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## **Antibacterial activity and phenolic content of propolis extracts obtained by different extraction methods**

### **Actividad antibacteriana y contenido fenólico de extractos de propóleos obtenidos por diferentes métodos de extracción**

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**Palabras clave:** flavonoides; examen fitoquímico; polifenoles totales

**Keywords:** flavonoids; phytochemical screening; total polyphenols

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#### **Resumen**

**Introducción:** Los datos disponibles sobre la composición química y los procedimientos para la extracción del propóleo son actualmente inconclusos. En este estudio se identificaron los grupos químicos presentes en los extractos de una mezcla de propóleos obtenidos por diferentes métodos. Además, se determinó su contenido de polifenoles y flavonoides y se estudió su actividad antioxidante y antibacteriana.

**Método:** Los extractos se obtuvieron por maceración, por el método ultrasónico y mediante microondas. Los compuestos fenólicos y flavonoides, así como la medición de la actividad antioxidante, se cuantificaron por métodos espectrofotométricos. La actividad antibacteriana se estudió a partir del efecto inhibitorio de cada extracto contra *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* y *Proteus mirabilis*, así como por el porcentaje de actividad y el Índice de Susceptibilidad Bacteriana (IBS).

**Resultados:** El tamiz fitoquímico evidenció la presencia de abundantes fitoquímicos biológicamente activos. Se encontró una diferencia significativa ( $p < 0.05$ ) en la actividad antioxidante y antimicrobiana de los extractos de propóleos obtenidos por diferentes métodos.

**Conclusión:** La presencia de grupos químicos de compuestos bioactivos y su actividad antioxidante y antibacteriana justifica el uso de estos extractos en la medicina tradicional.

#### **Abstract**

**Introduction:** The data available regarding the chemical composition and procedures for the extraction of the propolis are currently inconclusive. In this study the chemical groups present in the extracts of a propolis mixture obtained by different methods were identified. Additionally,

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their content of polyphenols and flavonoids was determined, and antioxidant and antibacterial activity was studied.

**Method:** The extracts were obtained by maceration and for the ultrasonic and microwave method. Phenolic and flavonoid compounds, as well as the measurement of antioxidant activity, were quantified by spectrophotometric methods. The antibacterial activity was studied from the inhibitory effect of each extract against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Proteus mirabilis*, and by the percentage of activity and the Index of Bacterial Susceptibility (IBS).

**Results:** The phytochemical screening evidenced the presence of abundant compounds with important biological activity. It was found a significant difference ( $p<0.05$ ) in the antioxidant and antimicrobial activity of the extracts of propolis obtained by different methods.

**Conclusion:** The presence of chemical groups of bioactive compounds and their antioxidant and antibacterial activity justifies the use of these extracts in traditional medicine.

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## **Introduction**

Propolis is a bee product of complex composition, which is obtained by the bees *Apis mellifera* L., by addition of wax and salivary secretions to the resinous, gummy or balsamic material that they collect from various plants (Ríos *et al.*, 2014). In the hive, bees use propolis for different purposes, such as closing cracks, minimizing access routes, coating and isolating animal remains that have been introduced into the hive, consolidating structural components, varnishing the inside of the hive cells for disinfectant purposes, and avoiding vibrations (Chaillou *et al.*, 2004).

The chemical component and biological activity of propolis depend on the flora area of bee collection and bee species (Vongsak *et al.*, 2015). It consists of 50–55% of resins and balms, 30–40% of beeswax, 5–10% of essential or volatile oils, 5% of pollen, and 5% of several organic and mineral materials (Toreti *et al.*, 2103). More than 160 compounds were identified, of which 50% are phenolic compounds to which biological properties are attributed. These compounds are a heterogeneous set of molecules with antioxidant activity that includes acid phenols and flavonoids (Drago-Serrano *et al.*, 2006) that contribute to preventing diseases linked to oxidative stress and to controlling the development of microorganisms (López-Airaghi *et al.*, 2013; Mayta-Tovalino *et al.*, 2012).

