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Bioactive Compounds, Antioxidant and Antimicrobial Activity of Propolis Extracts during In Vitro Digestion

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Abstract: The objective of this research was to determine the content of total phenols, total flavonoids, and the antioxidant and antimicrobial activity of the ethanolic extracts of propolis obtained by two methodologies during in vitro digestion. Ethanolic extracts of propolis were obtained by ultrasound and maceration and the yield and content of the bioactive compounds, as well as their antimicrobial and antioxidant activity, were evaluated. Yields higher than those reported in other investigations (71.6%) were obtained. The highest content of phenols and flavonoids in the ethanolic extracts was 34,406.6 mg GAE/100 g in propolis from San Pedro, obtained by maceration (SP M), and 19,523.2 mg QE/100 g in propolis from Teotitlán, obtained by ultrasound (TU), respectively, being higher than what is established in Mexican regulations. The antioxidant and antimicrobial activity of the extracts was not affected by the method of obtaining. At the end of the in vitro digestion there was an 80% loss of the phenolic content and a 90% loss of the flavonoid content. Therefore, antioxidant activity was affected. On the other hand, ultrasound improves the obtaining of bioactive compounds. In vitro digestion decreases the content of bioactive compounds; therefore, their functional properties are affected. Thus, it is important to consider technologies that allow extracts to be protected from in vitro digestion conditions.

Keywords: propolis; phenolic compounds; flavonoid; ultrasound; maceration; microbial inhibition

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1. Introduction

The word propolis is derived from the Greek, "pro" meaning "at the entrance of" and "polis" meaning "city"; it is a substance produced by bees in order to protect the bee's larvae, honey stores and combs from microbial infections [1,2]. According to the Mexican Official Standard [3], propolis is the natural product produced by the bees. It consists of resinous substances collected and processed by forage bees from the vegetation surrounding the apiary. With a resinous appearance, its color can vary depending on its floral origin and range from red, reddish-yellow, dark-yellow, brownish-green, brown or black, with a bitter and slightly spicy flavor. Bees produce propolis to use it as a thermal insulation material, sealing cracks present in the hive. This helps to control humidity and temperature inside the hive, soften the internal walls and protect the hive against microbial infections, as well as to mummify dead bees inside the hive [4].

Propolis is composed of 50% resins and vegetable balm, 30% beeswax, 5% pollen and 10% essential oils, among other organic compounds, mainly primary and secondary plant metabolites, such as amino acids, vitamins, minerals, phenolic compounds, terpenoids, tannins and alkaloids [5]. Nearly 300 chemical compounds have been identified. These compounds are not necessarily present in all propolis, because their presence depends

mainly on the botanical and geographical origin, the species of bee and the harvest season, as well as of various environmental factors [6–8]. In general, propolis contains flavonoids, terpenes, phenols and their esters, sugars, alcohols, ketones, hydrocarbons, amino acids, vitamins and minerals [7].

Since ancient times, propolis has been used in traditional medicine for the treatment of various conditions in human and animal health. Today propolis is consumed in various parts of the world, due to its health benefits, among which are antiseptics, antimicrobials (antibacterial, antifungal, antiprotozoal and antiviral), spasmolytics, anti-inflammatories, anesthetics, antioxidants, antidiabetic, antiulcer, antimutagenic, anticancer, immunomodulatory and antihepatotoxic, in addition, it is excellent for the treatment of skin burns and oral health care [9–11]. Phenolic compounds are those mainly responsible for antioxidant activity, due to their ability to capture free radicals with the formation of more stable molecules [2]. Furthermore, flavonoids and phenolic acids exert a strong antimicrobial activity [12–14]. For many years, tincture has been the traditional way to use propolis (alcohol extraction); however, in order to obtain a pure propolis extract, various extraction methodologies have been developed [4]. Ultrasound-assisted extraction is one of the most promising methods due to extraction time, yield and profitability [15]. On the other hand, ultrasound can be an excellent alternative for the decrystallization of honey, without affecting quality parameters [16]. In addition to reducing the size of the crystals and the content of products of the Maillard reaction, the honeys treated with the ultrasound increase the content of bioactive compounds, consequently, an improvement in their functional properties, mainly due to the liberation of bioactive compounds from the crystallized honey matrix [17].

The aim of the present investigation was to determine the content of phenols and flavonoids and the antioxidant and antimicrobial activities of ethanolic extracts of propolis obtained by ultrasound or maceration in a simulated in vitro digestion.

2. Materials and Methods

2.1. Propolis Samples

Three samples of propolis from hives *Apis mellifera*, from the Mazatec area belonging to the Oaxaqueña Cañada were used: Teotitlán de Flores Magón (18°07′57″ N, 97°04′20″ O), San Jerónimo Tecóatl (18°10′00″ N, 96°55′00″ O) and San Pedro Ocopetatillo (18°12′00″ N, 96°55′00″ O), Oaxaca (Table 1). Propolis collection was performed with the support of the following beekeeper organizations: "Miel Nectar Mazateco" and "Sociedad de Apicultores de Eloxochitlán". The samples were collected directly from the apiary, in hermetically sealed containers protected from light, and stored at −30 °C until use.

