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Evaluating bioactivity and bioaccessibility properties of the propolis extract prepared with L-lactic acid: An alternative solvent to ethanol for propolis extraction

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ABSTRACT

Ethanol readily solubilizes the active components of propolis. Due to certain disadvantages of ethanolic extracts, developing new non-ethanolic extraction methods is highly desirable. This study aimed to determine the content of phenolic compounds and antioxidant activity of propolis by using lactic acid extraction compared to ethanol. Various concentrations of ethanol and lactic acid were utilized to produce extracts, and an *in vitro* digestion procedure was applied to the extracts to compare the bioaccessibility of phenolic compounds by using LC-MS/MS in extracts and HPLC-DAD in digested samples. The compounds detected in the lactic acid-based propolis solution had similar values to those in the ethanol solution at a propolis concentration of 10%. The propolis-lactic acid solution had a significantly higher antioxidant activity against DPPH radical than the one prepared with ethanol at the same concentration ($p < 0.05$). Chrysin and naringenin were found as the most abundant phenolic compounds in the initial samples during *in vitro* digestion experiments. Pinocembrin was the most bioaccessible component among the dialyzed IN fractions of ethanol and lactic acid-based solutions, whereas some compounds were not detected. The results suggest that lactic acid may be used as an alternative to ethanol for propolis extraction.

1. Introduction

In an effort to tackle nutrient deficiency, the number of foods fortified with bioactive compounds is growing steadily, especially after the COVID-19 pandemic (Galanakis, Aldawoud, Rizou, Rowan, & Ibrahim, 2020). This trend has prompted researchers to search for new bioactive compound effects on human health. Recovering functional compounds from food processing by-products, medicinal plants, and other natural substances is possible (Galanakis, 2021). Propolis is a honeybee product that has recently gained attention as an integrative medicine due to its documented therapeutic effects, including antitumoral, anti-inflammatory, anticancer, antioxidant, and antimicrobial properties (Braakhuis, 2019). Moreover, propolis is regarded as a risk-free product, with the exception of those who might experience an allergy to it. As a result of its known biological functions, it can reduce the risk and severity of the infection and be used as an adjunct to treatment with the main drugs without the risk of potentiation or inactivation (Berretta, Silveira, Capcha, & De Jong, 2020). Honeybees produce propolis by modifying resins and balsamic compounds found in floral buds or the bark of deciduous trees with wax and apian gland secretions (Bankova, Popova, & Trusheva, 2016). Consequently, a high concentration of functional compounds such as flavonoids and other polyphenols and being toxic residue free are the indicators of a suitable extraction method (Idrus et al., 2018). Up to this point, ethanol

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extraction has been the most prevalent approach for the manufacturing of propolis extracts. This method is appropriate for obtaining propolis extracts rich in bioactive compounds and low in wax (Kubiliene et al., 2015). Using 70% ethanol for extraction is the most widely used technique because it is simple and ideally suited for the production of polyphenol-rich deparaffinated extracts (Pobiega, Kraśniewska, Derewiaka, & Gniewosz, 2019). However, its strong residual flavor, as well as certain disadvantages such as its solid, sticky nature and unsuitability for use in pediatrics or individuals with alcohol intolerance, are limiting factors for the use of ethanolic propolis extracts. Therefore, it is highly desirable to develop propolis preparations based on safe and effective non-ethanolic extraction methods (Kubiliene et al., 2015).

Water, oil, propylene glycol, and glycerol are the other solvents commonly used for propolis extraction (Arslan, Perçin, & Silici, 2010). However, numerous studies have reported that the concentration of biologically active compounds was found to be higher in ethanol-based extracts (Moura et al., 2011). In a previous study on commercial samples of propolis extracts with water and oil, the polyphenol contents were very low; consequently, investigations have begun to identify more efficient co-solvents that increase the solubility of these substances (Kubiliene et al., 2015). Although olive oil or other oil-based propolis extracts are optimal for dermatological pharmaceuticals and cosmetic preparations, no conclusion could be made about the superiority of oil-based propolis extracts over ethanolic extracts (Ramanauskienė & Inkėnienė, 2011). In a previous comparative study, the highest yield was obtained with ethanol as the solvent, while olive oil extracts were reported to be more advantageous because they can be used directly without removing the solvent (Pujirahayu, Ritonga, & Uslinawaty, 2014). Silici and Baysa (2020) reported that very high phenolic content was obtained in Turkish propolis with an extraction method using olive oil as the solvent. The benefits obtained from olive oil-based propolis might be due to the bioactive components of both olive oil and propolis. Glycerin (glycerol) and propylene glycol (PG) have also been used as solvents for propolis extraction. Although glycerin (glycerol) does not have an ADI value, it has been observed that its potential to dissolve propolis is considerably less than ethanol (Bakkaloğlu & Arıcı, 2019). Although it is generally regarded as safe for use, adverse effects of PG have also been reported previously by several authors noting infants being at a particularly increased risk (De Cock et al., 2014). Moreover, the American Contact Dermatitis Society named PG "an allergen of the year" in 2018 (McGowan, Scheman, & Jacob, 2018). According to epidemiological data, 0.8%–3.5% of people are allergic to PG, and these people should avoid using it. For this reason, the presence of PG in some water-based propolis drop products may pose a risk in atopic individuals (Jacob, Scheman, & McGowan, 2018).

