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Enhanced *In Vitro* Bioaccessibility and Anticancer Activity of Brazilian Propolis Extracted with L-Lactic Acid

Caglar Doguer[1], Fatma Duygu Ceylan[2], Esra Capanoglu[2], Nabil Adrar[2], Esra Bölük[3], Ali Timucin Atayoglu[4], Suzan Uzun[3], and Ibrahim Palabiyik[3]

[1]Department of Nutrition and Dietetics and [3]Department of Food Engineering, Faculty of Agriculture, Tekirdag Namik Kemal University, Tekirdag 59030, Türkiye
[2]Food Engineering Department, Istanbul Technical University, Istanbul 34475, Türkiye
[4]Traditional & Complementary Medicine Department, Istanbul Medipol University, Istanbul 34815, Türkiye

ABSTRACT: This study aimed to investigate whether L-lactic acid (LA), as a better solvent than ethanol (EtOH), enhances the bioaccessibility of phenolic compounds in red and green Brazilian propolis and to evaluate their potential anticancer effects *in vitro*. *In vitro* gastrointestinal digestion was performed by sequentially subjecting propolis extracts to buccal, gastric, and intestinal digestion phases, and samples were collected at each stage for analysis. The total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent with gallic acid as a standard. The total antioxidant capacity was evaluated using the cupric ion reducing antioxidant capacity and 2,2-diphenyl-1-picrylhydrazyl assays. The phenolic compounds of propolis samples were determined by high-performance liquid chromatography with diode-array detection analysis. The anticancer effects of propolis samples were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results showed that the TPC and antioxidant activity were markedly higher in the gastric and intestinal digestive products of LA propolis extracts than in those of ethanolic extracts. Treatment with red propolis LA extracts (RP-LA) resulted in a dose-dependent reduction in the viability of lung and colon carcinoma cells. Specifically, treatment with 1,750 ppm of RP-LA decreased the survival rate of carcinoma cells by 85% to 90%, whereas treatment with ethanolic propolis extracts at the same concentration did not exhibit any cytotoxic effect on cell viability. These findings suggest that LA is a more effective solvent than EtOH for extracting bioactive compounds from propolis, enhancing its antioxidant and anticarcinogenic potential.

Keywords: antineoplastic activity, biological availability, extraction, lactic acid, propolis

INTRODUCTION

An increasing number of studies have investigated the potential health benefits of natural products; among them, propolis has emerged as a particularly promising candidate (Zullkiflee et al., 2022). Natural products contain a diverse array of chemical compounds, such as phenolic compounds, which have been the subject of extensive research because of their robust biological properties and health benefits, including antioxidant (Fernandez-Panchon et al., 2008), anticancer (Roleira et al., 2015), and anti-inflammatory activities (Taofiq et al., 2015). Propolis is a natural resinous substance comprising resin, essential oils, waxes, pollen, and phenolic compounds (including aromatic acids, flavonoids, and their esters). It is collected by honey bees from plants, particularly the flowers and buds (Amin et al., 2023). The composition of propolis obtained from different geographical regions (e.g., Asia, Europe, and North and South America) varies because of the distinct characteristics of the local vegetation in each area. Moreover, propolis exhibits anticancer effects by inhibiting the growth of cancer cells. Several studies have reported the influence of propolis on cancer cell lines, including human gastric carcinoma (Desamero et al., 2019), colon (Ishihara et al., 2009), skin (Chen et al., 2007), breast (Shaker et al., 2023), and lung cancer (Ghazy and Hanafy, 2024). Many bioactive compounds present in propolis, including phenolic acids, flavonoids, and caffeic acid phenethyl ester, exert inhibitory effects on tumor growth (Fu et al., 2022). These anticancer properties of propolis have led to increased interest in understanding its chemical composition. Therefore, elucidat-

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Correspondence to Caglar Doguer, E-mail: cdoguuer@nku.edu.tr

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ing the cytotoxic effects of polyphenolic compounds and their derivatives found in propolis on cancer cells is essential for understanding their potential clinical applications.

