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The antimicrobial activity of a propolis extract
Activitatea antimicrobiană a unui extract de propolis

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Abstract

Propolis, along with honey, royal jelly, pollen, pasture, is a product whose prophylactic and therapeutic properties are known and used in folk medicine since ancient times. Propolis is actually the generic name for the resinous substance collected by honey bees from various plant sources. It is made by bees to defend the colony physically, chemically and biologically. The objectives of the research were: Determination of total polyphenols by UV-VIS spectrometry, Determination of the total flavonoid content by colorimetric method and Identification of individual polyphenols in propolis extract. LC-MS analysis of propolis extract was performed within the Interdisciplinary Research Platform

Rezumat

Propolisul, alături de miere, lăptișor de matcă, polen, păstură, este un produs ale cărui proprietăți profilactice și terapeutice sunt cunoscute și utilizate în medicina populară din cele mai vechi timpuri. Propolisul este de fapt denumirea generică pentru substanța rășinoasă colectată de albinele melifere din diferite surse vegetale. Este fabricat de albine pentru a apăra colonia sub raport fizic, chimic și biologic. Obiectivele cercetării au fost: Determinarea polifenolilor totali prin spectrometrie în UV-VIS, Determinarea conținutului total de flavonoide prin metoda colorimetrică și Identificarea polifenolilor individuali din extractul de propolis. Analiza LC-MS a extractului de propolis a fost realizată în cadrul Platformei de Cercetare Interdisciplinară

1. Propolis in therapy

The biochemical composition of propolis gives it unique therapeutic virtues.

Contains pollen, resin, wax, amino acids, lipids, sugars, volatile oils, organic and aromatic acids (cinnamic, caffeic, ferulic acids) active against Gram-positive and Gram-negative organisms.

It also contains vitamins (provitamin A, vitamins B, C, E, PP), minerals (iron, copper, zinc, silicon, calcium, magnesium), flavonoids (quercetin with antioxidant activity), plant hormones, enzymes, and antimicrobial substances.

The development of human society has allowed the knowledge of some very valuable natural resources in bee products (Fig. 1).

Many ancient healers used honey, bee pollen, propolis, royal jelly, beeswax, and venom to treat some diseases [5].

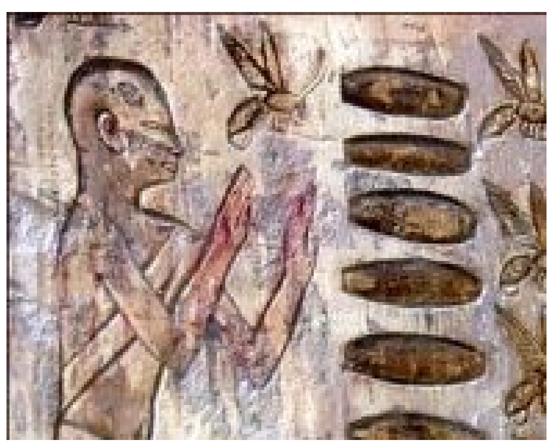

Figure 1. Hieroglyph in Ancient Egypt
Source: https://koreanskincaretips.com/benefits-of-propolis-on-skin/

In the modern era, studies and research on natural resources capable of ensuring the human body's nutritional balance and proper functioning have highlighted the biostimulator, nutritional and therapeutic value of these products.

Of these, propolis is currently one of the most important challenges for the world of nutritionists and the medical world [18].

The healing properties of propolis have been known since ancient times. The product was very well known in ancient Egypt.

A few millennia before Christ, propolis was known to the high priests, the only ones who possessed knowledge of medicine and chemistry and even the art of embalming corpses.

The fact that propolis was also known by the ancient Greeks is proved even by the Greek name of the product: pro (before), polis (fortress) [24].

There is very old evidence that directly refers to propolis.

The famous Greek philosopher Aristotle built a transparent hive because he wanted to know closely the activity of bees.

However, the bees did not want to reveal their secret and covered the inside of the transparent wall with a dark substance - probably propolis.

The origin of propolis was the subject of a controversy between two Roman writers - Pliny and Dioscorides.

The former believed that bees harvest propolis from resins secreted by willow, poplar, chestnut, and other plants, while the latter claimed that it is harvested from styrax.

Thereafter data on propolis is found in the works of Galen and Varron.

In the well-known work *"The Canon of Medical Science",* Abu Ali Ibn Sina (Avicenna) speaks about two types of wax: pure wax and black wax.

In his opinion, pure wax is the one used in the construction of honeycomb walls in which bees grow their young and store honey; black wax is the part of debris in the hive.

It is clear that black wax is nothing but propolis [32].

2. Classification of bee products

Many authors have analyzed the types of bee products [5, 8, 9, 21, 29, 32].

The main classes of bee products are:

Honey is the product made by bees exclusively from flower nectar or sweet juices from other parts of green plants, which they collect, enrich with their substances and process them in a specific way obtaining the product thus defined, which stores it in the cells of the honeycombs in the hive to constitute their energy food.