In recent years, the use of propolis in natural medicine has increased, being a valuable raw material in the cosmetics and food industries, which is why knowledge of the bioactive compounds present in this product is essential. The chemical composition of propolis is highly variable and complex due to the biodiversity of the vegetation of each region visited by bees (Mohammadzadeh *et al.*, 2007). However, even within the same country, the propolis composition may be qualitatively and quantitatively different depending on the region and time of collection (Koo, 1999), which makes it difficult for a universal standardization of their solutions (Popova *et al.*, 2010).

Although most studies on the antibacterial activity of propolis have been made from the extracts obtained with organic solvents (Omer *et al.*, 2016), the aqueous extracts have also shown some bactericidal activity (Rodríguez, 2000). Most studies on the antimicrobial activity of plants have focused more on the effect of solvent used and less on the method used to obtain the extracts. For example, Ertürk *et al.* (2016) investigated antibacterial and antioxidant activities of propolis samples from the Rize province of Turkey in different solvents. They discovered that the method used to obtain the extracts may affect the content and biological activity of chemical compounds such as polyphenols. This opens up the possibility of using the resources of the laboratory in a more efficient way, since it will allow to choose the method with which a better extraction of active compounds of the substance under study is achieved. In addition, the chemical composition of propolis is highly variable and complex due to the biodiversity of the vegetation of each region visited by bees (Mohammadzadeh *et al.*, 2007). For the empirical treatment of various diseases, propolis is prepared by maceration; So, it is interesting to know if the method used to obtain the extract affects the content of bioactive compounds and their biological activity. Therefore, the objective of this work was to identify chemical groups of bioactive compounds and to determine the content of total phenolic compounds and flavonoids, as well as to study the antioxidant and antimicrobial capacity of the extracts of the mixture of propolis of the Comarca Lagunera in the Northern Mexico obtained by different methods.

## **Material and Methods**

### **Reagents**

All the chemical reagents that were used as solvents and standards in the procedures were of the analytical grade of the Sigma-Aldrich® brand (St. Louis, MO, USA); for the microbiological

assays, the Mueller-Hinton Agar and Mueller-Hinton Broth culture media from Merck® (Darmstadt, Germany) were used.

### Collection of samples

Four samples of native propolis were collected from beehives using a standard plastic mesh trap of the apiaries located in the municipalities of Torreón and Matamoros, Coahuila state, and the municipalities of Gómez Palacio and Lerdo, in the Durango state. These municipalities form part of the Comarca Lagunera, which is located in northern and central Mexico (25° 05' and 26° 54' N and 101° 40' and 104° 45' W). It has an average of 235 mm of rain, an elevation of 1,139 m and an average annual temperature of 18.6 °C (CIGEL, 2018). All samples were kept in the darkness and stored at 4°C until use. With the samples from the four places, a mixture was formed, since that is how the population traditionally consumes them. This mixture was dried in a conventional oven (Linderberg/Blue, USA) at 55°C for 48 h. Moisture content was determined according the method 925.09 of the AOAC (2014).

### Obtaining extracts

The extracts of the propolis mixture were obtained by the classical method of maceration, by microwave-assisted extraction (Alonso-Castro *et al.*, 2016) and by ultrasound (Muñiz *et al.*, 2013).

**Classical method of maceration.** 20 g of the dry mixture of raw propolis was placed in a flask, and 200 mL of 70% ethanol was added; it was kept at 35°C at 150 rpm for 48 h on a shaker mixer (IKA KS 4000). **Ultrasound-assisted extraction.** 20 g of the dry mixture of crude propolis was placed in a flask, and 200 mL of ethanol (70%) was added; the flask was placed in an ultrasonic bath (Branson®) at 30°C to 300 W for 30 min. **Microwave-assisted extraction.** 20 g of the powdered dried stem of propolis was extracted with 315 mL of 70% ethanol using a closed system of microwave-assisted extraction (Multiwave 3000 Solv, Anton Paar, Graz, Austria). The extraction was performed at 70°C and 90 bars during 17 min. The extracts obtained by the three methods were filtered using filter paper 125 mm, No. 3 (Whatman®), and concentrated under reduced pressure in a rotary evaporator (BUGI®) to remove the solvent. The extracts were resuspended in distilled water and sterilized by membrane filtration with a 0.45 µm pore (Whatman®) and stored at 4°C until use. From the stock solution of each extract, the

chemical groups were identified in each of them, and their antioxidant and antimicrobial activities were studied.