Table 1. Origin and conditions for obtaining propolis extracts.

Sample	Origin	Treatment	Time
T U	Teotitlán de Flores Magón	Ultrasound	30 min
T M	Teotitlán de Flores Magón	Maceration	17 days
SP U	San Pedro Ocopetatillo	Ultrasound	30 min
SP M	San Pedro Ocopetatillo	Maceration	17 days
SJ U	San Jerónimo Tecóatl	Ultrasound	30 min
SJ M	San Jerónimo Tecóatl	Maceration	17 days

T U = Teotitlán de Flores Magón, Ultrasound; T M = Teotitlán de Flores Magón, Maceration; SP U = San Pedro Ocopetatillo, Ultrasound; SP M = San Pedro Ocopetatillo, Maceration; SJ U = San Jerónimo Tecóatl, Ultrasound; SJ U = San Jerónimo Tecóatl, Maceration.

2.2. Obtaining Extracts/Extraction Methods/Preparation of Extracts

The extracts were obtained/prepared according to Osés et al. [18], with some modifications. Frozen propolis (−30 °C) was ground with the help of a mill (Jiawanshun, Model HC-2000Y, China). From the powdered propolis 6 treatments were prepared (Table 1). In all treatments, the solvent used was 85% ethanol, in a 1:30 v/v ratio, at a temperature of 20 °C. Ultrasounds were applied at a frequency of 42 kHz in an ultrasonic bath (Branson 3510,

Mexico). The maceration was carried out with magnetic stirring at 200 rpm. Subsequently, the extracts were centrifuged at 18,510× *g* for 15 min at 4 °C. The liquid extracts were concentrated in a rotary evaporator at 40 °C (Büchi R-215, Switzerland). For the total evaporation of the extraction medium, the extracts were placed in a vacuum oven at 40 °C. The dry extracts were stored at 4 °C, in glass containers protected against light, until use.

2.3. In Vitro Digestibility of Propolis Extracts

The in vitro simulation of the gastrointestinal digestion of the extracts was performed according to Minekus et al. [19], with some modifications. Digestion was divided into two stages: (a) Gastric phase; a solution of 20 mg/mL dry extract in 80% ethanol was prepared, later this solution was diluted (1:5 *v*/*v*) with distilled water, and the solution adjusted to pH 2 with 6 M HCl, 20 mL of gastric fluid (40,000 units of porcine pepsin in 0.3 M $CaCl_2$ in 0.1 M HCl) were added, and the mixture was incubated in a water bath at 37 °C, with agitation, for 120 min. (b) Intestinal phase, the pH was adjusted to 7 with 0.5 M $NaHCO_3$, then 10% pancreatic fluid was added (0.4 g of pancreatin and 2.5 g of bile salts in 100 mL of 0.1 M $NaHCO_3$), the mixture was incubated in a water bath at 37 °C, with agitation, for 120 min. Finally, the mixture was heated to boiling for 4 min in order to inactivate the enzymes. Four samples were taken during the digestion: from the initial extract, at the end of the gastric phase, at the end of the intestinal phase and after boiling. All samples were centrifuged at 18,510× *g* for 10 min at 4 °C with a centrifuge (Hermle, Z 36 HK, Gosheim, Germany) and they were later kept in refrigeration until analysis. Total phenols, flavonoids, antioxidant activity with the reagent 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) and 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^+$) and antimicrobial activity were further determined.

2.4. Determination of Bioactive Compounds

2.4.1. Quantification of Total Phenolic Compounds

Phenols were quantified according to the Folin–Ciocalteu method described by Rababah et al. [20], with some modifications: a dilution of propolis extract (1:1000 *w*/*v*) was made using 85% ethanol, it was homogenized until complete solubility using a Vortex, then the extracts were centrifuged at 18,510× *g* for 15 min at 4 °C (Hermle, Z 36 HK, Gosheim, Germany). For this assay, 0.3 mL of the supernatant were taken in a test tube and mixed with 1.5 mL of Folin–Ciocalteu 0.2 N reagent, incubated for 8 min at room temperature and in the absence of light. Then 1.2 mL of 0.7 M Na_2CO_3 were added, they were homogenized and incubated at room temperature for 120 min in the absence of light. Absorbance was measured at 765 nm using a spectrophotometer (Jenway 6715, Ware, UK), against a water blank. The total phenolic content was determined from a gallic acid standard curve (0 to 100 mg/L). The amount of total phenols was expressed as mg equivalents of gallic acid (mg GAE/100 g propolis extract), according to the following equation. All samples were analyzed in triplicate.

$$\text{Total phenolic compounds (mg GAE/100 g)} = (\text{GAC} \times \text{V} \times \text{DF} \times 100)/(\text{weight of the sample (g)})$$

where:

GAC = Gallic acid concentration from the standard curve (mg/mL);
V = Volume of sample (mL);
DF = Sample dilution factor.