According to the origin of the raw material, the honey can be of:

- *Vegetable origin (flower honey):*
- *Monofloral origin (acacia, lime, sunflower, mint, raspberry, etc.);*
- *Polyfloral origin (seasonal, associated with many flowering plants).*
- *Animal origin (manna honey).*

The wax is secreted by the cerigene glands of working bees aged between 13-18 days.

At the moment of secretion, the wax is liquid and in contact with the air, it solidifies in the form of clear white scales, very small, which after a certain time becomes yellow [5,33].

Beeswax (produced by *Apis mellifera L.*) is classified in:

- *apiary wax;*
- *press wax;*
- *extractive wax.*

Royal jelly, a secretion of the pharyngeal and mandibular glands of worker bees, is used by them to feed the larvae in the first 3 days, the queen larvae throughout the period until the captivity, and the queens.

Pollen and bee bread

The pollen is collected by the bees from the polleniferous or nectaro-polleniferous plants and stored by them in the honeycomb cells of the hive constituting the bee bread, which represents the protein part necessary to feed the bee family and the young.

The transformation of pollen into bee bread is related to the natural process of conservation of pollen which following some fermentative biochemical processes under the action of certain microorganisms, becomes bee bread.

Propolis is the generic name for the resinous substance collected by honey bees from various plant sources.

The venom is a mixture of the secretion of the venom glands of the worker bees, being stored in the venom bag and eliminated on the outside at the time of the sting.

Apilarnil, a bee product obtained from drone larvae and nutrient content from the honeycomb cells.

3.1. Description of propolis

The word propolis is derived from Greek, in which *pro* = *for* (or in defense) and *polis* = *fortress*, which means the defense of the fortress (in this case, the hive) (Fig. 2).

Another etymology of this word can also be considered, attributing to it a Latin origin (the term is used by Pliny) [19].

Figure 2. Propolis granules
Source: https://www.csid.ro/sanatate/medicina-alternativa/

The construction of the word comes from the assembly of the prefix pro with the meaning *"in purpose, favorable for"* and polis, a form of conjugation of the verb polire (polir in French) = to cover, to glaze, and by deformation lisser = to smooth.

Propolis refers to a whole series of resinous, gummy, and balsamic substances with a viscous consistency, collected from different parts (buds and bark, in particular) of vegetables (especially certain trees) by bees who bring to the hive, and modify them by the contribution of its secretions (mainly wax and salivary secretions) [5, 2, 22].

The bees use propolis as a "plastic" material with which they make the grouting of cracks (smaller than 3-5 mm) and smoothing the rough surfaces inside the hive, fixing and consolidating the combs, polishing the cells especially those from the old combs in which several generations of broods have grown, as well as for covering and embalming the large pests that have entered in the hive, which they kill but cannot evacuate them.

Apart from its plastic role, propolis is used for sanitizing and asepticizing the microclimate in the hive.

3.1.1. The origin of propolis

Regarding the origin of propolis, some theories confirm its double origin:

Internal origin

Propolis is a resinous residue from the first phase of pollen digestion, a process that takes place in a small organ located between the goiter and the middle intestine *("pollen stomach" or "chylus magen")* [15, 30].

The outer shell of the pollen grain is transformed into a balm and regurgitated by the bee in the form of drops, deposited in the hive. The presence of traces and fragments of exine (the outer layer of pollen) in propolis was an argument in this regard.

As a counterargument, some authors show that bees locked in a greenhouse and supplied with abundant pollen did not deposit any propolis [5].

Another striking proof against this theory comes in the case of an apiary located in a place where the vegetation is predominantly composed of *Populus balsamifera*.

The pollen of this species is harvested in spring when the bees consume it immediately, or propolis is not harvested from this tree until the end of the season. It is currently accepted that bees collect propolis from various plant sources in temperate areas in the north.

The main wood tree species producing propolis are:

- poplar (*Populus spp*.),
- beech (*Betula spp*.),
- pine (*Pinus spp*.),
- fir (*Abies spp*.),
- plum (*Prunus spp*.),
- willow (*Salix spp*.),
- elm (*Ulmus spp*.),
- oak (*Quercus spp*.),
- indian chestnut (*A. hippocastanum L*.),
- ash (*Fraxinus excelsior L*.),
- cherry (*Prunus avium*) as well
- sunflower (*Helianthus spp*.,) [5].

Bees probably visit other species of wood tree to harvest this valuable material, but they are still far from being fully inventoried.

The propolis found in the hive does not have the same appearance, the same consistency, and the same smell as the vegetable resins harvested by the bees and examined after the bees were captured in flight; they do not harvest it as a finished product, and bees may add several salivary secretions or enzymes in the same way they do for honey.

Hives located near or in forests propolize much more than those located in plain areas.

Also, bees propolize in autumn and spring more than in summer.

Pollen can hold a few percent, and foreign bodies a variable amount depending on the attention paid to harvesting operations in the hive [18, 33].