Processing propolis with alternative solvents raises concerns about their use. When the available solvents are evaluated, solvents with high dissolving efficiency may cause negative consequences for human health. On the other hand, the phenolic content provided by propolis with harmless solvent alternatives is reported as being lower, and the therapeutic preparations produced from these samples were observed to be less effective (Bakkaloğlu & Arıcı, 2019). Conventional chemical solvents that dissolve high phenolic compounds are often extremely flammable and toxic. Hence, much research focuses on replacing dangerous solvents with more environmentally friendly alternatives. Developing new, eco-friendly, and benign solvents is one of the critical topics in Green Chemistry. Chemicals derived from biomass have been offered promising opportunities in searching for predictable, "sustainable," or "green" solvents. The choice of solvent for propolis extraction directly affects the amount of bioactive substances in the final product. Therefore, total phenolic content and antioxidant capacity may depend on solvent selection. Recently, lactic acid has been used to extract phenolic compounds from plant materials (Azmir et al., 2013). Lactic acid is an organic acid that is used as a food preservative. It is a standard metabolic product of the body, and one of the oldest known biological preservatives, as a weak organic acid produced by certain microorganisms (Ameen & Caruso, 2017). Besides skin and gastric mucosa irritation at high concentrations, using lactic acid at the recommended amount and concentration does not pose a severe safety risk to health. Lactic acid is present in the structure of an optical isomer (enantiomer), and compared to L-lactic acid, D-lactic acid does not play a role in the fundamental metabolic processes of most organisms. Enzymes involved in lactate metabolism, such as L-lactate dehydrogenase, are incapable of converting D-lactic acid due to their specificity for the L-isomer. Therefore, L-lactic acid is considered safe, while the other isomers may have safety concerns (Pohanka, 2020).

The bioaccessibility of phenolics in the digestive tract and circulatory system are critical factors in interpreting their health benefits (Shahidi & Peng, 2018). *In vitro* digestion methods are frequently preferred because they are quick, safe, inexpensive, and relatively simple. However, only a few studies have been carried out to assess the bioaccessibility of different propolis samples in the literature (Ozdal et al., 2019; Yen et al., 2017; Yesiltas et al., 2014). Furthermore, another critical aspect of this study is to propose a novel solvent for propolis extraction to overcome the disadvantages of ethanolic extracts since it is highly desirable to explore novel non-ethanolic extraction techniques. In this study, we compared and evaluated the phenolic contents and profiles, antioxidant capacities, and the impact of *in vitro* gastrointestinal digestion on the antioxidant activity of propolis samples prepared using L-lactic acid or ethanol.

2. Material and methods

2.1. Extraction of propolis samples

The propolis-solvent samples were prepared in proportions of 10%, 20%, 30%, and 40% (g/g) by using 70% ethanol (Merck KGaA, Darmstadt, Germany) or 80% L-lactic acid (Merck KGaA, Darmstadt, Germany) and raw propolis samples from an apiary in the Mersin region were used (southern part of Turkey). Then the samples were homogenized using a homogenizer (IKA T 25 digital ultra-turrax, Germany) for 30 min, and propolis samples were kept at room temperature in the dark for 15 days. Next, the prepared solutions were filtered using Whatman No. 4 filter paper (Millipore, USA) by gravity filtration. Finally, the extracts were stored at +4 °C until analysis.

2.2. Determination of polyphenol contents with LC-MS/MS procedure

Mass spectrometer measurements were performed with a Thermo Orbitrap Q-Exactive mass spectrometer (Paisley, UK) using electrospray ionization (ESI) as the ion source. LC separations were conducted on a C18 analytical column (Troyosil, 5 μm particle size, 150 mm × 3 mm) using the mobile phases consisting of methanol (A, 0.5% formic acid) and water (B, 0.5% formic acid). The gradient program was conducted as 0–1 min 50% A and 50% B and 1.01–30 min 100% A and at the end of the program, 30.01–35.00 50% A and 50% B. The temperature of the column was adjusted to 30 °C, and the flow rate of the mobile phase was 0.3 ml/min. Samples were taken into a 5 ml volumetric flask, adjusted to a final concentration of 3 ppm from 100 mg/L internal standard solution, and curcumin was used as an internal standard. The analysis was conducted after the filtration of samples with 45 μ filters. The volume of injection was 10 μl (Gülçin, Bursal, Şehitoğlu, Bilsel, & Gören, 2010).

2.3. Determination of antioxidant capacity with 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

According to the preliminary test results, the prepared solutions were diluted at specific proportions. The samples were taken between 20 and 300 μl, and 600 μl of 1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) radical solution was added, and the total volume was completed to 6 ml with methanol. After mixing the tubes, the samples were kept in the dark and at room temperature for 30 min, and the absorbances were read at

517 nm by using a UV–vis spectrophotometer against the blank prepared with methanol (Bölük, Sözeri Atik, Kolaylı, Demirci, & Palabiyik, 2021; Malkoç, Çakir, Yakup, Zehra, & Kolayli, 2019). The results were given as mM Trolox Equivalent (TE)/L, and the calibration curve was given as y = 0.4555x-10.04; $R^2 = 0.996$. Analyses were carried out in duplicate.

2.4. Determination of cupric reducing antioxidant capacity (CUPRAC) assay

Before analysis, copper (II) chloride solution at a concentration of 10–2 M, ammonium acetate (NH_4Ac) buffer with pH 7.0, and a solution of neocuproine (Nc) in ethanol was prepared at a concentration of 7.5×10^{-3} M. Nc solution in ethanol was prepared daily at a concentration of 7.5×10^{-3} M. By mixing 1 ml of copper (II) chloride solution, 1 ml of Nc solution, 1 ml of ammonium acetate buffer, 100 μl of sample extract or standard extracts (for the calibration curve) and 1 ml of Milli-Q water, and the absorbance was obtained at 450 nm after 30 min of incubation (Apak et al., 2007). The calibration curve was constructed using the Trolox equivalent (y = 13.3770x-0.0218; $R^2 = 0.9999$). The results were represented as mM TE/L. Analyses were carried out in duplicate.

2.5. Total phenolic content analysis

The total phenolic contents of the samples were evaluated using Folin-Ciocalteu's phenol reagent (Merck KGaA, Darmstadt, Germany). First, 100 μl of sample filtrates were taken, 500 μl of the Folin-Ciocalteu reagent and 7.5 ml of distilled water were mixed, mixed, and stood for 1 min. Then, 1 ml of the saturated Na_2CO_3 solution was added, and the total volume was completed to 10 ml. After 60 min, it was read against the blank at 720 nm (Slinkard & Singleton, 1977). The calibration curve was constructed using gallic acid (Merck KGaA, Darmstadt, Germany) (y = 0.0007x+0.0072; $R^2 = 0.9995$). The results were represented as in g gallic acid equivalents (GAE)/L of the sample. Analyses were carried out in duplicate.