2.4.2. Total Flavonoid Quantification

The total flavonoid content was determined according to the Dowd method adapted by Arvouet-Grand et al. [21] with some modifications: a dilution of propolis extract (1:1000 *w*/*v*) was made using pure methanol, it was homogenized until complete solubility using a Vortex, then the extracts were centrifuged at 18,510× *g* for 15 min at 4 °C (Hermle, Z 36 HK, Gosheim, Germany). A solution of 2% aluminum chloride ($AlCl_3$) in methanol was prepared. For this assay, 2 mL of the supernatant with 2 mL of $AlCl_3$ were placed in a

test tube, homogenized and left to stand at room temperature for 20 min in the absence of light. The absorbance was measured at 415 nm in a spectrophotometer (Jenway 6715, Ware, UK) using methanol as a blank. Quercetin was used for the elaboration of the standard curve (0 to 100 mg/L). The total content of flavonoids was expressed in equivalent mg of quercetin/100 g of propolis extract (mg QE/100 g of propolis extract), according to the following equation. All samples were analyzed in triplicate.

$$\text{Total flavonoids (mg QE/100 g)} = (\text{QC} \times \text{V} \times \text{DF} \times 100)/(\text{weight of the sample (g)})$$

where:

QC = Quercetin concentration from the standard curve (mg/mL);
V = Volume of sample (mL);
DF = Sample dilution factor.

2.5. Antioxidant Activities

2.5.1. ABTS Free Radical Inhibition Activity

The antioxidant activity was determined using the chromogenic compound 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) according to Pimentel-González et al. [22]. A dilution of propolis extract (1:1000 w/v) was made using 85% ethanol, it was homogenized until complete solubility using a vortex, then the extracts were centrifuged at 18,510× g for 15 min at 4 °C (Hermle, Z 36HK, Gosheim, Germany). Of 7 mM ABTS stock solution (36 mg of ABTS reagent), 20 mL was prepared by adjusting to 10 mL with distilled water and 10 mL of 2.45 mM potassium persulfate (5.83 mg of potassium persulfate in 10 mL of distilled water) was added. To generate the $ABTS^+$ free radicals, the mixture was kept under magnetic stirring for 24 h in total darkness. Before starting the reading of the samples, the $ABTS^+$ reagent was standardized at an absorbance of 0.7 ± 0.01 using 20%ethanol. An amount of 0.2 mL of the supernatant with 2 mL of standardized $ABTS^+$ was placed in a test tube, homogenized and left to stand at room temperature for 6 min in the absence of light. The samples were read at 734 nm in a spectrophotometer (Jenway 6715). Gallic acid was used to prepare the standard curve (0 to 100 mg/L) and 20% ethanol was used as a blank. Antioxidant activity was expressed as mg equivalents of gallic acid in 100 g of extract, according to the following equation. All samples were analyzed in triplicate.

$$\text{Antioxidant activity (mg GA/100 g)} = (\text{GA} \times \text{V} \times \text{DF} \times 100)/(\text{Sample weight (g)})$$

where:
GA = Gallic acid concentration from the standard curve (mg/mL);
V = Sample volume (mL);
DF = Sample dilution factor.

2.5.2. DPPH Free Radical Inhibition Activity

The antioxidant activity of DPPH (radical 1,1-diphenyl-2-picrylhydrazyl) was determined according to Turkmen et al. [23]. The propolis extract was diluted (1:1000 w/v) using methanol, homogenized until complete solubility using a vortex, then the extracts were centrifuged at 18,510× g for 15 min at 4 °C (Hermle, Z 36 HK, Gosheim, Germany). One hundred mL of a stock solution of 0.2 mM DPPH (7.9 mg of DPPH were dissolved and adjusted to 100 mL with 80% methanol) was prepared to generate $DPPH^+$ free radicals, and the mixture was kept under magnetic stirring for 2 h in total darkness. Before starting the reading of the extracts, the $DPPH^+$ reagent was standardized to an absorbance of 0.7 ± 0.01 using 80% methanol. Two and half mL of the standardized $DPPH^+$ reagent was added to 0.5 mL of the supernatant, and they were incubated for 1 h in the absence of light. Samples were read at 517 nm with a spectrophotometer (Jenway 6715, Ware, UK), and methanol was used as a blank. The antioxidant activity was expressed as mg equivalents of gallic acid in 100 g of extract, according to the following equation. All samples were analyzed in triplicate.

Antioxidant activity (mg GA/100 g) = (GA × V × DF × 100)/(Sample weight (g))

where:
GA = Gallic acid concentration from the standard curve (mg/mL);
V = Sample volume (mL);
DF = Sample dilution factor.

2.6. Antimicrobial Activity of Propolis Extracts

Four microbial strains were used: *Staphylococcus aureus* (ATCC 13709), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 43971) and *Candida albicans* (ATCC 1023). The strains were activated in a tube with 10 mL of Müller Hinton broth and incubated at 37 °C for 24 h. This operation was performed a total of three times. Finally, the turbidity of the microorganisms was standardized at 0.5 on the McFarland scale (1×10^8 CFU/mL).