Impurities considered normal are small plant particles, harvested by bees with the raw material and hairs on the body of bees, and unwanted particles are wood particles, other mechanical impurities, and fragments of honeycombs, incorporated into propolis during its collection from the hive to beekeepers.

Propolis can be counterfeit mainly with wax, raw conifer resin, or saccharin (rosin).

3.1.2. Propolis production

The tendency of bees to propolize is a breed or ecotype character and even depends on the individual characteristics of some bee families (Fig. 3) [5].

Apis florea and *Apis dorsata* species do not use propolis, also some breeds of *Apis mellifera* species such as *Apis mellifera lamarki,*

Romanian bee (*Apis mellifera carpatica*) have a moderate tendency to propolize compared to bee breeds with more pronounced tendencies to propolize such as:

- Caucasian bee (*A. mellifera caucasica*),
- Saharan bee (*A. mellifera sahariensis*),
- Anatolian bee (*A. mellifera anatolica, A. melifera scutelata)* and others.

Figure 3. Propolis-producing bees
Source: https://www.csid.ro/sanatate/medicina-alternativa/

The bees identify the resin particle to be harvested with the help of antennas, then with their jaws take the piece, pull it, and stretch it in the form of a thin wire until it breaks.

With their feet, they handle the broken particle and store it. This operation is repeated until the propolis grains reach the required size.

During the processing of this sticky material, bees do not stick to it because they secrete a certain substance or a series of substances that help them to deal with this situation.

When unloading it, the bee is helped by other bees, which pull and break the propolis with their jaws and store it where it is needed.

Disposal of propolis can often be done right on the flight board and can take several hours. In the operation of propolis harvesting and unloading, the tongue is not used by bees [5, 12].

Propolis is harvested only at a high temperature, above 20°C, when the viscous matter is soft, easier to collect and handle.

As the temperature drops, the bees stop harvesting propolis.

Propolis-collecting bees are specialized for this work, are small in number and can sometimes be younger than 15 days; rarely performs other operations, but they are also engaged in the cementing activity inside the hive [5].

3.1.3. Propolis harvesting and storage

Homogeneous propolis, intended for valorization, is harvested in the active season, namely at the end of the budding period of each plant species or group of plants with a certain vegetative specificity, located in the same pedoclimatic microzone.

The amount of propolis that can be obtained from a hive varies from one region to another and can be from 100 to 400 grams.

Harvesting propolis for human needs is done by scraping it from the frames, from the gaps, from the plateaus or with the help of propolis collectors.

Scraping propolis is done with a chisel with a knife or sharp scrapers, taking care to obtain a product as clean as possible, without impurities.

Harvesting is usually done during the control of the hives or every 15-20 days when the ambient temperature is at least 20°C.

The harvested propolis will be stored in small spherical lumps that form from harvest, wrapped in foil or plastic bags, then placed in well-lined boxes of waxed paper and kept in a cool place [5, 18, 33]

It is also recommended to keep in colored glass jars, closed with glass stopper, and then paraffined.

Propolis must be protected from moisture and direct exposure to sunlight or other heat sources. It is recommended to keep propolis at a maximum temperature of 20°C, in ventilated rooms, free of moisture and external smells.

The packaging, transport, and delivery of propolis are done in plastic bags placed in wooden crates, with a capacity of 5-20 kg.

3.1.4. Properties of propolis

Organoleptic and physical properties

Propolis is of various categories:

- *common propolis* from different plants
- *polyphyte propolis* and *uniplant* or *monophyte propolis*, harvested from a single plant (species) or from a group of plants located in a certain pedoclimatic zone.

Presentation and identification

Propolis is a resinous substance with a sticky and heterogeneous appearance, it has the appearance of a solid mass of brown color (darker or lighter) or gray-green, and in section, the appearance can be homogeneous or marbled (Fig. 4) [5].

Figure 4. Fresh propolis appearance
Source: https://www.verywellhealth.com/

The *consistency of propolis* is viscous, sticky, hard, brittle, and kneaded between the fingers leaves marks. It softens at a temperature of 30-37 °C, below 15°C it is hard and odorless, at 50-60 °C it melts, and above 78°C it becomes liquid.

Slightly heated in the water bath, it is divided into two very distinct parts: one viscous which settle, and another one liquid (propolis wax) which floats on the surface and is used differently in beekeeping.

The smell is pleasant, characteristic of natural resins and is appreciated organoleptically at room temperature by smelling a whole piece or a sample taken with the drill in the middle of it [5, 18].

The taste is pleasant, often hot, sometimes bitter, slightly astringent, different depending on the origin.

The density is between 1,120-1,136, being partially soluble in alcohol, gasoline, turpentine, acetone, chloroform, ether, ammonia, benzene, trichloroethylene and only a suitable mixture of various solvents allows the almost complete dissolution of its components.

It is insoluble in cold water but is partially soluble in it using various processes.

The most useful solvent for practical needs is 95°, concentrated ethyl alcohol.