### **Phytochemical screening**

The identification of chemical groups in the extracts of the propolis mixture was carried out by means of a phytochemical screening. Chemical groups corresponding to: alkaloids, sterols, unsaturations, sesquiterpenelactones, flavonoids, saponins, coumarins, and phenolic oxydrils were identified by colorimetric reactions according to the techniques described by Ramman (2006) and Buvaneswari *et al.* (2011). **Alkaloids:** Mayer Test: To 0.5 mL of the extract, 6 drops of Mayer's reagent were added by carefully adding one side of the test tube. A white-creamy precipitate indicated the test as positive. Distilled water was used as a negative control and as positive control caffeine. **Sterols:** Liebermann–Burchard reaction: 1.0 mL of extract dissolved with 1.0 mL of chloroform was mixed with 1.0 mL of acetic anhydride and 0.5 mL of concentrated H<sub>2</sub>SO<sub>4</sub>. A series of color changes showed the presence of phytosterols. A blue-green ring indicated the presence of terpenoids. Distilled water was used as a negative control and as positive control almond oil. **Unsaturations:** A solution of 2% KMnO<sub>4</sub> was added dropwise in water to 0.5 mL of the extract; the test was positive if discoloration or formation of a brown precipitate was observed resulting from the formation of manganese dioxide. Distilled water was used as a negative control and as positive control olive oil. **Sesquiterpenelactones:** Baljet test: two solutions were used which were mixed in equal volumes before use. Solution A: 0.5 g of picric acid was placed in 50 mL of ethanol. Solution B: 5.0 g of NaOH in 50 mL of water was added. For the test, 50 µL of the extract and 4 drops of the reagent were added. The test was positive if the mixture becomes orange or dark red. Distilled water was used as a negative control and as positive control celery extract. **Flavonoids:** H<sub>2</sub>SO<sub>4</sub> test: 50 µL of the extract and 10 µL of concentrated sulfuric acid were placed. The test was positive if yellow colorings were observed for flavones and flavonols; orange-icing for flavones; red-blue for chalcones and red-purple for quinones. Distilled water was used as a negative control and as positive control guava extract. **Saponins:** Shinoda test: Magnesium filings were added to 0.5 mL of the extract, and then a few drops of concentrated HCl were added. The test was considered positive with the appearance of colors orange, red, pink, and pink-blue to violet. Distilled water was used as a negative control and as positive control cassava. **Coumarins:** Sodium hydroxide test: 50.0 µL of the extract was

placed and 10.0  $\mu\text{L}$  of 10% NaOH solution was added; the test was positive when a yellow color appeared that disappeared when acidulating with 10.0  $\mu\text{L}$  HCl. Distilled water was used with a negative control and as positive control cinnamon oil. **Phenolic oxydriles:**  $\text{FeCl}_3$  test: 0.5 mL extract and 5%  $\text{FeCl}_3$  drops were added together. The appearance of a red, violet-blue or green precipitate was considered positive. Distilled water was used with negative control and as positive control vanillin.

### **Antioxidant properties**

**Sample preparation.** For the determination of the polyphenols and the antioxidant capacity of each propolis extract, 1 g was weighed and placed in a flask with 4.0 mL of water (ratio 1:5); the mixture was homogenized by constant stirring for 2 h at 500 rpm, protected against the light. It was then centrifuged at 10,000 rpm and stored in eppendorf tubes, and the respective analyses were performed immediately.