2.6. Simulated in-vitro gastrointestinal (GI) digestion

In order to simulate the *in vitro* GI digestion conditions, the applied model was adapted from the work of Minekus et al. (2014) with slight modifications and performed in triplicate for 10% ethanolic and lactic acid-based solutions. The formulations of the salivary, gastric, and intestinal fluids are described in Table 1. Briefly, 5 ml of propolis extracts were mixed artificial saliva medium made up of amylase solution, saliva liquid, distilled water, and $CaCl_2$. After incubating at 37 °C in a shaking water bath (Memmert SV 1422, Memmert GmbH & Co. Nürnberg, Germany) for 2 min, the gastric phase was directly started without collecting aliquots from the buccal phase. In the gastric phase, the prepared stomach fluid was mixed with $CaCl_2$, pepsin solution, and distilled water, and pH was adjusted to 3 using HCl. Samples were incubated in a shaking water bath (Memmert, Nürnberg, Germany) at 37 °C for 2 h in a stomach medium. After the gastric phase, 5 ml aliquots were collected for further analysis. The intestinal phase was simulated in two different ways. Firstly, for the intestinal medium, the prepared intestinal fluid was mixed with bile solution, pancreatin solution, $CaCl_2$, and distilled water, and pH was adjusted to 7 with NaOH. 5 ml of samples were collected after being incubated in a shaking water bath (Memmert, Nürnberg, Germany) at 37 °C for 2 h in the intestinal medium for further analyses. Secondly, segments of dialysis bags were cut to a specified length and filled with sufficient $NaHCO_3$ (20 ml) to neutralize the titratable acidity of the sample and placed into the same intestinal medium as the first way. Finally, after simulating 2 h of intestinal digestion, 5 ml aliquots were collected from the inside (Intestinal IN) and outside (Intestinal OUT) dialysis bags for further analysis. Aliquots collected from the gastric and intestinal phases were centrifuged (Hettich, Tuttlingen, Germany) at 14,000 rpm and 4 °C for 5 min, and the supernatants were kept at 20 °C until further analyses.

Table 1
Simulated digestion fluids used for *in vitro* gastrointestinal system simulation.

Constituents	Concentration (mol/L)	Salivary Fluid (pH:7) (mL)	Gastric Fluid (pH:3) (mL)	Intestinal Fluid (pH:7) (mL)
KCl	0.5	15.1	6.9	6.8
KH_2PO_4	0.5	3.7	0.9	0.8
$NaHCO_3$	1	6.8	12.5	42.5
NaCl	2	–	11.8	9.6
$MgCl_2(H_2O)_6$	0.15	0.5	0.4	1.1
$(NH_4)_2CO_3$	0.5	0.06	0.5	–
HCl	6	0.09	1.3	0.7

*All digestion fluids should be fulfill to 400 ml with distilled water.

2.7. HPLC-DAD analysis of polyphenols in digested samples

The profiles of phenolic compounds from samples were determined following the method of Capanoglu, Beekwilder, Boyacioglu, Hall, and De Vos (2008). Sample extracts were filtered using a 0.45 μm membrane filter. A Waters 2695 HPLC system with a PDA (Waters 2996) detector was used to conduct analyses. A Supelcosil LC-18 (25 cm × 4.60 mm, 5 m column Sigma-Aldrich, Steinheim, Germany) was used. The mobile phase is composed of solvent A, Milli-Q water with 0.1% (v/v) Trifluoroacetic acid (TFA), and solvent B, acetonitrile with 0.1% (v/v) TFA. A linear gradient was used as follows: At 0 min, 95% solvent A and 5% solvent B; at 45 min, 65% solvent A and 35% solvent B; at 47 min, 25% solvent A and 75% solvent B; and at 54 min returning to initial conditions. The flow rate was 1 ml/min. Detection was done at 280, 312, and 360 nm. Retention times and characteristic UV spectra were employed for identification. The quantification was performed using external standards.

2.8. Statistical analysis

Statistical analyses were performed by one-way ANOVA using JMP 5.0.1 (SAS Institute) program. The Tukey multiple comparison test determined a significant difference between the data according to the $p < 0.05$ value.

3. Results

3.1. The total phenolic content determination and comparison

In this study, the potential of lactic acid was evaluated as a solvent for propolis extraction. The total phenolic contents determined in solutions with 10%, 20%, 30%, and 40% propolis prepared either in ethanol or lactic acid solvents are given in Fig. 1. The total phenolic contents of all samples were found to be between 17.3 g GAE/L and 136.2 g GAE/L, and when the solvents were compared, it was observed that as the propolis concentration increased, the total amount of phenolic contents increased significantly, as expected. When different solvents with the same concentration were compared, it was found that the total phenolic contents of the samples prepared with ethanol in 20% and 30% solutions were statistically higher. In comparison, the samples prepared with lactic acid in 10% and 40% solutions contained a higher amount of total phenolic contents than the samples with ethanol ($p < 0.05$).

The changes in the total phenolic contents during *in vitro* GI digestion are shown in Table 2. There was a significant decrease from initial extracts to digested phases for ethanolic and lactic acid extracts ($p < 0.05$). The total phenolic contents of 10% propolis-ethanolic extract varied between 1598 and 17397 mg GAE/L, whereas between 783 and 23787 mg GAE/L for 10% propolis-lactic acid extract. The lactic acid solution had statistically higher total phenolic content at all stages of digestion,