One condition is that the propolis to be pure, admitting impurities "barely visible to the naked eye."

Purity is highlighted by solubilization in 95° alcohol.

Physical-chemical properties

Specialized research shows that propolis has a complex but variable biochemical composition, depending on the plant source from which the product was collected by bees [2, 5, 12].

Chemical analysis of propolis (performed by high-fine analysis methods: gas chromatography coupled with mass spectrophotometry, electrophoresis, U.V. spectrophotometry) revealed the following components:

- resins,
- flavonoids
- wax (majority components);
- volatile oils,
- tannins,
- balms,
- enzymes,
- fat-soluble vitamins,
- mineral substances (minority components).

In propolis, there are also few pollen grains with variable amounts of impurities, and soluble sugars and nitrogenous substances are in insignificant quantities.

The proportion of propolis components (identifying over 149 substances) is shown in table 1.

Table 1
The proportion of propolis components

Components	Proportion
Resins and balms	55 %
Wax	30 %
Essential oils	10 %
Pollen	5 %

Resins constitute the majority fraction and they are composed mostly of free monocarboxylic acids with the formula $C_{20}H_{30}O_2$ (resin acids) of the type of abietic and pimaric acids [16, 17].

Also, in the composition of the resin were identified:

- aromatic acids in a free state and small quantities:
  - benzoic acid, (role in the antibacterial and antifungal activity of propolis),
  - cinnamic acid, caffeic acid, ferulic acid (with specific action: bactericidal and bacteriostatic) [22, 25];
- coumarin and terpenoid substances (esculetol, scopoletol)
- aromatic aldehydes, vanillin, and isovanillin;
- esters of benzoic, cinnamic, coumaric acids;
- aromatic alcohols, benzyl alcohol.

Aromatic esters are thought to have an important contribution to the antimicrobial action of propolis.

The presence of free acids determines at propolis a pronounced chemical reaction, clearly different from that of beeswax.

Wax, the second component of crude propolis, has a double origin, so it consists of two fractions:

- *vegetable fraction* consisting of the wax from the structure of the sticky substance on the tree buds which is the raw material of propolis.
- the second fraction is made up of the *wax itself* that the bees add in the process of transforming the raw material into propolis.

It is estimated that the ratio of the two fractions of propolis wax is 1: 3.

Propolis wax (due to its origin) has a different composition and organoleptic and physico-chemical properties (the values of the main indices) than beeswax.

The consistency is relatively soft and sticky, crystalline structure weakly highlighted, color brown with greenish reflections and a pronounced aromatic smell.

As a non-valuable component, wax is insoluble in cold alcohol, so in this way, it can be separated from the other valuable components.

Flavonoid substances are abundantly represented in crude propolis so that quantitatively they occupy the next place after resins and wax [30].

Along with resins and other alcohol-soluble components, they are considered therapeutically valuable substances.

In practical activity, they are extracted together and used in the form of various preparations, such as tinctures.

The qualities of crude propolis are assessed by reference to a flavonoid, a component of crude propolis.

The international literature shows that in propolis the percentage of flavonoids is 15-20% [16].

The same data show that in propolis a certain type of flavones predominates and the most important is chrysin.

3.2. Pharmacological action of propolis

Propolis has various natural properties, with the following activities:

- antimicrobial,
- antioxidant,
- antitumoral,
- antiviral,
- anti-inflammatory,
- antibacterial,
- anaesthetic,
- antifungal,
- antiprotozoal,
- antihepatotoxic, and
- antiseptic [3, 10, 13, 14].

The antibacterial activity of propolis has been studied *in vitro* against several types of Gram-positive and Gram-negative bacteria and also the synergism between different propolis compounds, mainly galangin-flavonoids and pinocembrin [4, 13, 21, 26].

The antioxidant and antimicrobial properties of propolis are also extremely important for the food industry due to its potential to delay lipid oxidation and, as such, increase the shelf life of food [15].

Toreti et al., [31] reported data on the efficacy of poplar propolis against gram-positive and Gram-negative microorganisms, such as multidrug-resistant bacteria, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA).

Quercetin and its derivatives have antibacterial efficacy against MRSA, *Staphylococcus epidermidis*, and *S. aureus*.

It has also been shown in clinical trials that the antibacterial potential of propolis is related to the large number of active compounds (over 200 substances) [29].

Flavonoids, which are the main polyphenols in propolis, are affected by the source and the environment in which the bee lives [31].

For the most part, the composition of propolis is inconsistent due to the variety of plant species that grow around the apiary, from which the bees collect the necessary exudates [3].

The primary constituents present in propolis are flavonoids, phenolic compounds, and mixtures of aromatic materials [10].

Propolis is also rich in cinnamic acid, which has powerful efficacy against several bacteria:

- *Bacillus spp.,*
- *Streptococcus pyrogenes,*
- *Aeromonas spp.*
- *Micrococcus flavus,*
- *Pseudomonas aeruginosa,*
- *Yersinia ruckeri,*
- *Vibrio spp.,*
- *E. coli,*
- *Mycobacterium tuberculosis;*
- *Listeria monocytogenes* [37].