However, because of the low solubility of its bioactive compounds, the biological activities of propolis are limited (Kubiliene et al., 2015). A significant proportion of commercial propolis extracts are prepared in aqueous ethanolic solutions, which are rich in phenolic bioactive compounds (Yesiltas et al., 2014). Nevertheless, the use of ethanolic propolis extracts is restricted because of their adhesive texture, unsuitability for pregnant women and pediatric or alcohol-intolerant individuals, and religious concerns. In addition to ethanol (EtOH), other solvents commonly used to extract propolis include glycerol, oil, water, and propylene glycol (Kubiliene et al., 2015). However, the extraction efficiency of these solvents is generally low compared with that of EtOH. Recently, L-lactic acid (LA), an organic acid, has been reported to be the best alternative extraction medium for phenolic compounds in propolis (Atayoglu et al., 2023; Lazović et al., 2024). Atayoglu et al. (2023) reported that the total phenolic content (TPC) of propolis samples extracted using LA was higher than that of samples extracted using EtOH. They also found that the antioxidant activity in digested propolis samples extracted with LA as a solvent was remarkably higher than that of samples extracted with EtOH.

Although there is considerable evidence regarding the beneficial role of ethanolic propolis extracts in carcinogenesis, the anticarcinogenic properties of propolis extracted with LA remain largely unexplored. The bioaccessibility of phenolics in LA propolis extracts within the digestive tract and circulatory system and their relationship with their anticarcinogenic effect require further investigation. Therefore, the present study aimed to evaluate and compare the effects of LA and EtOH as solvents for the extraction of red and green propolis on the phenolic profiles, TPC, and antioxidant and anticancer activity before and after *in vitro* digestion.

In this study, red and green propolis extracts were prepared from LA or EtOH. The phenolic profiles, TPC, and antioxidant activity of the extracts were evaluated before and after *in vitro* digestion. In addition, the cytotoxic effects of propolis LA and ethanolic extracts on lung cancer (A549) and colon cancer (Caco-2) cells were assessed and compared.

MATERIALS AND METHODS

Materials

Brazilian red and green propolis samples with a purity of 20% were provided by Bioessens Limitada. All chemicals used in the analyses were obtained from Sigma and Merck.

Extraction of propolis samples

The Brazilian propolis-solvent samples were prepared in proportions of 10% (g/g) using 70% EtOH (Merck KGaA) or 80% LA (Merck KGaA). Then, the samples were homogenized using a homogenizer (IKA T 25 Digital Ultra-Turrax) for 30 min, and the propolis samples were kept in the dark at room temperature for 15 days. Next, the prepared solutions were filtered using Whatman No. 4 filter paper (Millipore) by gravity filtration. Finally, the extracts were stored at 4°C until analysis.

Simulated *in vitro* gastrointestinal (GI) digestion

In vitro GI digestion has been used to simulate the conditions of the human digestive system in order to study the behavior of various compounds (e.g., polyphenols) during digestion. To simulate the GI digestion of red and green propolis extracts, we used the model adapted from Minekus et al. (2014) with slight modifications (Fig. 1). The compositions of salivary, gastric, and intestinal fluids are shown in Table 1.