The antibacterial activity was determined by the agar diffusion technique per well [24]. One mL of the microbial suspension adjusted to 0.5 in the McFarland scale was placed in a Petri dish and 20 mL of Müller–Hinton agar was added, the inoculum was homogenized and allowed to solidify, then the wells were made with a sterile punch (5 mm). From the pure extract, different dilutions (540 mg, 270 mg, 135 mg and 67.5 mg/mL) were prepared using dimethyl sulfoxide (DMSO) as diluent. Twenty-five µL of each of the concentrations, negative control (DMSO) and positive control (An antifungal–antibiotic solution (100×), consisting of 10,000 units of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per mL (Sigma-Aldrich Química, S. de R.L. de C.V. A5955)) were placed in the wells. The boxes were incubated at 37 °C for 24 h. All analyses were performed in triplicate. For inhibitory activity, the diameter of the inhibition halos (mm) was measured with a vernier calliper.

2.7. Analysis of Results

A completely randomized design was used. The results are expressed as the mean ± standard deviation. When ANOVA had significant differences ($p \leq 0.05$), Tukey's technique of comparison of means was used. All analyses were performed using the Statgraphics Centurion XVI software version 16.1.11 (Stat Point Technologies, Inc., The Plains, VA, USA).

3. Results

3.1. Obtaining the Extracts

Performance/Yield

In general, the extraction method did not influence the yield of the extracts; however, the place of origin of the propolis does influence the yield. This could be due to the type of vegetation in which the bees collect the propolis. On the other hand, very high yields were obtained, ranging from 63.84 to 71.59% (Figure 1). The propolis from San Jerónimo Tecoalt was the one that presented the highest yield (71.34 to 71.54%), in addition there was no significant difference ($p \leq 0.05$) in the extraction method. On the other hand, the propolis from San Pedro Ocopetatillo was the one with the lowest yield (63.84–66.05%), and, again, the extraction method did not influence the yield.

3.2. Behavior of Bioactive Compounds during In Vitro Digestion

3.2.1. Total Phenols

Table 2 shows the content of total phenols in the propolis extracts, as well as in the different stages of in vitro digestion: gastric phase (GP), intestinal phase (IP) and intestinal phase after boiling (IPB). In general, no significant differences ($p < 0.05$) were found in the content of total phenols in the different propolis extracts and production methods. The content of phenolic compounds was from 30,824.9 to 34,406.6 mg GAE/100 g. There was a significant difference in the TU and TM propolis, with the ultrasound treatment having a higher phenol content (33,433.3 mg GAE/100 g). However, the treatments SP U and SP M and SJ U and SJ M were not affected by the method of acquisition. Therefore, the use of

ultrasound can be an excellent option for obtaining propolis extracts, reducing the time it takes to acquire them.

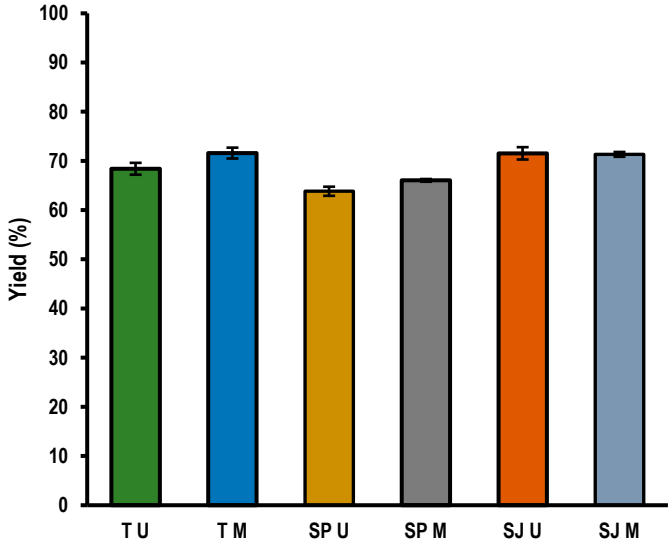

Figure 1. Yield in propolis extracts, from three places and two extraction methods. (T U = Teotitlán de Flores Magón, Ultrasound; T M = Teotitlán de Flores Magón, Maceration; SP U = San Pedro Ocopetatillo, Ultrasound; SP M = San Pedro Ocopetatillo, Maceration; SJ U= San Jerónimo Tecóatl, Ultrasound; SJ U = San Jerónimo Tecóatl, Maceration).

Table 2. Behavior of total phenols during the in vitro digestion of different propolis extracts.

Phase	Total Phenolic Content (mg GAE/100 g)					
	T U	**T M**	**SP U**	**SP M**	**SJ U**	**SJ M**
Ext	33,433.3 ± 293.9 ^{a,A}	31,642.4 ± 1110.0 ^{a,B}	33,316.5 ± 178.4 ^{a,A}	34,406.6 ± 116.7 ^{a,A}	31,253.1 ± 233.5 ^{a,B}	30,824.9 ± 526.6 ^{a,B}
GP	7262.6 ± 189.9 ^{b,B,C}	6746.7 ± 321.1 ^{b,A}	6921.9 ± 87.5 ^{b,A,B}	7476.7 ± 58.3 ^{b,C}	7447.5 ± 87.5 ^{b,C}	7291.8 ± 73.4 ^{b,B,C}
IP	7572.8 ± 245.2 ^{b,A,B}	7245.8 ± 88.1 ^{b,A}	7187.4 ± 245.2 ^{b,A}	7315.9 ± 88.1 ^{bc,A}	7374.3 ± 123.0 ^{b,A}	8098.4 ± 455.5 ^{b,B}
IPB	7273.8 ± 157.9 ^{b,A}	7495.8 ± 420.4 ^{b,A}	6958.5 ± 20.2 ^{b,A}	7180.4 ± 0.0 ^{c,A}	7472.4 ± 438.1 ^{b,A}	8208.2 ± 72.9 ^{b,B}