### **Determination of total content phenolic compounds**

Total content phenolic compounds were determined using Folin–Ciocalteu reagent following the method described by Singleton *et al.* (1999). From each propolis extracts, dilutions in ratio 1:10 were made with deionized water. 1 mL of this dilution was obtained and placed in a test tube, and 5 mL of Folin–Ciocalteu reagent (diluted 1:10 with deionized water) was added. It was allowed to stand for 8 min, and then 4 mL sodium bicarbonate solution (7.5%, w/v) was added, until a homogeneous mixture was obtained. The tubes were covered with foil to protect them from light and were incubated for 2 h at room temperature. The absorbance of each mixture was measured at 740 nm using a spectrophotometer (*Genesys 10uv, USA*).

To determine the total phenol content, the absorbance value obtained for each of the evaluated samples was replaced in the equation of a standard curve of gallic acid (Skerget *et al.*, 2005). The content of total phenolic compounds was expressed as milligrams of gallic acid equivalents per liter of extract ( $\text{mg GAE} \cdot \text{L}^{-1}$ ).

### **Estimation of total flavonoids content**

Estimation of flavonoids was done as described by Lagouri *et al.* (2014). The concentration of total flavonoids in the propolis extracts was measured spectrophotometrically at 415 nm. Half a

milliliter of the sample was transferred to a 10-mL volumetric flask containing 2 mL of  $\text{AlCl}_3$  (to 10% in ethanol) and 3 mL of  $\text{CH}_3\text{COONa}$  ( $50 \text{ g}\cdot\text{L}^{-1}$  in ethanol). The controls contained all the reagents except for the extract. After 2.5 h at  $20^\circ\text{C}$ , the absorbance was measured at 440 nm. The same procedure was repeated for the standard quercetin solutions (25–250 ppm), and the results were expressed in milligrams of quercetin per gram of propolis and were presented as means of triplicates.

### **Antioxidant activity**

Was performed according to the methodology described by Brand *et al.* (1995), which it is based on inhibition of the stable radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH). A calibration curve was done from a methanolic stock solution of DPPH radical, with a concentration of  $20 \text{ mg}\cdot\text{L}^{-1}$  and the ( $\pm$ )-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox reagent). In a Falcon tube, 990  $\mu\text{L}$  of the DPPH radical and 10  $\mu\text{L}$  of the solution of the ethanolic extract of propolis added, the mixture was shaken with vortex and allowed to stand for 30 min in the absence of light, and its absorbance was read in a spectrophotometer at 517 nm.

### **Antibacterial activity**

The antibacterial activity was studied from the inhibitory effect of each extract against *Staphylococcus aureus* ATCC 35556, *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 14028 and *Proteus mirabilis* ATCC 9150. From the revitalized strains, the inoculum was prepared as described by Cockerill *et al.* (2012). The percentage of activity of each extract and the rate of bacterial susceptibility were also calculated.

### **Inhibitory effect**

The inhibitory effect of each extract was measured from the zones of inhibition observed when using the disk diffusion technique (Souto *et al.*, 2006). 10  $\mu\text{L}$  ( $1 \times 10^6$  CFU) of bacteria were inoculated on the surface of Muller-Hinton agar. After that, filter paper discs (Whatman®) of 6.0 mm diameter were impregnated with 10  $\mu\text{L}$  of each extract. Moxifloxacin (50 mg), a broad-spectrum antibiotic, was used as a positive control, and distilled water was used as a negative control. All tests were performed in triplicate.



### Percentage of activity

The percentage of activity (equation 1) indicates the total antimicrobial potency of each extract (López-Malo *et al.*, 2005).

$$\text{Activity (\%)} = \frac{100 \times \text{Number of strains susceptible to the extract}}{\text{Total strains tested}} \quad (\text{Equation 1})$$

### Index bacterial susceptibility (IBS)

The IBS (Equation 2) shows the number of microorganisms susceptible to the extract, assessing ranges from 0 (resistance extract all samples) to 100 (susceptible to the whole extract) (López-Malo *et al.*, 2005; Panghal *et al.*, 2011).

$$\text{IBS} = \frac{100 \times \text{Number of effective extracts for each bacterial strain}}{\text{Total number of bacterial strains}} \quad (\text{Equation 2})$$

### Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) to test differences between extraction methods, and a (Student-Newman-Keuls) comparison of means was used to determine the differences between groups when necessary. Statistical analyses were conducted using Statistica software. Differences were considered statistically significant at 95% of confidence level.