Propolis flavonoids have fungicidal activity against *C. pelliculosa, C. parapsilosis, Pichia ohmeri, C. famata, C. glabrata* [33].

Other authors have reported that propolis constituents such as

- 3-acetylpinobanksin acetate,
- pinobanksin-3-acetate,
- pinocembrin,
- p-coumaric acid and
- caffeic acid has antifungal activity against *Mycobacteria, Candida, Trichophyton, and Fusarium* [12].

The antitumor activity of propolis is due to the combined function of polyphenolic constituents such as caffeic acid and phenolic compounds [29, 35, 36].

According to many studies, propolis has antiprotozoal activity against:

- *Leishmania donovani,*
- *Trypanosoma cruzi,*
- *Giardia lamblia,*
- *Trichomonas vaginalis,*
- *Toxoplasma gondii și*
- *G. duodenalis [3, 12].*

Propolis components also have the therapeutic ability to repair and regenerate damaged tissues [18].

These are due to their immunomodulatory, anti-inflammatory, and antimicrobial characteristics.

Moreover, the presence of bioflavonoids, arginine, vitamin C, provitamin A, complex B, along some minerals increase the activity of wound healing.

Crişan et al. [8], following the experimental results, support the existence of significant antiviral activity of propolis extract on HSV (Herpes Simplex Virus) infection "*in vitro*".

The action of propolis is manifested by a decrease in the infectious titer of HSV, by a virucidal action and by inhibitory effects on the multiplication of HSV.

The multiplication of HSV is maintained at a low level, becoming normal after the removal of propolis from the environment.

The authors believes that propolis acts nonspecifically, stimulating the cellular capacity of defense, considering that propolis may induce the formation of interferon.

Cristina et al. [6] in a study on comparative antimicrobial efficacy showed that propolis tinctures (alcohol dilutions 5-10% of propolis monophyte and polyphyte) have good activity on *Micrococcus lysodeiticus, Bacillus subtilis, Sarcina lutea, Corynebacterium equi, Proteus, and Mycobacterium*. This activity was comparable to that of Rifampicin, Chloramphenicol, Penicillin G, Ampicillin, and Flumequine.

Propolis tinctures had medium activity on *Staphylococcus aureus, Streptococcus spp. And Listeria monocytogenes,* and very low on the genera *Salmonella and Escherichia*.

The analgesic properties of propolis have been the subject of numerous works, the authors initially studied the anaesthetic effect of this product in alcoholic solutions on rabbit cornea.

They showed, like other authors, that its effect was stronger than that of cocaine and more importantly that of procaine.

The synergistic effect of propolis and procaine was also observed.

Research on animal tolerance to propolis by *Velescu and Marin* (50) revealed that oral administration to the dog of 100 ml of propolis emulsion (10% alcohol) for 10-20 days, does not change the general condition of the animals.

After slaughter, no changes in the digestive and urinary mucosa were found, and the liver and kidneys looked normal.

An important role in the isolation and identification of the main components of the propolis composition was played by *Popravko* [24].

Below are the main components identified in propolis, which form at least 1/3 of this substance, dissolved in alcohol.

The main components of propolis are those of the flavonoid type, especially flavones, flavonols, and flavonones (Fig. 5).