Propolis extracts were mixed with artificial saliva medium containing amylase solution, saliva liquid, calcium chloride ($CaCl_2$), and distilled water. Following incubation at 37°C in a shaking water bath (SV 1422, Memmert GmbH & Co.) for 2 min, the gastric phase was initiated immediately after the buccal phase without collecting

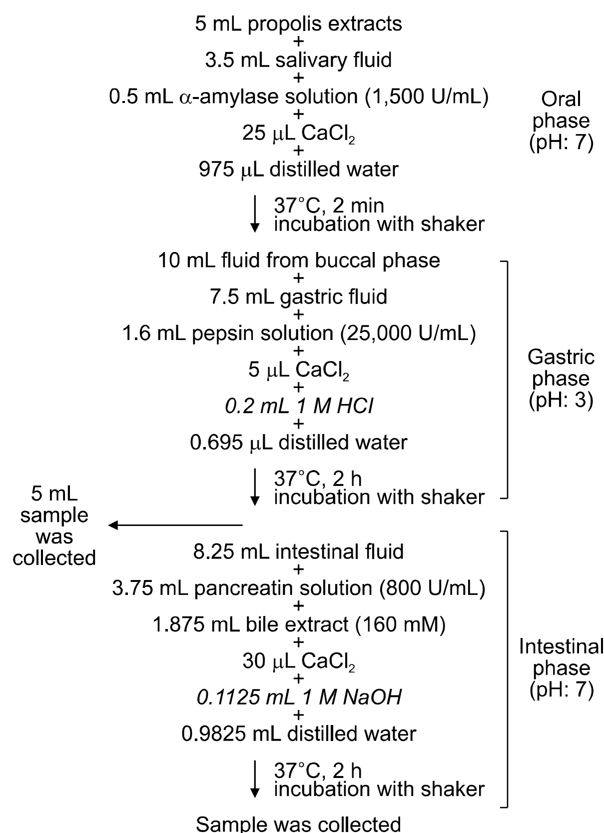

Fig. 1. Flow diagram of the simulated *in vitro* gastrointestinal digestion.

Table 1. Composition of the digestion fluids used to simulate the gastrointestinal system

Constituent	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
$NaHCO_3$	1.0	6.8	12.5	42.5
NaCl	2.0	0	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	0
HCl	6.0	0.09	1.3	0.7

All digestion fluids were filled with distilled water to a total volume of 400 mL.

aliquots.

To prepare the stomach medium for the gastric phase, the previously prepared stomach fluid was mixed with pepsin solution, $CaCl_2$, and distilled water. Then, the pH was adjusted to 3.0 using 1 M hydrochloric acid. The mixed samples were incubated in stomach medium in a shaking water bath (Memmert) at 37°C for 2 h. Following the completion of the gastric phase, 5 mL aliquots were collected for further analysis.

Afterward, the intestinal medium was prepared by combining intestinal fluid with pancreatin and bile solutions, $CaCl_2$, and distilled water. Then, the pH was adjusted to 7.0 by adding sodium hydroxide (NaOH) to the mixture. Following incubation in intestinal medium in a shaking water bath (Memmert) at 37°C for 2 h, samples were collected. After simulated intestinal digestion, 5 mL aliquots were collected for further analysis.

An aliquot of the sample from each digestion phase was centrifuged (Hettich) at 32,800 *g* and 4°C for 5 min to separate the supernatant. Subsequently, the supernatant was stored at 20°C until further analysis.

Determination of the TPC and antioxidant capacity of propolis samples

To determine the TPC, colorimetric assay was performed using the Folin-Ciocalteu reagent in accordance with the method of Turkmen et al. (2006) with slight modifications. Gallic acid was used as a standard, and the phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram. The total antioxidant capacity was assessed using cupric ion reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays as previously described (Kumaran and Joel karunakaran, 2006; Apak et al., 2007). The results were quantitatively expressed in terms of milligrams of Trolox equivalent (TE) per 100 g of sample using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as the standard.