Different lowercase letters in the columns represent a significant difference ($p < 0.05$) with a comparison of means using Tukey's test. Different capital letters in the rows represent a significant difference ($p < 0.05$) with a comparison of means using Tukey's test. Ext = Extract, GP = Gastric Phase, IP = Intestinal Phase, IPB = Intestinal Phase after Boiling. T U = Teotitlán de Flores Magón, Ultrasound; TM = Teotitlán de Flores Magón, Maceration; SP U = San Pedro Ocopetatillo, Ultrasound; SP M = San Pedro Ocopetatillo, Maceration; SJ U = San Jerónimo Tecóatl, Ultrasound; SJ U = San Jerónimo Tecóatl, Maceration.

During in vitro digestion (GP), there was a decrease of around 80% in the content of total phenols in all treatments. This could indicate that the acid pH or pepsin may directly affect these compounds. However, in the next stage of digestion (IP and IPB) the phenols remained constant. The phenol content at the end of the digestion was from 6958.5 to 8208.2 mg GAE/100 g.

3.2.2. Total Flavonoids

The propolis extracts from TU (Teotitlán de Flores Magón), presented the highest content of flavonoids with a range of 18,107.7 to 19,523.2 mg QE/100 g, followed by the extracts from SP (San Pedro Ocopetillo) with 3361.0 to 4152.3 mg QE/100 g and the extracts with the lowest content were those from SJ (San Jerónimo Tecóalt) with 2859.8 to 3237.9 mg QE/100 g (Table 3). All the extracts obtained by ultrasound have the highest content of flavonoids. In general, during in vitro digestion, the flavonoid content was modified in each of the phases. At the end of the digestion, the treatments SJ U and SJ M were the most affected with a reduction greater than 98%, and TU and TM presented a reduction of 91.6%. Therefore, we can deduce that the digestion process negatively affects the bioavailability of bioactive compounds (flavonoids) of propolis extracts, therefore its functional properties could be affected.

Table 3. Behavior of total flavonoids during the in vitro digestion of different propolis extracts.

Phase	Total Flavonoid Content (mg QE/100 g)					
	T U	**T M**	**SP U**	**SP M**	**SJ U**	**SJ M**
Ext	19,523.2 ± 0.0 ^{aE}	18,107.7 ± 108.8 ^{aD}	4152.3 ± 46.1 ^{aC}	3361.0 ± 39.5 ^{aB}	3237.9 ± 82.4 ^{aB}	2859.8 ± 82.4 ^{aA}
GP	1170.4 ± 0.0 ^{dA}	1258.3 ± 57.8 ^{cA}	741.8 ± 32.9 ^{bB}	670.4 ± 9.5 ^{bB}	340.6 ± 25.1 ^{bC}	412.1 ± 49.4 ^{bC}
IP	1345.2 ± 39.5 ^{cA}	1325.4 ± 39.5 ^{cA}	553.9 ± 0.0 ^{cB}	184.6 ± 30.2 ^{dD}	296.7 ± 0.0 ^{bC}	487.9 ± 11.4 ^{bB}
IPB	1628.7 ± 11.4 ^{bA}	1496.8 ± 11.4 ^{bB}	389.0 ± 11.4 ^{dC}	296.7 ± 0.0 ^{cD}	59.3 ± 0.0 ^{cE}	19.7 ± 0.0 ^{cF}

Different lowercase letters in the columns represent a significant difference ($p < 0.05$) with a comparison of means using Tukey's test. Different capital letters in the rows represent a significant difference ($p < 0.05$) with a comparison of means using Tukey's test. Ext = Extract, GP = Gastric Phase, IP = Intestinal Phase, IPB = Intestinal Phase after Boiling. T U = Teotitlán de Flores Magón, Ultrasound; TM = Teotitlán de Flores Magón, Maceration; SP U = San Pedro Ocopetatillo, Ultrasound; SP M = San Pedro Ocopetatillo, Maceration; SJ U = San Jerónimo Tecóatl, Ultrasound; SJ U = San Jerónimo Tecóatl, Maceratio.