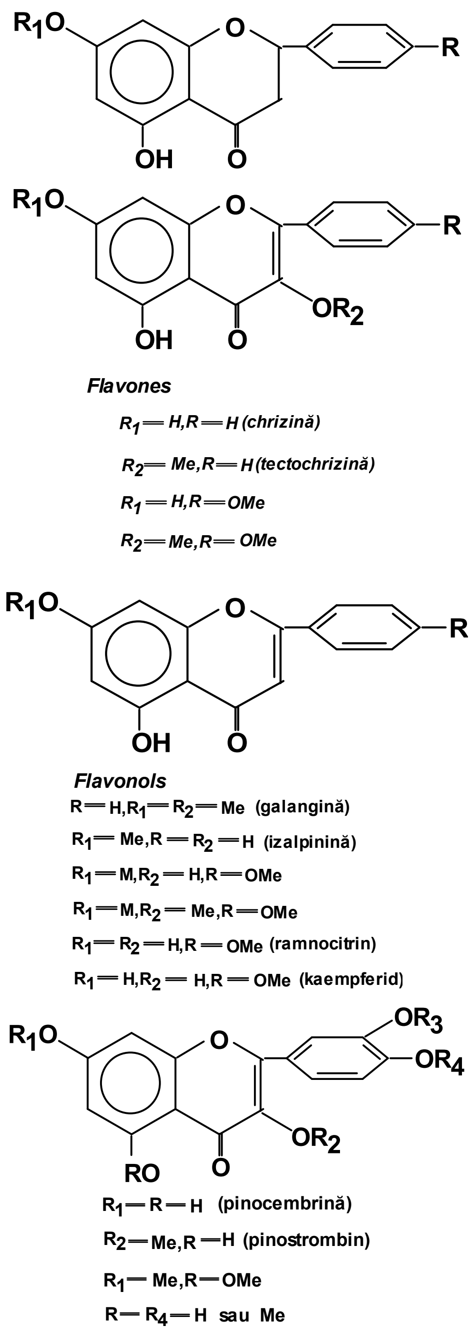

Figure 5. The main components of propolis

3.3. Alcoholic extractive solutions

3.3.1. Tinctures

FR X. defines tinctures as: *"liquid extractive pharmaceutical preparations, in the form of alcoholic, hydroalcoholic or heteroalcoholic solutions, obtained by extraction of plant or animal products"*.

The name comes from the Latin *tingere* = to color, because tinctures[1] are colored solutions.

The drug-solvent ratio is 1:10 (m/m) for tinctures prepared from plant products containing strongly active substances (10%) and 1:5 (m/m) for tinctures prepared from other vegetable products (20%) [7].

The solvent used for extraction is generally diluted alcohol (70%). Also are used hydro-alcohol-ether mixtures, other concentrations of alcohol (50%), sometimes acidified (usually with hydrochloric acid), diluted formic acid 1%, phosphoric acid 59%, especially for vegetable products containing alkaloids.

Vegetable products that have a lipid content will be degreased beforehand to obtain an optimal yield and prepared with appropriate stability.

The humidity of dry drugs can be between 2-20% (under the conditions in which that drug, although dry, contain a high percentage of moisture). Even if prepared correctly, the tinctures may be more diluted due to the water contained. In this situation, it is recommended to use a larger amount of drugs or use more concentrated alcohol.

The preparation methods are:

- maceration,
- repeated maceration and
- percolation.

Tinctures with reduced stability are prepared by dissolving dry extracts or by diluting fluid extracts.

When another method of preparation is used, the tinctures obtained must comply with the conditions laid down in the F.R.

[1] Alcoholic solutions obtained by dissolving certain chemicals in alcohol (eg alcoholic iodine-iodide solution, improperly called, iodine tincture) cannot be considered tinctures.

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Description: are clear, colored liquids with a characteristic odor and taste of the components of the plant product and the solvent used in its preparation. By dilution with water, some tinctures become opalescent or cloudy. In F.R. limits are provided for:

- Fe^{3+} (0,001%) detected as potassium hexacyanoferrate (II) complex (blue), compared to a standard solution;
- heavy metals (0.001%) detected as sulphides and compared with the standard solution;
- alcohol content determined according to the provisions of F.R. X to *"Alcohol concentration of pharmaceutical preparations";*
- evaporation residue: 10g of tincture is evaporated on the water bath, in a weighing ampoule with d = 4cm and a height of 2cm, previously weighed; dry at 105 °C for 3 hours, weigh and report to 100 g of the tincture;
- dosage: it is done according to the methods indicated in each monograph; the concentration being expressed per 100g of tincture;

4. Materials and methods

4.1. Product description

In this study was used one commercial propolis tincture (Fig.6).

Figure 6. Propolis tincture (Dacia Plant)

Ingredients:

- Bee products: propolis (30%)
- Solvents: ethyl alcohol 85%.
- Bee product / ethyl alcohol ratio - 1:3
- The product does not contain artificial preservatives and dyes.

The product contains:

- flavones (acacetin, chrysin, apigenin),
- flavanols (galangin, isalpinin, kaempferol),
- flavonoids (pinocembrine, pinostrobin, pinobaksin),
- phenolic acids (caffeic, cinnamic),
- nicotinic acid,
- polyphenols,
- phenolic aldehydes,
- sesquiterpene,
- coumarin,
- steroids,
- phenylpropanoid,
- alcohol,
- ketone,
- carbohydrates,
- essential oils,
- balms,
- vitamins (A, C, E, PP and components of the B complex),
- trace elements,
- amino acids,
- tannins,
- pollen, etc.

Propolis tincture is the alcoholic extract of propolis obtained by cold preparation.

Among the vegetable and bee products administered orally, the alcoholic and hydroalcoholic solutions are the most easily assimilated by the body, the effect appearing in a short time.

Compared to the classic production method, Dacia Plant uses a modern percolation process, which allows obtaining a high-quality product.

4.2. Determination of total polyphenols by UV-VIS spectrometry

The UV-VIS spectrum was performed on the UV-VIS Shimadzu Spectrophotometer, Pharma - SPEC 1700, and the absorbance was measured at 760 nm in the range 190-1100 nm, scanning speed 240 nm / min, at steps of 1 nm (Fig 7).

To determine the total amount of polyphenols, 20 µl of propolis tincture (1 mg / ml) was mixed sequentially with 300 µl of distilled water and 100 µl of Folin Ciocalteau reagent.

After 4 minutes, 1000 µl of distilled water and 400 µl of 20% sodium carbonate were added. The reaction mixture was kept in the darkness for 2 h at room temperature and the absorbance was measured at 760 nm.