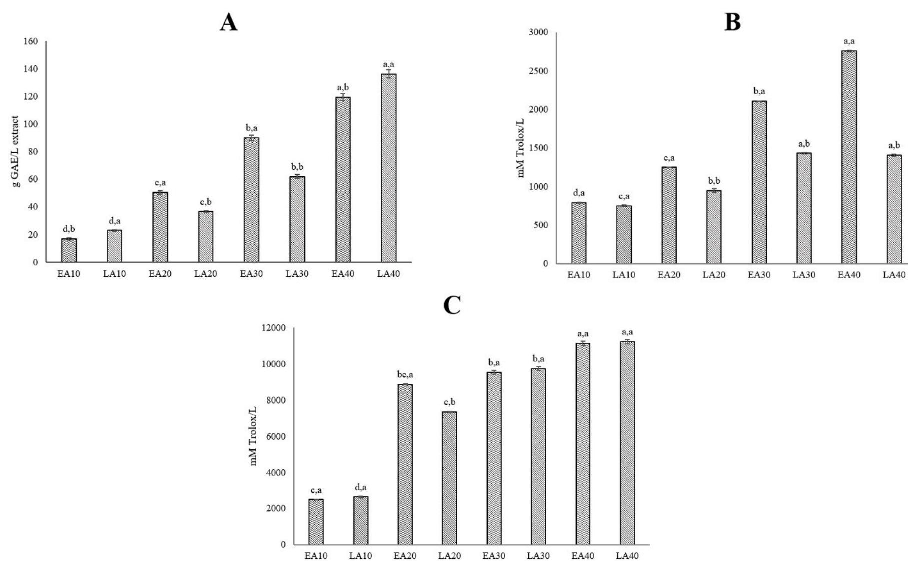

Fig. 1. A: Total phenolic content of the samples (mg GAE/L); **B:** Results of antioxidant activity analysis of samples with *2,2-Diphenyl-1-picrylhydrazyl* (DPPH) radical method; **C:** The antioxidant activity analysis results of samples with cupric reducing antioxidant capacity (CUPRAC) method.
GAE: Gallic Acid Equivalent, EA: Ethanolic Extract, LA: Lactic Acid Extract
First letters present the differences between the solvent ratio of samples, and second letters show differences between the samples with the same solvent ratio ($p < 0.05$).

Table 2
Total phenolic contents of gastric and intestinal phases of propolis extracts after *in vitro* GI digestion.

Sample	Total phenolic content of digested propolis extracts (mg GAE/L)			Total phenolic content of dialyzed propolis extracts (mg GAE/L)	
	Initial	Gastric Phase	Intestinal Phase	Intestinal IN	Intestinal OUT
Propolis-Ethanolic Solution (10%)	$17397 \pm 146^{B,a}$	$2159 \pm 231^{B,b}$	$2020 \pm 110.^{B,b}$	$1598 \pm 45^{A,b}$	$2054 \pm 93^{B,b}$
Propolis-Lactic Acid Solution (10%)	$23787 \pm 662^{A,a}$	$5552 \pm 424^{A,b}$	$5960 \pm 92^{A,b}$	$783 \pm 33^{B,c}$	$3847 \pm 39^{A,bc}$

*Data represent average values ± standard deviation of three independent samples. Uppercase superscripts represent statistically significant differences among extraction method and lowercase superscripts represent statistically significant differences among digestion steps ($p < 0.05$).

except for the dialyzed IN fraction ($p < 0.05$). The highest total phenolic content was found in the initial lactic acid extract, and the lowest was observed in the dialyzed IN fraction of the lactic acid extracts. The amount of phenolic compounds reaching the intestinal phase was higher in the lactic acid solution ($p < 0.05$). However, the recoveries of these compounds after dialysis were found as 9.3% for ethanolic extracts and 3.3% for lactic acid extracts.

3.2. Antioxidant activity determination and comparison

The results of the antioxidant activity analysis performed by the DPPH scavenging activity method of solutions prepared with two different solvents, lactic acid and ethanol containing 10, 20, 30, and 40% propolis, are given in Fig. 1 as mM TE/L. It was observed that the antioxidant activity increased when the propolis concentration increased. When propolis samples with different solvents in the same concentration were compared, it was found that the 10% propolis-lactic acid solution had a statistically similar level of antioxidant capacity to the one prepared with ethanol ($p < 0.05$). However, this was not the case at higher concentrations (Fig. 1). The results of the antioxidant activity analysis of the solutions measured by the CUPRAC method are given in Fig. 1 as mM TE/L. As the concentration of propolis increased, a statistically significant increase was observed in antioxidant activity for both solvents ($p < 0.05$). In addition, when the solutions containing lactic acid and ethanol solvents are compared for the same amount of propolis, ethanolic extracts of 20% solution showed statistically higher antioxidant activity than lactic acid solutions. However, no difference was observed between samples prepared with three solvents in 10%, 30% and 40% solutions.

For evaluating the effect of *in vitro* GI digestion on total antioxidant capacity, CUPRAC and DPPH assays were performed. The total antioxidant capacities of initial extracts and gastric and intestinal phases are presented in Table 3. The total antioxidant capacities of propolis extracts throughout the digestive tract varied from 30 to 790 mM TE/L propolis

Table 3
Total antioxidant capacities of gastric and intestinal phases of propolis extracts after *in vitro* GI digestion.

Sample	Antioxidant activities of digested propolis extracts (mM TE/L)			Antioxidant activities of dialyzed propolis extracts (mM TE/L)	
	Initial	Gastric Phase	Intestinal Phase	Intestinal IN	Intestinal OUT
DPPH					
Propolis-Ethanolic Solution (10%)	$790 \pm 0.09^{A,a}$	$520 \pm 0.00^{A,b}$	$420 \pm 0.03^{B,c}$	$30 \pm 0.00^{B,e}$	$100 \pm 0.01^{A,d}$
Propolis-Lactic Acid Solution (10%)	$750 \pm 0.04^{A,b}$	$370 \pm 0.01^{B,c}$	$820 \pm 0.015^{A,a}$	$40 \pm 0.00^{A,d}$	$60 \pm 0.00^{B,d}$
CUPRAC					
Propolis-Ethanolic Solution (10%)	$2490 \pm 0.15^{B,c}$	$2940 \pm 0.03^{A,b}$	$2080 \pm 0.13^{B,d}$	$1550 \pm 0.13^{A,e}$	$4320 \pm 0.33^{A,a}$
Propolis-Lactic Acid Solution (10%)	$2640 \pm 0.08^{A,b}$	$2490 \pm 0.17^{B,c}$	$2790 \pm 0.12^{A,a}$	$680 \pm 0.01^{B,e}$	$2230 \pm 0.09^{B,d}$