3.3. Antioxidant Activity during In Vitro Digestion

The antioxidant capacity (ABTS) of the extracts was higher in the extracts obtained by maceration, with the exception of the SJ U and SJ M treatments, where there is no significant difference ($p \leq 0.05$) due to the extraction method (Table 4). However, during in vitro digestion in the gastric phase, all extracts showed a decrease in antioxidant activity: TU 86%, TM 89%, SP U 92%, SP M 93%, SJ U 91% and SJ M 91%. This could indicate that the gastric conditions cause some type of hydrolysis or reaction with other compounds that affects the antioxidant capacity. At the end of the intestinal phase, an increase in antioxidant capacity is seen in all treatments, with some of them showing a significant difference ($p \leq 0.05$). In general, the antioxidant capacity by DPPH is greater than the antioxidant capacity by ABTS. In the gastric phase there was an increase in the antioxidant capacity by DPPH of close to 50%, probably due to the hydrolysis of larger phenolic compounds, forming some smaller ones. However, at the end of the intestinal phase there was again a reduction in antioxidant capacity, which could affect the functional properties of the propolis extracts.

Table 4. Antioxidant activity of propolis extracts and their behavior during in vitro digestion.

Phase	ABTS (mg GAE/100 g)					
	T U	**T M**	**SP U**	**SP M**	**SJ U**	**SJ M**
Ext	8333.2 ± 355.7 ^{aA}	10,793.8 ± 333.7 ^{aB}	14,647.7 ± 578.0 ^{aC}	16,530.2 ± 867.2 ^{aD}	13,180.3 ± 933.8 ^{aC}	13,002.4 ± 533.6 ^{aC}
GP	1133.4 ± 294.1 ^{cA}	1152.0 ± 462.8 ^{cA}	1126.0 ± 169.8 ^{cA}	1100.1 ± 222.3 ^{dA}	1100.1 ± 111.1 ^{dA}	1092.7 ± 128.3 ^{dA}
IP	3977.3 ± 607.6 ^{bA}	3779.7 ± 876.7 ^{bA}	3092.4 ± 3100.2 ^{bAB}	2827.4 ± 2964.5 ^{cB}	2899.3 ± 2358.4 ^{cB}	3856.0 ± 876.7 ^{cA}
IPB	3821.0 ± 2156 ^{bAB}	3812.0 ± 473.2 ^{bAB}	3996.2 ± 134.7 ^{bBC}	3663.8 ± 77.8 ^{bA}	4054.6 ± 473.2 ^{bBC}	4157.9 ± 1078.0 ^{bC}
	DPPH antioxidant activity (mg GAE/100 g)					
Ext	20,706.4 ± 648.8 ^{bA}	18,883.8 ± 1223.7 ^{bA}	19,315.5 ± 299.5 ^{bA}	13,368.3 ± 843.0 ^{bC}	17,013.3 ± 83.0 ^{bB}	16,773.5 ± 0.0 ^{bB}
GP	35,817.7 ± 1438.8 ^{aAB}	39,534.8 ± 2651.4 ^{aA}	29,342.9 ± 1438.8 ^{bC}	38,455.6 ± 1774.4 ^{aA}	36,777.0 ± 1361.8 ^{aAB}	32,580.3 ± 1648.4 ^{aBC}
IP	1471.9 ± 436.0 ^{cA}	3383.4 ± 0.0 ^{cB}	1203.4 ± 872.0 ^{cC}	1494.0 ± 251.7 ^{cC}	2366.1 ± 907.6 ^{cBC}	2656.7 ± 666.0 ^{cBC}
IPB	1813.8 ± 1572.0 ^{dA}	360.4 ± 251.0 ^{cA}	796.4 ± 666.0 ^{cA}	1232.4 ± 251.7 ^{cA}	2685.8 ± 1744.0 ^{cA}	1184.2 ± 755.2 ^{dA}

Different lowercase letters in the columns represent a significant difference ($p < 0.05$) with a comparison of means using Tukey's test. Different capital letters in the rows represent a significant difference ($p < 0.05$) with a comparison of means using Tukey's test. Ext = Extract, GP = Gastric Phase, IP = Intestinal Phase, IPB = Intestinal Phase after Boiling. T U = Teotitlán de Flores Magón, Ultrasound; TM = Teotitlán de Flores Magón, Maceration; SP U = San Pedro Ocopetatillo, Ultrasound; SP M = San Pedro Ocopetatillo, Maceration; SJ U = San Jerónimo Tecóatl, Ultrasound; SJ U = San Jerónimo Tecóatl, Maceration.

3.4. Antimicrobial Activity

Figure 2 shows the antimicrobial activity of the six propolis extracts obtained by ultrasound and maceration against four pathogenic strains. It should be mentioned that the antimicrobial activity was evaluated in the extracts before and at the end of in vitro digestion. However, no treatment at the end of the simulated in vitro digestion showed inhibitory activity. On the other hand, the higher the concentration of extract, the greater the halos of inhibition. The inhibitory activity is a function of the components of the extract, the place of origin of the propolis and in some cases of the extraction method. The SP U

and SP M treatments did not present activity against *S. aureus*. The greatest inhibition for *S. aureus* was the SJ U treatment. In this case, the ultrasound (SJ U) treatment was better than the maceration (SJ M). All treatments presented inhibitory activity for *C. albicans*. In addition, this strain was the one that presented the highest inhibition halos; therefore, we consider that these extracts could be an excellent source of bioactive compounds in the preparation of formulations for control of urogenital infections. Regarding *E. coli*, the TU and TM treatments presented the highest inhibitory activities, followed by the SJ U and SJ M treatments. It is worth mentioning that these treatments did not present inhibition halos at concentrations lower than 270 and 135 mg/mL, respectively, being able to be considered as the minimum inhibitory concentration. In addition, the SP U and SP M treatments did not present inhibition halos at any of the concentrations evaluated. For *S. typhimurium*, all the extracts presented halos of inhibition, the largest being the extracts obtained by maceration. Finally, we consider that the analysed propolis can be used in traditional medicine for the treatment of various health conditions.