## Results and discussion

### Phytochemical screening

The moisture of the dry propolis mixture from which the extracts was 1.66% ± 0.29. The phytochemical screening of the hydroalcoholic extract of propolis revealed the presence of important secondary metabolites. Likewise, there was no difference in the presence of metabolites analyzed in relation to the extraction method used.

The bioclimate and flora of the Comarca Lagunera, whose vegetation is composed of *Prosopis* spp., *Abies alba* Mill., *Acacia farnesiana* (L.) Willd, Phoenix spp. and *Larrea tridentata* (Sessé and Moc. ex DC.) Coville, varieties (Estrada-Rodríguez, 2004), favor the presence of bioactive compounds in propolis. The result of phytochemical screening of propolis samples coincides with that reported by Soltani *et al.* (2017), who by means of gas chromatography and

mass spectrometry, detected: aromatic acid ester, saturated hydrocarbon alkaloids, saturated hydrocarbons, and aromatic heterocyclic organic compounds, in ethanolic extracts of Algerian propolis. Among the numerous groups of substances identified in propolis samples from different localities, the most common are aromatic acids and esters, chalcones, flavonoids, terpenoids, and waxy acids. Furthermore, most of the biological activities of propolis have been attributed to these compounds, especially the flavonoids (Trusheva *et al.*, 2006). A significant number of reports describe its anti-inflammatory, immunomodulatory, antioxidant, anticancer, and hepatoprotective properties, as well as many other biological activities of propolis (Borges *et al.*, 2011).

### Total content phenolic compounds and antioxidant activity

When quantifying the content of phenols in the extracts of a mixture of propolis obtained by maceration, assisted by ultrasound and by microwaves methods, it was observed that the method used to obtain the extracts does affect the content of phenols and flavonoids as well as the antioxidant capacity on which phenols have more influence than flavonoids (Tabla 1). The traditional maceration method was better for the extraction of phenolic compounds because it was observed a 40 and 28% more with respect to the other 2 methods; however, the extraction assisted by ultrasound was better to extract flavonoids, since it was obtained 54 and 52% more than the methods of maceration and assisted by microwaves respectively. Although the antioxidant capacity of the extract obtained by ultrasound was reduced by 8%, in relation to the other two methods, which suggests that the antioxidant capacity it is not only due to flavonoids, but to a synergistic effect with other bioactive compounds present in the sample

**Table 1.** Phenols content, flavonoids and antioxidant capacity in the propolis extracts.

Extract (1:10 w/v)	Phenols content (meq Gallic acid)	Flavonoids content (meq quercetin)	Percentage of antioxidant capacity (meq Trolox)
Maceration	35.32 ± 0.42 <sup>a</sup>	1.39 ± 0.16 <sup>b</sup>	12.34 ± 0.93 <sup>a</sup>
Ultrasound-asisted extraction	21.22 ± 0.15 <sup>c</sup>	2.57 ± 0.07 <sup>a</sup>	11.40 ± 0.06 <sup>b</sup>
Microwave- assisted extraction	25.55 ± 0.11 <sup>b</sup>	1.33 ± 0.46 <sup>b</sup>	12.39 ± 0.02 <sup>a</sup>

\*Values are the average of three replicates ± SD. (p < 0.05).

The interest in quantifying phenolic compounds and flavonoids present in propolis is because they are responsible for the physiological activity of this product (Delgado *et al.*, 2015). Farré *et al.* (2004) consider that the phenolic compounds act synergistically with flavonoids, and of these, quercetin is one of the flavonoids of interest to quantify.

### Antibacterial activity

The extracts obtained by the three methods showed 100% of antimicrobial activity and IBS was of 100%. The inhibitory effect of the extracts (Table 2) varied, not only regarding the extraction method, but also depending on the bacteria studied. It was observed that the inhibitory effect was less for *E. coli* than for the other studied bacteria.

