

The common scientific approaches to study the antibacterial properties of propolis regards the correlation between antibacterial activity and, in some cases, the metabolic profile (Popova et al., 2017), in others, the isolation of a single compound (Savka et al., 2015; Cushnie et al., 2007), or the raw propolis from a specific region without any chemical characterization (Villanueva et al., 2015; Ambia et al., 2017; Oliveira et al., 2017). However, it is well established that the physicochemical composition of propolis depends on the geographical and botanical origin, season and bee species (as it is reviewed in Huang et al., 2014) and this variability, in turn, could be reflexed on differences of biological activities. For instance, it was demonstrated that MIC values of EEP for S. *aureus* were different according to collection time and locations (Lu et al., 2004). Despite the extracts A, B and C, were made starting from propolis with different geographical origins, they showed a comparable activity against the majority of bacteria strains tested. This result was due to the similar chemical profile in which some polyphenols molecules were always present within a specific percentage range (Volpi & Fachini, 2017; Cowan, 1999; Takaisi et al., 1994). The results suggested that propolis was active against many different bacterial and fungi spp which causes skin, respiratory, vaginal and gastrointestinal infections. The main infective agents responsible for skin

disease are those of the genus Staphylococci (Hanses, 2017) for which propolis M.E.D.® extracts were particularly active (MIC value: 156-625 µg/ml) in both antibiotic susceptible and resistant strains. No activity was found, for the concentrations tested, against *P. acnes* (MIC value: >5000 µg/ml) a common skin organism, usually associated to acne vulgaris, but also to postoperative and device-related infections (Perry & Lambert, 2011).

Traditionally propolis is used in cold flu diseases involving upper respiratory tract infections (Wagh, 2013) in fact propolis M.E.D.® extracts highlighted a strong activity against Streptococci spp in resistant and susceptible strains to Penicillin, and resistant to Clindamycin and Erithromycin, Macrolide and Erythromycin (MIC value: 20-40 µg/ml). *M. catarrhalis* is involved in the upper respiratory tract infections as well as in otitis (Ren et al., 2016). Against these bacteria, propolis M.E.D.® extracts were particularly active (MIC value: 39-78 µg/ml). *A. niger* is a mold reported as one of the cause of pneumonia (Person et al., 2010) against which tested extracts were very active (MIC value: 79-156 µg/ml).

Lactobacilli spp are commonly used as probiotics to maintain vaginal and gastrointestinal health and microflora (Sungur et al., 2017). Propolis extracts did not shown any antibacterial activity against these microorganisms (MIC value: > 5000 µg/ml). On the contrary, propolis M.E.D.® extracts, had very strong activity against vaginal and gastrointestinal pathogens such as *N. gonorrhoeae, G. vaginalis, A. vaginae* isolates (MIC value: 78-312 µg/ml) and good activity against candida spp (MIC value: 1250-2500 µg/ml). These results taken together could suggest a synergic effect of antibacterial activity of propolis M.E.D.® extracts against vaginal and gastrointestinal pathogens and the probiotic activity of lactobacilli whose growth is not inhibited. Even if

propolis M.E.D.® extracts were not active against Clostridium spp strains, it was demonstrated that Lactobacilli are able to prevent *Clostridium difficile*-associated diarrhea (Goldenberg et al., 2013).

Conclusions

The antimicrobial activity of the three brown propolis M.E.D.® extracts was comparable, even if each extract was composed of a mixture of raw materials with different geographical origins. This result could be explained by the presence of the same amount of total polyphenols including quercetin, apigenin, pinobanksin, chrysin, pinocembrin, galangin in all three extracts. Considering the obtained MIC values, brown propolis M.E.D.® extracts can be used in products as natural treatment for high respiratory tract, skin, gut and intimate area infections. Moreover, no information are present in literature on microorganisms previously susceptible to propolis that acquired resistance mechanisms. Thanks to the standardization of propolis extraction method, it is possible to obtain reproducible extract profiles that can be used in future to establish the correlation between quantity of extracted total polyphenols containing a specific fingerprint and the antibacterial activity. Then the use of these propolis extracts could be useful to reduce the use of antibiotics in humans and animals.

FINAL CONCLUSIONS

Propolis is a heterogeneous matrix and the study of its activities, especially for the high variability of its chemical composition, and bioavailability is very complex.

For this reason, M.E.D.® method was developed and setup to prepare standardized extracts with constant composition that could be taken as reference for future studies to obtain comparable results. In addition, using M.E.D.®, it is possible to obtain low alcoholic grade, glycerin and water soluble products with high content of bioactive molecules.

Despite several biological activities are attributed to propolis, the mechanisms of action and the relation between chemical composition and activity is still unknown. The most studied propolis properties are: antioxidant, anti-inflammatory and antimicrobial.

Using standardized M.E.D.® propolis extracts, it was possible to clarify the *in vitro* anti-inflammatory and antioxidant mechanisms, the *in vivo* antioxidant activity and the bioavailability, and the *in vitro* antibacterial activity.

Based on the results of this research, the antioxidant and anti-inflammatory effects attributed to green and brown propolis could be due to modulation of the levels of certain miRNAs involved in these pathways. Brown propolis, which is richer in flavonoids than in hydrocinnamic acid derivatives, was active on all miRNAs tested suggesting that brown propolis has greater epigenetic activity. In turn, the brown propolis showed a superior modulatory capacity in

modifying the expression levels of mRNAs as miRNAs target, and the corresponding proteins as TNF-α, NFE2L2 and GPX2.

The antioxidant activity was also highlighted in *in vivo* studies, in which chronic administration of brown propolis was able to increase the endogenous antioxidant defenses in mice. However, propolis bioavailability studies confirmed that galangin is immediately absorbed and modified as galangin glucuronide, as literature data and neither galangin nor its metabolites are accumulated in the liver.

The most known and studied activity of propolis is the antibacterial one. Using a panel of many microorganisms from clinical isolates and ATCC library, different species and strains, both sensitive and resistant to antibiotics, it was evaluated the antibacterial activity of propolis M.E.D.® extract in which propolis with different geographical origins were mixed to obtain three extracts that shared similar chemical profile and total polyphenol content, in fact the antimicrobial activity of these extracts was comparable.

The results obtained in this thesis, thanks to the standardization of propolis extraction method allowed to obtain reproducible extract profiles, overcoming some limits of the current scientific studies. Moreover, this approach could be taken as reference for future studies to obtain comparable results.

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