Standard solutions of gallic acid between 0.5 and 20 µg / ml were used for the calibration curve (y = 0.0082x, r2 = 0.9980, interception = 0.0018).

Figure 7. Shimadzu Spectrophotometer, UV-VIS, Pharma-SPEC 1700

The total amount of polyphenols was expressed as gallic acid equivalent (mg) per gram of extract (mg GAE / g dry extract). All analyzes were performed using three determinations from each sample and calculating the mean value.

4.3. Determination of the total flavonoid content by the colorimetric method

The total flavonoid content was determined by the aluminum-based colorimetric method and using quercetin as the reference standard. 100 µl of propolis tincture was used over which 1 ml of methanol, 3.5 ml of water for HPLC, 2% (w/v) AlCl3 (200 µl) were added sequentially, and 200 µl potassium acetate.

After 30 minutes of storage at room temperature, the absorbance was measured at 435 nm using a spectrophotometer.

The total amount of flavonoids was expressed as µg equivalent of quercetin per mg of dry matter.

4.4. Determination of individual polyphenols by LC-MS

To determine the individual polyphenols in propolis tincture, the Shimadzu chromatograph equipped with SPD-10A UV detector I and LC-MS 2010, EC 150/2 Nucleodur C18 Gravity SB 150 x 2mm x 5 µm column was used (Fig. 8).

Figure 8. Shimadzu chromatograph

The chromatographic conditions were as follows:

- mobile phase A: water acidified with formic acid at pH-3,
- B: acetonitrile acidified with formic acid at pH-3,
- gradient program: 0.01-20 min. 5% B, 20.01-50 min 5-40% B, 5-55 min, 40-95% B, 55-60 min 95% B.
- Solvent flow 0.2 ml / min,
- temperature 20 °C.
- The monitoring wavelength was 280 nm and 320 nm.
- Calibration curves were performed in the range of 20-50 µg / ml.

5. Results and discussion

5.1. Determination of total polyphenols by UV-VIS spectrometry

The total polyphenol content of propolis tincture was determined by the spectrophotometric method and is presented in table 2.

Table 2.
Total polyphenol content for propolis tincture

ID sample	mg GAE/g Repeat 1	mg GAE/g Repeat 2	mg GAE/g Repeat 3	Media
15	129,8	130,7	131,2	130,56

The average of the three determinations was 130.56 mg GAE / g.

5.2. Determination of the total flavonoid content by the colorimetric method

The total flavonoid content expressed in mg quercetin / g obtained after the three repetitions is presented in table 3.

Table 3.
Total flavonoid content of propolis tincture

ID sample	mg quercetin / g	mg quercetin/ g	mg quercetin/ g	Mean
15	43,68	49,7	45,12	46,16

The average of the three determinations for the total flavonoid content was 46.16 mg quercetin / g.

The total content of polyphenols and flavonoids may vary due to different factors, such as flora, resin collection period, queen genetics, and collection period (Fig. 9-10).

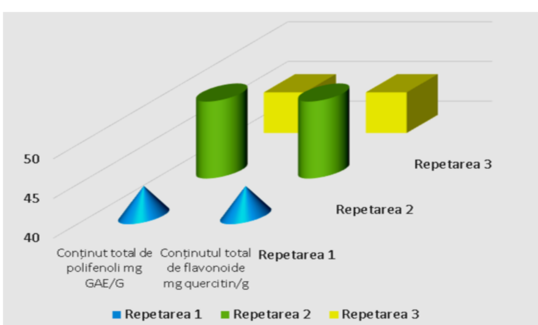

Figure 9. Values of the three determinations for total polyphenols and flavonoids

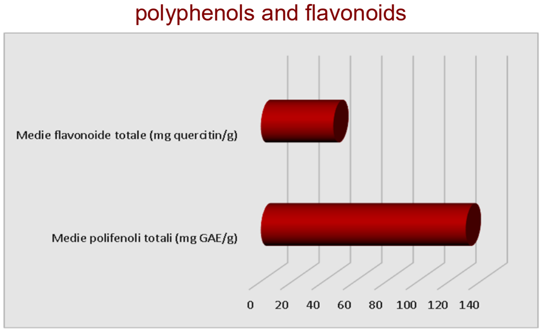

Figure 10. Mean of the three determinations for total polyphenols and flavonoids

This high variability in the total content of polyphenols and flavonoids occurs due to different sources of plant exudate, as well as the location of the apiary [31].

Last but not least, the season in which propolis is collected by bees is also a determining factor for its composition.

5.3. Identification of individual polyphenols in propolis tincture by LC-MS

Following the analysis by liquid chromatography coupled with mass spectrometry, in the commercially propolis tincture we identified the following compounds presented in Fig. 11 and table 4.