High-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis of phenolic compounds

The phenolic profile of each sample was determined in accordance with the method previously described by Capanoglu et al. (2008). The Waters 2695 HPLC System with a photodiode array detector (Waters 2996, Milford) and a Supelcosil LC-18 column (25 cm×4.60 mm, 5 m column Sigma-Aldrich) were used to analyze the sample extracts. The mobile phase components comprised Milli-Q water with 0.1% (v/v) trifluoroacetic acid (TFA, solvent A) and acetonitrile with 0.1% (v/v) TFA (solvent B). The following linear gradient was used throughout the experiment: at 0 min, 95% solvent A and 5% solvent B were used; at 45 min, 65% solvent A and 35% solvent B were used; at 47 min, 25% solvent A and 75% solvent B were used; and at 54 min, the initial conditions returned. The flow rate was 1 mL/min. Three distinct wavelengths were used for detection: 280, 312, and 360 nm. Sample identification was achieved by using retention times and characteristic ultraviolet spectra. Then, the results were quantified using external standards, which are reference substances of known concentration analyzed under the same conditions to ensure accurate quantification.

Cell culture conditions

Human lung (A549) and colon (Caco-2) adenocarcinoma cells (#CCL-185 and #HTB-37, respectively; American Type Culture Collection) were cultivated in minimum essential medium with Earle's salt (#M4655, Sigma-Aldrich) supplemented with 15% (v/v) fetal bovine serum (#FBS-11A, Capricorn Scientific), 1% antibiotic mixture containing amphotericin B, streptomycin, and penicillin (#A5955, Sigma-Aldrich), 1% sodium pyruvate (#S8636, Sigma-Aldrich), and 1% nonessential amino acids (#M7145, Sigma-Aldrich). The cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2).

Determination of the anticancer effects of propolis samples

To determine whether propolis samples extracted by LA exert anti-viability effects in A549 and Caco-2 cells compared with ethanolic extracts, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously (Yıkmış et al., 2024). A549 and Caco-2 cells were plated into 96-well tissue

culture dishes at a density of 1×10^5 cells per well and allowed to attach for 24 h. Then, the cells were treated with various concentrations of either ethanolic or LA extracts of red or green propolis (750–1,750 ppm) for 24 h. Cells exposed to LA or EtOH (70%) alone, under the same conditions as the experimental group, served as the negative control. Before the treatments, samples containing LA were neutralized by adding 1 mM of NaOH at a rate of 4% of sample volume. Following incubation for 24 h, 5 mg/mL of MTT (#A3338, Biomatik) prepared by dissolving MTT powder in sterile phosphate buffered saline was added to each well, representing 10% of the culture volume. Subsequently, the cells were maintained in a cell culture incubator at 37°C for 3 h. After the MTT solution was carefully discarded from the wells, 100 μL of dimethyl sulfoxide was added to each well to solubilize the formazan crystals. Then, the optical density (absorbance) of the dissolved solution was measured at 570 nm using a microplate reader (UV-2600 Spectrophotometer, Shimadzu). The mean percentage of viable cells was calculated using the following formula: % cell viability$=[(OD_{treatment}-OD_{blank})/(OD_{control}-OD_{blank})]\times100$.

A nonlinear regression model was used to fit the relative cell viability values in order to estimate the half maximal inhibitory concentration (IC_{50}) using the GraphPad Prism software (v6.0, GraphPad Software).

Statistical analysis

All experiments were conducted in triplicate, and the results are presented as the mean±standard deviation. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($P\leq0.05$). For the MTT assay, two-way ANOVA was performed to evaluate the interaction between the treatment dose and cell viability. Since there was a significant interaction between variables, Tukey's post hoc test was performed to determine the significance of differences between pairs of group means. Statistical analyses were performed using GraphPad Prism software (v6.0, GraphPad Software).

RESULTS

Simulated *in vitro* GI digestion

***LA extraction increases the TPC and total antioxidant capacity of propolis samples*:** The results showed that LA is a more effective solvent for propolis extraction than EtOH. The TPC obtained from the red and green propolis extracts following the *in vitro* digestion process are presented in Table 2.