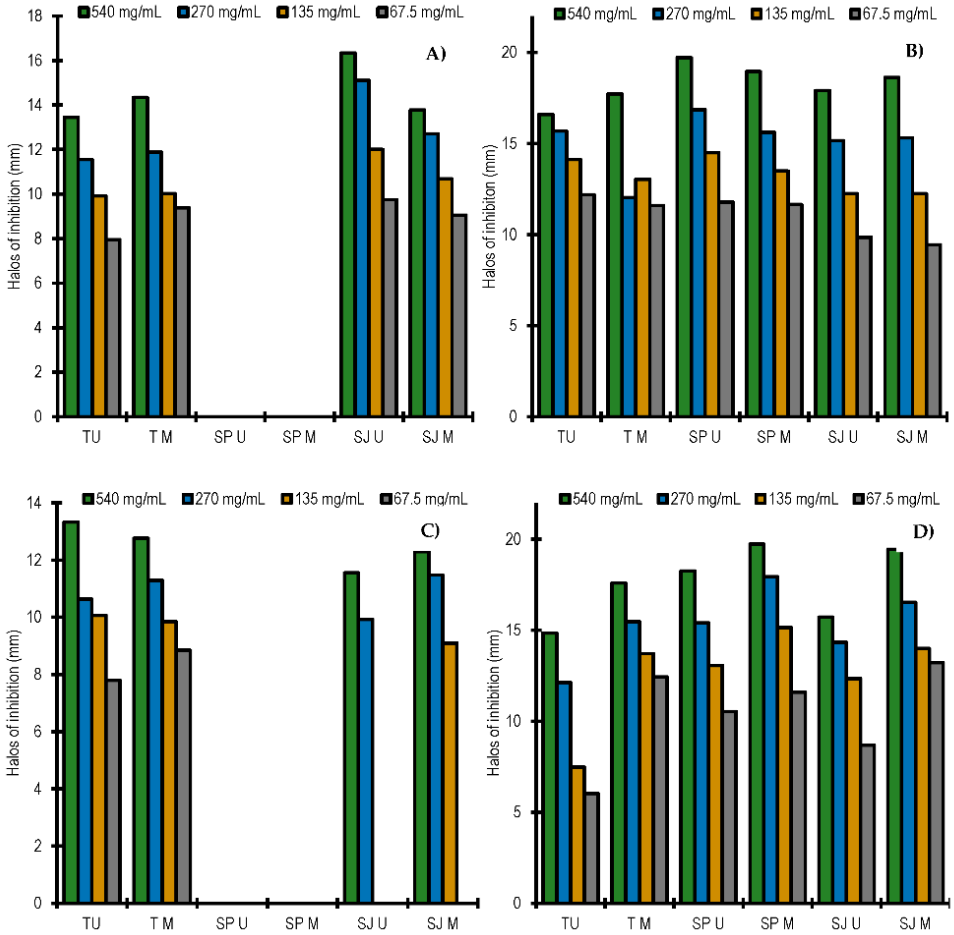

Figure 2. Inhibitory activity of propolis extracts against different microbial strains. (**A**) *Staphylococcus aureus* (ATCC 13709), (**B**) *Candida albicans* (ATCC 1023), (**C**) *Escherichia coli* (ATCC 25922) and (**D**) *Salmonella typhimurium* (ATCC 43971).

4. Discussion

In general, raw propolis is not used in the preparation of food or medicine due to its composition. Extracts are its most common form, and it is important to consider that the performance of the extracts is variable due to different factors, such as the extraction method (maceration, Soxhlet, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluids with CO_2 and high pressure), the nature of the solvent (according to its polarity; low, medium or high), ratio or proportion of solvent and time and tempera-

ture of extraction [4,15]. Our extracts presented a yield of 63.8 to 71.5%, being higher than the 2.57 to 22.67% [25] and 18.2% [26] of various propolis simples.