*Data represent average values ± standard deviation of three independent samples. Uppercase superscripts represent statistically significant differences among extraction method and lowercase superscripts represent statistically significant differences among digestion steps ($p < 0.05$).

extract for 10% ethanolic extracts and 40–820 mM TE/L propolis extract for 10% lactic acid extracts according to the DPPH method. After intestinal digestion, 53.2% and 109.3% of the antioxidant components were found in the intestinal fraction for ethanolic extract and lactic acid extract, respectively. However, 3.8% and 5.3% of these compounds were available in the dialyzed fraction, respectively. The total antioxidant capacities of propolis samples during digestion varied from 1550 to 4320 mM TE/L propolis extract for 10% ethanolic extracts and 680–2790 mM TE/L propolis extract for 10% lactic acid extracts according to the CUPRAC method. After intestinal digestion, 83.5% and 105.7% of the compounds were found in the intestinal fraction for ethanolic and lactic acid extract, respectively. Contrary to the DPPH method, the recoveries in the dialyzed fraction were higher in the CUPRAC method, as 62.3% for the ethanolic and 25.8% for the lactic acid solution ($p < 0.05$).

3.3. Phenolic profile determination by using LC-MS/MS and HPLC-DAD and comparison

The phenolic profile of 10% ethanol and lactic acid-propolis extracts was determined by LC-MS/MS. Table 4 shows the phenolic content of propolis solutions prepared with lactic acid and ethanol at a propolis concentration of 10%. Ascorbic acid, chlorogenic acid, fumaric acid, caffeic acid, trans taxifolin, *p*-coumaric acid, rutin, apigenin, caffeic acid phenethyl ester (CAPE), kaempferol were determined in higher amounts in lactic acid-propolis solution when compared to ethanol-based propolis solution. As seen in Table 4, epigallocatechin and lutcolin-7-rutinoside compounds detected in the lactic acid-based propolis solution have similar values to those in the ethanol solution, which are 0.37 and 0.28 for the ethanolic solution and 0.41 and 0.35 for the lactic acid-based solution, respectively. Furthermore, naringin and isosakuranetin were below the detection limit for the lactic acid-based propolis solution.

The abundant phenolic components of initial and digested ethanolic and lactic acid extracts were determined by HPLC-DAD analysis. A comparison of the phenolic profiles of propolis extracts is shown in Table 5. Up to 22 individual phenolic compounds were identified in the samples. Chrysin and naringenin were found as the major phenolic compounds in the initial solutions. The highest amounts detected in the intestinal phase were pinocembrin for ethanol and lactic acid-based solutions.

Similarly, pinocembrin was the most bioaccessible component among the dialyzed IN fractions. On the other hand, Gallic, protocatechuic, 4-hydroxybenzoic, vanillic acids, cynarin, and rutin were not detected in the initial extracts. On the other hand, hesperedin, phlorizin, quercitrin, and luteolin were only observed in the initial solutions. Interestingly, quercetin was detected in the initial and dialyzed IN fraction of ethanol-based and initial and gastric fraction of lactic acid-based solutions. Similarly, kaempferol was only detected in the initial ethanol-based solution and the gastric fraction of lactic acid-based solutions.

Table 4
Results of liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Phenolic Compounds Detected	m/z	Propolis-Ethanolic Solution (10%) mg/L	Propolis-Lactic Acid Solution (10%) mg/L
Ascorbic acid	175.02	103.28 ± 11.43[b]	261.03 ± 28.90[a]
(−)-Epigallocatechin	307.08	0.37 ± 0.04[a]	0.41 ± 0.05[a]
Chlorogenic acid	353.08	6.82 ± 0.76[b]	24.49 ± 2.73[a]
Fumaric acid	115.00	354.49 ± 39.49[b]	1498.67 ± 166.95[a]
Caffeic acid	179.03	1476.18 ± 163.41[b]	3887.92 ± 430.39[a]
(+)-Trans Taxifolin	303.05	14.14 ± 1.58[b]	46.85 ± 5.24[a]
Lutcolin-7-rutinoside	593.15	0.28 ± 0.03[a]	0.35 ± 0.04[a]
Naringin	579.17	0.01 ± 0.00	<LOD[a]
p-Coumaric acid	163.04	5285.42 ± 626.32[b]	8367.11 ± 991.50[a]
Hesperidin	609.18	0.10 ± 0.01[a]	0.9 ± 0.1[a]
Rutin	609.14	1.35 ± 0.16[b]	11.68 ± 1.38[a]
Hyperoside	463.08	2.51 ± 0.29[b]	13.95 ± 1.60[a]
Dihydrokaempferol	287.05	185.47 ± 21.05[a]	95.47 ± 10.84[b]
Apigenin 7-glucoside	431.09	9.14 ± 1.09[b]	17.71 ± 2.11[a]
Ellagic acid	300.99	1.59 ± 0.18[b]	16.05 ± 1.84[a]
Quercitrin	447.09	4.86 ± 0.57[b]	22.25 ± 2.60[a]
Nepetin-7-glucoside	479.11	1.19 ± 0.14[b]	6.08 ± 0.69[a]
Quercetin	301.03	134.45 ± 15.35[b]	441.53 ± 50.42[a]
4-Hydroxy benzoic acid	137.02	8.26 ± 0.92[b]	12.37 ± 1.38[a]
Salicylic acid	137.02	8.52 ± 0.97[b]	12.03 ± 1.37[a]
Naringenin	271.06	4350.94 ± 480.34[b]	7338.62 ± 810.18[a]
Luteolin	285.04	27.46 ± 3.41[b]	67.35 ± 8.36[a]
Nepetin	315.05	159.76 ± 17.96[b]	314.14 ± 35.31[a]
Genistein	269.04	341.63 ± 37.68[b]	478.30 ± 52.76[a]
Kaempferol	285.07	155.46 ± 18.50[b]	329.88 ± 39.26[a]
Hispidulin	301.07	263.13 ± 29.55[b]	389.12 ± 43.70[a]
3′-O-Methyl quercetin	315.05	183.08 ± 21.53[b]	383.92 ± 45.15[a]
Apigenin	269.04	1215.69 ± 140.29[b]	2306.94 ± 266.22[a]
Isosakuranetin	285.07	108.17 ± 12.42	<LOD[a]
Caffeic Asit Phenethyl Ester	283.09	511.57 ± 58.22[b]	888.08 ± 101.06[a]
Chrysin	253.05	300.73 ± 33.35[b]	481.72 ± 53.42[a]
Rhamnocitrin	301.07	132.47 ± 14.86[b]	225.07 ± 25.25[a]
Acacetin	283.09	551.97 ± 62.70[b]	814.21 ± 92.49[a]
Hederagenin	473.36	456.22 ± 50.23[a]	157.40 ± 17.33[b]