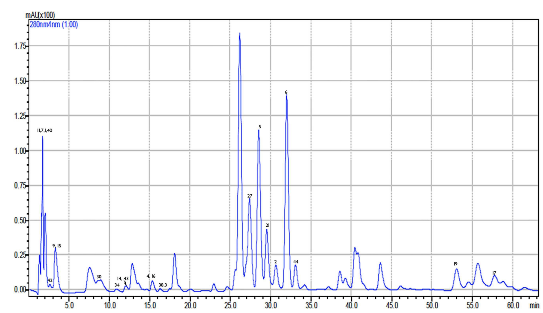

Figure 11. Chromatogram of propolis tincture

Table 4.
Volatile compounds identified in propolis tincture

Identified compound	µg/g
Acacetin	14,6
Apigenin	83,5
Galagin	28,6
Kaempferol	8,21
Caffeic acid	21,6
Pinocembrin	134,5
Pinobanksin	52,1
Rutin	39,2
Catechin	7,6
Elagic acid	5,9
P-coumaric acid	34,2
Pinostrobin	18,2
Galic acid	32,7
Izofeluric acid	4,6

Following the chromatographic gas analysis in the propolis tincture tested by us, were identified 14 components shown in Fig.12.

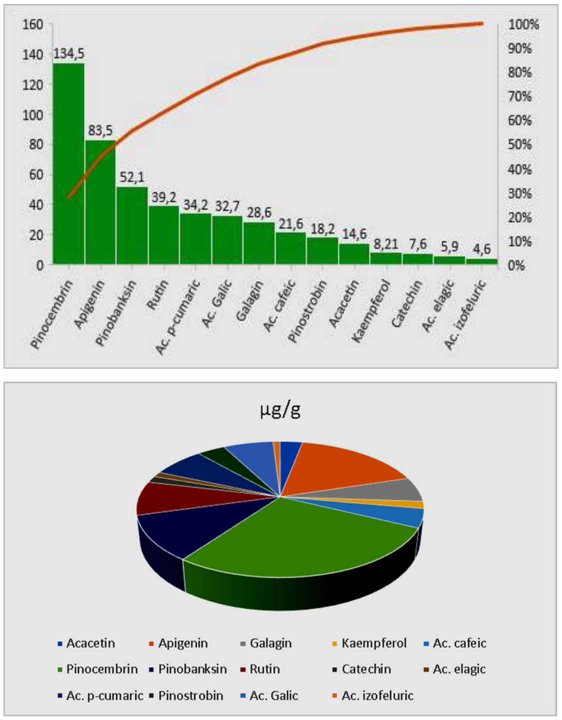

Figure 12. Representation of the main compounds identified in propolis tincture

Many of these components are also listed by the manufacturer in the product data-sheet. In addition to other compounds, those identified by us have been identified by other researchers in propolis from different regions of Europe [16].

Following the gas chromatographic analysis, we can say that the biological activity of propolis is attributed to its chemical composition which mainly includes phenolic compounds. Plant polyphenols are known for their beneficial health effects.

Some authors have identified over 300 constituents in various propolis samples and claim that the proportions depend on the place and time of harvesting [2,23].

The results we obtained are similar to those of other researchers who also found coumaric acid, ferulic acid, and caffeic acid in Canadian propolis (Nagar).

Previous studies have also described the presence of caffeic acid [19].

The most important active compounds are flavonoids, terpenoids, phenylpropanoids, aromatic acids, and phenolic compounds [22, 29].

Afrouzan et al., [1] claim that coumaric acid is responsible for the antimicrobial activity of propolis, Acacetin is an aglycone that can be derived from the hydrolysis of the respective flavonoid glycoside from the leaves of some plants, such as *Pseudacacia robinia* [17].

Wagh [33] argues that the mode of action of propolis is due to the interaction between phenolic compounds and other compounds, such as pinocembrin, galangin, and pinobanksin.

Components of propolis, such as pinocembrin, have antibacterial activity against *Streptococcus spp*., and p-coumaric acid on *Helicobacter pylori*.

Caffeic acid, diterpenoids, and phenolic compounds have a destructive capacity against tumor cells. The antitumor effect of propolis is due to the combined function of polyphenolic constituents [29].

Previous studies have reported that propolis constituents such as 3-acetylpinobanksin, pinobanksin-3-acetate, pinocembrin, p-coumaric acid, and caffeic acid show anti-fungal activity [3, 9].

It seems that due to its complex composition propolis possesses a wide range of pharmacological actions, including antibacterial, antifungal, antiprotozoal, hepatoprotective, antioxidant, anti-inflammatory, antiviral, and antitumor.

6. Conclusions

Following the study on the chemical composition of propolis extract, we can conclude:

- increased amounts of total polyphenols and flavonoids were determined;
- by liquid chromatography coupled with mass spectrometry were identified 14 compounds, the largest amounts being recorded for pinocembrin, apigenin, and pinobaksin;
- the total content of polyphenols and flavonoids may vary due to different factors, such as flora, resin collection period, queen genetics, and collection period;

- all pharmacological activities of propolis are due to the complex chemical composition.

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