According to the Official Mexican Standard, propolis marketed in Mexico must have a minimum of 5000 mg EGA/100 g of phenolic compounds [3]. The propolis analysed had a total phenol content of 30,824.9 to 34,406.6 mg EGA/100 g, thus complying with Mexican regulations. In addition, these results are higher than those reported in ethanolic extracts of propolis from China (8934.3 to 20,178.6 mg EGA/100 g). These extracts were obtained with ultrasounds, and the difference in the content of total phenols could be related to the type of vegetation from which the bees collected the propolis and the collection season, as well as various environmental factors [27,28]. Ethanolic extracts (70%) of propolis from Turkey have a very variable total phenol content, ranging from 2748 to 19,970 mg EGA/100 g. The authors mention that this variability is associated with the geographical origin of the propolis. In addition, these values are lower than ours [29]. On the other hand, aqueous extracts of propolis from Algeria have a total phenol content of 9688.3 to 45,883.3 mg EGA/100 g, the variation was mainly due to the place of origin of the propolis [30]. On the other hand, there are various methods for the purification of extracts, which can influence the content of bioactive compounds. In the elaboration of propolis extracts from Greece, four purification methods were used, obtaining a total phenol content of 11,385 to 29,811 mg EGA/100 g, centrifugation being the most effective method [31]. The total phenolic content in ethanolic extracts of propolis from Turkey was 25,000 mg EGA/100 g. However, at the end of in vitro digestion only 8.9% were bioavailable. It should be noted that in vitro digestion was performed in crude propolis [29], thus the low bioavailability of phenols could be due to acid conditions and possible isomerization reactions of the phenolic compounds during the digestion process [32]. The bioavailability of our extracts at the end of digestion was 20.88 to 26.62%.

The range of total flavonoids in our extracts was from 2859 to 19,523 mg EQ/100 g of propolis. These results are higher than those proposed by the Mexican regulations (min. 500 mg EQ/100 g). Various investigations report a total flavonoid content of 3823 to 4745 mg EQ/100 g [31]; 304.7 to 527.3 mg EQ/100 g [30]; 16,622 to 51,977 mg EQ/100 g [27]; 1100 to 15,000 mg EQ/100 g [7]; 4946.5 mg EQ/100 g [33]; and 55,758 mg EQ/100 g [26]. As we can see, the flavonoids' content is highly variable; therefore, biological activity could vary. As mentioned above, the composition of phenolic compounds (flavonoids) depends on factors, such as floral and geographical origin. However, for the biological properties of the extracts to be preserved, it is important to assess the bioavailability at the end of the digestion. The bioavailability of total flavonoids from our extracts at the end of in vitro digestion ranged from 0.68 to 9.36%. Therefore, it is important to look for alternatives for the protection of bioactive compounds during gastrointestinal conditions.

The propolis extracts presented strong antioxidant activity, using the ABTS and DPPH methods, ranging from 8333.2 to 16,530.2 and 13,368.3 to 20,706.4 mg EGA/100 g, respectively. We can observe that the antioxidant activity is directly related to the content of phenolic compounds (Tables 2 and 3), and the significant difference ($p \leq 0.05$) that the extracts present is mainly due to the place of collection of the propolis [8] and, in some cases, to the method of obtaining. On the other hand, the antioxidant activity of propolis extracts can be increased using various purification methods, centrifugation being one of the most effective [31]. Around the world there are several investigations on the biological properties and characterization of bioactive compounds in propolis extracts with very promising results. The extracts obtained by the ultrasound-assisted method have better biological properties than the extracts obtained by traditional methods [27,34], which is why, in recent years, it has become the most popular method in the areas of medicine, biochemistry, food industry, among others [35]. On the other hand, ethanol is the best extraction solvent for obtaining propolis extracts by ultrasounds [36]. During in vitro digestion, the antioxidant activity by both the ABTS and DPPH methods was significantly affected, as was the content of phenolic compounds (total phenols and flavonoids). At the end of the digestion there was a decrease in antioxidant activity from 77.83% for ABTS and from 98.09% for DPPH.

As we have been able to observe, the antimicrobial activity of the evaluated extracts is variable, and its effect depends mainly on the place of origin rather than on the extraction method. It has been proposed that the antimicrobial activity of propolis extracts acts in two ways: (1) Direct effect on the microorganism, affecting the permeability of the cell membrane, cell membrane disruption, decreased ATP production and decreased mobility and (2) the stimulation of the immune system, activating the body's natural defenses [7,37]. There are various works available on the antimicrobial activity of propolis. These investigations vary according to the type of microorganism to be evaluated (bacteria, molds, yeasts, protozoa and viruses), extraction conditions (method, temperature, type of solvent, ratio or proportion of solvent, etc.), as well as the place of origin of the propolis [38]. The extracts obtained by ultrasounds have antimicrobial activity against the strains of *S. aureus*, *E. coli*, *C. krusei*, *C. glocosporioides*, *M. mucedo* and *A. solani*, ultrasounds being an excellent means of obtaining propolis extracts [39]. Propolis from Chile has antibacterial activity with strains of *S. mutans* and also decreases the formation of biofilms [39]. Rivero et al. [40] mentions that propolis from Guanajuato, Mexico, has an excellent antibacterial activity against oral pathogens (*S. mutans*, *S. oralis*, *S. sanguinis* and *P. gingivalis*).

5. Conclusions

The use of ultrasound in the production of ethanolic extracts of propolis can be an excellent option, since it provides better yields and increases its biological activity. In vitro digestion considerably reduces the content of bioactive compounds in the extracts analysed total phenols and total flavonoids thus, the antioxidant activity and antimicrobial activity. In what concerns the antimicrobial activity, the inhibition halos depend mainly on the concentration and the place of origin of the propolis.

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