Lower-case letters present the differences between the samples in the same line.
[a] LOD: limit of detection.

4. Discussion

The search for novel green solvents for the extraction of functional natural substances was one of the earliest fields of research in green chemistry (Bankova, Trusheva, & Popova, 2021). Green extraction techniques are simple and rapid methods to recover bioactive components from food sources (Kovacevic et al., 2018). The current study suggested that lactic acid might be a potential alternative solvent to ethanol. Notably, the bioactive compounds which are responsible for anticancer/antitumoral, antibacterial, antiviral, and anti-inflammatory effects, i.e., epigallocatechin gallate, caffeic acid, quercetin, naringenin, apigenin, kaempferol, chrysin, and CAPE were detected at significantly higher concentrations in propolis solution with lactic acid in comparison to the ethanolic propolis extract.

Previously, Kubiliene et al. (2015) used a mixture of polyethylene glycol (PEG 400) and water as a solvent in their study. They examined the composition and biological activity of various alternative propolis extracts and obtained comparable results with ethanol regarding phenolic components. Moreover, in the study of Funari et al. (2019), aqueous L-lysine was considered a promising solvent for green propolis extraction since it had the potential to replace water or ethanol. Also, CAPE is one of the most effective components of propolis with antioxidant capacity. In the current study, propolis with lactic acid indicated a much higher value of CAPE than ethanolic propolis (Russo, Longo, & Vanella, 2002). Furthermore, the components such as rutin, quercetin, apigenin, kaempferol, and chrysin are among the main phenolic components of propolis that are directly related to antioxidant activity. These and most other ingredients on the list have been detected in the lactic acid solution at higher concentrations (Escriche & Juan-Borrás, 2018). In the study of Sun, Wu, Wang, & Zhang (2015), they reported that the phenolic compounds of propolis are dependent on solvent concentration. In contrast to our study, the quercetin levels of different propolis extracts, including ethanol at a 25, 50, 75, 95, and 100% ratio, varied between 0.10 and 0.22 mg/g. In addition, in the present study, CUPRAC assay results were found to be higher when compared with the results of antioxidant capacity determination with DPPH scavenging activity. Similarly, Ozdal, Sari-Kaplan, Mutlu-Altundag, Boyacioglu, and Capanoglu (2018) obtained the highest antioxidant activity for propolis samples with the CUPRAC assay. This might be due to the difference in the dissolving capacity of the solvents used in the methods (Apak et al., 2007). Although it was determined that the antioxidant activity in the ethanolic solution was higher for some concentrations, most of the

Table 5
Phenolic profiles of initial gastric and intestinal phases of propolis solutions after *in vitro* GI digestion.