**Table 2.** Inhibition zones (mm) showing the inhibitory effect of propolis extracts.

Microorganisms tested	Extract types			
	Maceration	Assisted by microwave	Assisted by ultrasound	Moxifloxacin (50 mg)
<i>Escherichia coli</i>	7.0 ± 0.0 <sup>a</sup>	7.0 ± 0.0 <sup>a</sup>	7.0 ± 0.0 <sup>a</sup>	24.0 ± 0.5
<i>S. typhi</i>	10.3 ± 0.6 <sup>a</sup>	12.3 ± 0.6 <sup>a</sup>	11.0 ± 1.0 <sup>a</sup>	29.0 ± 0.0
<i>Staphylococcus aureus</i>	9.0 ± 0.01 <sup>a</sup>	9.0 ± 0.0 <sup>5 a</sup>	8.0 ± 0.0 <sup>a</sup>	19.0 ± 0.5
<i>Proteus mirabilis</i>	9.7 ± 0.6 <sup>a</sup>	10.2 ± 1.2 <sup>a</sup>	11.2 ± 0.3 <sup>a</sup>	24.5 ± 0.6

Values are the average of three replicates ± SD.

The different letters between columns indicate a significant difference ( $p < 0.05$ ).

There was no significant difference ( $p < 0.5$ ) between the diameters of the zone of inhibition of extracts obtained by different techniques, which supports the way in which the extract is traditionally used, that is, by maceration. The least sensitive microorganism was *S. typhi*, and the most sensitive was *E. coli*. Although the three extracts were active against the bacteria studied, none of them was as effective as the antibiotic used as a positive control. These results are similar from those reported by Ertürk *et al.* (2016) and for Koo *et al.* (2000), who reported that the ethanolic extract of crude extract of propolis was found the most sensitive microorganism to propolis was *E. coli* in the Gram negative group. This activity can be a synergy between flavonoids, apigenin, chrysin, or other components in raw propolis samples. According to Hegazi *et al.* (2014), the propolis samples show a different antimicrobial activity and attribute it to the

complex composition of the resin. In general, propolis is used to treat various diseases in humans. One of the most important properties of propolis is its antimicrobial activity, which is attributed mainly to flavonoids, the main biologically active compound in propolis, and it is a substance that has potential for the treatment of conditions caused by different microorganisms (Tolosa and Cañizares, 2002). Several trials have demonstrated the antibacterial activity of ethanolic extracts against *Staphylococcus aureus*, *E. coli* and *Pseudomonas* spp. (Ortega *et al.*, 2011; Díaz-Mena *et al.*, 2000), and it is currently investigated whether the propolis can be used as a natural food preservative (Vargas-Sánchez *et al.*, 2013). Dimov *et al.* (1992) correlated the antibacterial activity of propolis with the presence of flavonoids; and Farnesi *et al.* (2009) reported that the bactericidal effect of these compounds on the metabolic disturbance of ion channels due to paired phosphorylation / dephosphorylation reactions. The antimicrobial activity is also attributed to phenolic acids: ferulic, caffeic and cumaric, bioactive compounds found in propolis samples by Popova *et al.* (2010). The presence of phenolic compounds in the samples of propolis explain their antimicrobial activity, which is supported by Marcucci (1995), who reported that caffeic acid, as well as benzoic acid and cinnamic acid act on the microbial membrane, causing structural damage to the cells. Until now, no single propolis component has shown to possess more antibacterial properties than that of the total extract. Some authors cite the highly complex and variable composition of propolis as a reason for its antimicrobial activity and the data gathered to date suggest that such activity can be linked to multiple targets, with several constituents acting in synergy (Scazzocchio, 2006). Propolis affects the cytoplasmic membrane, inhibits bacterial motility and enzyme activity, exhibits bacteriostatic activity against different bacterial genera and can act as a bactericide at high concentrations (Mirzoeva, 1997).

## **Conclusions**

The method of obtaining the extract affects the content of phenols and flavonoids, as well as its antioxidant capacity; however, with the traditional method of maceration, phenols are extracted, which contributes to their antioxidant capacity. The abundant biologically active phytochemicals, and their antioxidant and bactericidal activities, seem to validate the classical medicinal uses of propolis extracts.

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