Compounds	Propolis-Ethanolic Solution (10%) mg/L					Propolis-Lactic Acid Solution (10%) mg/L				
	Initial	Gastric Phase	Intestinal Phase	Intestinal IN	Intestinal OUT	Initial	Gastric Phase	Intestinal Phase	Intestinal IN	Intestinal OUT
Gallic acid	<LOD*	<LOD*	26.5 ± 0.1^{cd}	38.22 ± 1.3^{bc}	34.3±0^{cd}	<LOD*	35.84 ± 0.1^{cd}	120.03 ± 6.4^{a}	17.6 ± 0.4^{de}	56.71 ± 0.6^{b}
Protocatechuic acid	<LOD*	91.72 ± 2.4^{c}	<LOD*	<LOD*	<LOD*	<LOD*	123.6 ± 0.3^{b}	180.1 ± 4.4^{a}	<LOD*	<LOD*
4-hydroxybenzoic acid	<LOD*	7.67 ± 2.7^{b}	55.18 ± 0.8^{ab}	28.49±4^{ab}	28.38 ± 0.8^{ab}	<LOD*	64.61 ± 1.4^{a}	53.43 ± 1.1^{ab}	28.82 ± 3.5^{ab}	64.4 ± 11.6^{a}
Vanilic acid	<LOD*	5.98±0^{c}	<LOD*	<LOD*	7.65 ± 0.6^{c}	<LOD*	42.25 ± 0.2^{b}	54.66 ± 1.9^{a}	5.95 ± 1.3^{c}	39.64 ± 1.1^{b}
***trans*-cinnamic acid**	143.63 ± 1^{a}	72.93 ± 0.6^{bc}	87.69 ± 1.2^{bc}	47.43 ± 0.4^{cd}	45.44 ± 0.2^{cd}	141.69 ± 29.8^{a}	74.87 ± 0.2^{bc}	104.1 ± 6.1^{ab}	19.14 ± 1.6^{d}	52.1 ± 0.5^{cd}
Hesperedin	932.63 ± 10.4^{a}	<LOD*	<LOD*	<LOD*	<LOD*	843.48 ± 176.0^{a}	<LOD*	<LOD*	<LOD*	<LOD*
Pinocembrin	1926.08 ± 9.0^{a}	792.05 ± 6.7^{bc}	823.35 ± 0.4^{bc}	463.93 ± 4.3^{bc}	596.45 ± 1.4^{bc}	2162.59 ± 46.7^{a}	1123.8 ± 4.7^{b}	1093.8 ± 75.7^{b}	188.41 ± 15.5^{c}	590.58 ± 7.6^{bc}
Phlorizin	82.83 ± 5.5^{a}	<LOD*	<LOD*	<LOD*	<LOD*	79.75 ± 33.1^{a}	<LOD*	<LOD*	<LOD*	<LOD*
Naringenin	3337.41 ± 12.1^{a}	88.48 ± 1.8^{b}	113.93 ± 1.9^{b}	210.46 ± 9.7^{b}	286.82±3^{b}	2795.39 ± 63.4^{a}	174.46 ± 1.1^{b}	91.52 ± 7.6^{b}	76.56 ± 0.1^{b}	155.67 ± 1.1^{b}
Chrysin	4436.61 ± 31.9^{a}	73.93 ± 0.6^{b}	48.76 ± 1.7^{b}	141.78 ± 9.6^{b}	260.12 ± 6.4^{b}	3934.77 ± 96.6^{a}	187.26 ± 2.1^{b}	102.62 ± 9.9^{b}	27.01 ± 0.7^{b}	89.98 ± 3.3^{b}
Chlorogenic acid	593.61 ± 2.9^{b}	432.12 ± 0.1^{bc}	225.77±2^{cd}	36.07 ± 1.7^{d}	98.92 ± 1.5^{cd}	1032.82 ± 29.1^{a}	744.23 ± 1.6^{ab}	712.07 ± 34.3^{b}	23.32 ± 1.5^{d}	77.43 ± 0.8^{d}
Cryptochlorogenic acid	199.47 ± 3.7^{b}	129.84 ± 0.2^{bc}	114.64 ± 1.3^{bc}	40.33±1^{c}	57.86 ± 1.2^{c}	290.36 ± 65.5^{a}	187.3 ± 0.3^{b}	201.3 ± 12.6^{ab}	30.28 ± 3.3^{c}	102.09 ± 1.6^{bc}
Caffeic acid	497.88 ± 3.6^{ab}	312.45 ± 0.7^{cd}	140.4 ± 1.3^{de}	113.87 ± 1.3^{de}	72.49 ± 32.6^{e}	512.18 ± 14.3^{a}	316.44 ± 0.2^{bcd}	348.99 ± 20.3^{abc}	67.73 ± 6.1^{e}	187.45 ± 1.6^{cde}
Ferulic acid	329.36 ± 3.7^{a}	135.39 ± 2.8^{cde}	180.47 ± 0.9^{cd}	88.68 ± 1.7^{de}	98.92 ± 0.8^{de}	322.21 ± 66.1^{ab}	145.76 ± 0.8^{cde}	224.81 ± 12.7^{bc}	37.73 ± 3.2^{e}	106.52 ± 0.8^{de}
Cynarin	<LOD*	19.68 ± 7.9^{a}	9.62 ± 1.7^{a}	<LOD*	4.27±0^{a}	<LOD*	13.48 ± 0.5^{a}	<LOD*	<LOD*	<LOD*
***p*-coumaric acid**	62.33 ± 0.9^{a}	15.7 ± 0.4^{cd}	25.04 ± 0.1^{bc}	13.86 ± 0.4^{cd}	13.7 ± 0.2^{cd}	58.81 ± 11.9^{a}	16.93±0^{cd}	39.3 ± 2.7^{b}	3.73 ± 0.4^{d}	11.1±0^{cd}
Apigenin	16.6 ± 0.7^{ab}	<LOD*	<LOD*	<LOD*	<LOD*	26.98 ± 7.7^{a}	6.63 ± 1.5^{bc}	<LOD*	<LOD*	<LOD*
Rutin	<LOD*	29.02 ± 1.3^{b}	<LOD*	<LOD*	<LOD*	<LOD*	57.37 ± 2.5^{a}	<LOD*	<LOD*	<LOD*
Quercitrin	122.83 ± 31.9^{a}	<LOD*	<LOD*	<LOD*	<LOD*	106.18 ± 33.5^{a}	<LOD*	<LOD*	<LOD*	<LOD*
Luteolin	88.2 ± 7.1^{a}	<LOD*	<LOD*	<LOD*	<LOD*	71.63 ± 15.9^{a}	<LOD*	<LOD*	<LOD*	<LOD*
Quercetin	80.91 ± 3.6^{a}	<LOD*	<LOD*	3.49 ± 0.3^{c}	<LOD*	60.28 ± 12.4^{b}	2.22±0^{c}	<LOD*	<LOD*	<LOD*
Kaempherol	19.01 ± 16.5^{a}	<LOD*	<LOD*	<LOD*	<LOD*	<LOD*	5.79 ± 0.3^{b}	<LOD*	<LOD*	<LOD*

Data represent average values ± standard deviation of three independent samples. Different letters in the rows represent statistically significant differences ($p < 0.05$). LOD: limit of detection.

essential specific phenolic components were detected at higher levels in the lactic acid solution. For example, naringin and isosakuranetin were not detected in the lactic acid solution by LC-MS/MS analysis, and hederagenin was determined in a more significant amount in the ethanolic solution.

Although solvents consisting of lactic acid: sodium acetate and lactic acid: ammonium acetate show relatively low efficiency, their value as solvents should not be ignored (Bakirtzi, Triantafyllidou, & Makris, 2016). In a study comparing the total phenolic contents of commercially sold propolis extracts and freshly prepared ethanolic propolis extract, ethanolic extracts had the highest amount of phenolic compounds, followed by glycol, olive oil, and water extracts. On the other hand, the phenolic components obtained from the lactic acid-propolis extract in the current study were significantly higher than the total phenolic values determined in the commercial propolis extracts (Kolayli, Yakup, & Zehra, 2020). Even though the significant components of propolis were found at significantly higher levels in propolis-lactic acid solution, higher antioxidant activities and total phenolic contents were observed in some ethanolic-propolis samples with different concentrations. It is possible that the presence of resins in the samples may cause misinterpretation in the analysis. It has been observed that lactic acid-based propolis extract is water soluble compared to ethanolic extracts. It has no bitter taste, is not so gummy and sticky, and appears to have a light yellow color in water. This also shows that lactic acid selectively dissolves phenolic components and does not dissolve the resin part that gives propolis its adhesive property compared to ethanol. Resin, by definition, is a dark substance that can be extracted with apolar solvents; its components, long-chain fatty acids, terpenes, resin acids, and ligands, have a pungent odor and a bitter taste (Satil, Selvi, & Polat, 2011). It is thought that the part that causes allergic reactions in propolis is the terpene component of the resin (Shi, Nedorost, Scheman, & Scheman, 2016), indicating that solvents such as lactic acid may be more advantageous with their lower levels of terpenes and is less allergic.

Propolis has both hydrophilic and hydrophobic compounds, and ethanol can dissolve both groups, including the resinous part of propolis, which could react with Folin-Ciocalteu (F–C) reagents, although they might have no beneficial health effects (Bastola, Guragain, Bhadriraju, & Vadlani, 2017). However, lactic acid is a polar solvent and has many sites to make hydrogen bonding with phenolics which increases the solubility of phenolics. Moreover, it is known that the solubility of phenols increases with an increase in the acidic strength of the solution. Therefore, the lactic acid solution selectively dissolves the phenolic compounds of propolis and does not dissolve the non-polar resinous part. This is an essential advantage of lactic acid over ethanol as the selectivity of the lactic acid solution might ease the standardization of propolis, which is an essential aspect from the economic and commercial point of view.

Differences in the patterns and correlations of total phenolic contents and antioxidant activities may be attributable to the heterogeneity of the reaction conditions, such as wavelength and required reaction time, under which the measurements are conducted (Ozdal et al., 2019). Moreover, da Silva, de Souza, Matta, de Andrade, and Vidal (2006) found a low correlation between total phenolic content and DPPH antioxidant activity method, while a higher correlation was found between antioxidant activity and total flavonoid contents. This might indicate that lactic acid could dissolve more flavonoids at low propolis concentrations, while at high concentrations, lactic acid could dissolve more phenolic acids than ethanol (da Silva et al., 2006; Ozdal et al., 2019).

In the present study, we also aimed to compare the bioaccessibility of propolis solutions in ethanolic and lactic acid-based forms. It has already been indicated in the literature that the extraction method has a significant impact on the antioxidant capacity of propolis (Kubiliene et al., 2015). The effect of *in vitro* simulated gastrointestinal digestion on the various extracts using ethanol, glycerol, and water was investigated by Yen et al. (2017), and the results indicated that ethanol showed better

antioxidant activities. On the other hand, Turkut et al. (2019) also evaluated the bioaccessibility of propolis extracts prepared with different solvents and found that aqueous extracts had the highest phenolic compounds in the intestinal phase. According to our findings, although there were some exceptions, lactic acid showed significantly higher antioxidant activity in digested samples than ethanol. Due to the very low pH values of lactic acid-based solutions, preliminary experiments were carried out to mimic the pH levels of the stomach and intestinal phases. As a result of these preliminary trials, it was observed that lactic acid-based solutions showed higher antioxidant activity when diluted by 50% before digestion.

The results indicated that lactic acid: water provided significantly better results than ethanol: water for extracting polyphenols. It may be attributed to a) Hydrogen bonding ability of lactic acid: lactic acid is a better hydrogen bond donor than ethanol. Huamán-Castilla, Mariotti-Celis, Martínez-Cifuentes, and Pérez-Correa (2020) compared the hydrogen bond stabilization energies of different solvent mixtures with gallic acid. The overall and carboxylic acid H-bond stabilization energies of the glycerol-water system compared to others were proposed to be an essential factor for higher extraction. Losada, Tran, and Xu (2008) showed that lactic acid undergoes self-association and forms bonds with the solvents such as water and methanol. These studies demonstrate the role of lactic acid as an H-bond donor during polyphenol extraction. b) The pH values of lactic acid-based propolis extracts with concentrations of 10, 20, 30, and 40% were determined as 0.12, 0.19, 0.60, and 0.61, respectively. Lactic acid significantly lowers the pH of the solutions. Many flavonoids show pH-dependent solubility and stability, which may be one of the reasons why we achieved a better extraction in the acidic solution. As the concentration of lactic acid increases, there are chances of a decrease in the solubility or even precipitation from the solution upon dilution during testing.

In the European Union Regulation assessment report No.528/2012, lactic acid is a naturally occurring alpha-hydroxy acid found in humans, animals, and plants. Lactic acid production as an intermediate metabolite in a 70 kg person at rest is 117–230 g/day, but this value has been reported to be much higher during exercise. Additionally, the estimated total intake through food in the EU and US is estimated as 1.65–2.76 g/person/day (DocIII6.2.01), and lactic acid is also approved in the EU as a food additive without an ADI value (Quantum satis; Dir 0.95/2/EC) ((EU), 2016). Furthermore, the high boiling point of lactic acid needs to be considered when it is used as an extraction solvent. Natural deep eutectic solvents (NADES) have higher boiling points, like lactic acid, than conventional organic solvents, such as ethanol. However, because of the volatility of ethanol, extractions using ethanol cannot be performed at high temperatures. Therefore, when using NADES as a solvent, the limiting upper temperature must be used to prevent the degradation of bioactive compounds in the sample (Mulia, Muhammad, & Krisanti, 2017). Also, the resin adsorption technique can be used to remove eutectic solvents from phenolic extracts without a significant loss in the solvent removal process (Rodríguez-Juan, Rodríguez-Romero, Fernández-Bolaños, Florido, & Garcia-Borrego, 2021).

5. Conclusion

To the best of our knowledge, this is the first study to show that L-lactic acid can be suggested as a safe and efficient solvent alternative to ethanol which is currently known as the best solvent for propolis extraction. However, when choosing solvents to be used in studies in which the antioxidant potential or biological activities of propolis are investigated, in addition to the *in vitro* methods, in vivo conditions and studies should also be taken into account for the comprehensive assessment of the final product. Further studies are needed to determine the most suitable concentrations of lactic acid-based propolis preparations for clinical usage compared to different types of extracts.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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