The TPC of red propolis-ethanolic extract (RP-EtOH) (45.6±2.9 mg GAE/g) was significantly higher ($P<0.05$) than that of RP-LA (30.5±4.1 mg GAE/g) (Table 2). Compared with red propolis, the TPC did not exhibit a significant variation between undigested propolis samples extracted with LA (70.4±3.4 mg GAE/g) or EtOH (75.4±7.9 mg GAE/g). The TPC was markedly increased in red and green propolis samples extracted with LA following the completion of the gastric and intestinal digestion phases. However, the TPC was significantly lower in the RP-EtOH samples obtained during the gastric (27.5±0.9 mg GAE/g) and intestinal (31.4±1.3 mg GAE/g) digestion phases than in the undigested control group (45.6±2.9 mg GAE/g). The TPC in green propolis EtOH extract (GP-EtOH) demonstrated a notable decrease (52.2±5.4 mg GAE/g) or increase (111.7±0.1 mg GAE/g) following gastric and intestinal digestion, respectively, compared with that in the undigested control group (75.4±7.9 mg GAE/g). Finally, the TPC of green propolis was consistently higher than that of red propolis in all cases, regardless of the solvent used for extraction and the phases of digestion ($P<0.05$).

In addition, *in vitro* GI digestion was evaluated in terms of the total antioxidant capacity using DPPH and CUPRAC assays. The total antioxidant capacities of the initial extracts and those during the gastric and intestinal phases are shown in Table 3.

For the CUPRAC assay, the total antioxidant capacities of propolis extracts throughout the digestive tract varied between 16.0±2.4 mg TE/g and 42.4±7.8 mg TE/g for RP-LA, 25.8±2.3 mg TE/g and 40.2±3.6 mg TE/g for RP-EtOH, 77.0±7.2 mg TE/g and 246.3±31.1

Table 2. The total phenolic content (TPC) of the gastric and intestinal phases of red and green propolis extracts after *in vitro* gastrointestinal digestion

Propolis type	Extraction solvent	TPC (mg GAE/g propolis)		
		Initial	Gastric	Intestinal
Red propolis	L-lactic acid	30.5±4.1^{Cb}	79.4±3.1^{Ba}	84.3±22.6^{Ca}
	Ethanol	45.6±2.9^{Ba}	27.5±0.9^{Db}	31.4±1.3^{Db}
Green propolis	L-lactic acid	70.4±3.4^{Ab}	146.3±7.4^{Aa}	173.3±25.0^{Aa}
	Ethanol	75.4±7.9^{Ab}	52.2±5.4^{Cc}	111.7±0.1^{Ba}

Values are presented as mean±SD of three independent samples.
Different uppercase letters in the columns represent statistically significant differences for each sample ($P<0.05$). Different lowercase letters in the rows represent statistically significant differences for each sample ($P<0.05$).
GAE, gallic acid equivalents.

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

Propolis type	Extraction solvent	CUPRAC (mg TE/g propolis)			DPPH (mg TE/g propolis)		
		Initial	Gastric	Intestinal	Initial	Gastric	Intestinal
Red propolis	L-lactic acid	16.0±2.4[Cb]	40.4±0.5[Ca]	42.4±7.8[Ca]	10.9±2.0[Bb]	13.8±0.3[Bb]	40.1±2.5[Ba]
	Ethanol	40.2±3.6[Ba]	25.8±2.3[Db]	28.3±0.4[Cb]	19.6±0.5[Aa]	2.7±0.1[Db]	1.4±0.5[Dc]
Green propolis	L-lactic acid	77.0±7.2[Ab]	214.6±4.3[Aa]	246.3±31.1[Aa]	9.2±0.1[Bc]	26.9±1.9[Ab]	58.4±2.0[Aa]
	Ethanol	82.8±15.1[Ab]	70.4±1.0[Bb]	124.2±14.0[Ba]	19.5±0.1[Aa]	7.6±0.1[Cc]	12.7±2.9[Cb]

Values are presented as mean±SD of three independent samples.
Different uppercase letters in the columns represent statistically significant differences for each sample (P<0.05). Different lowercase letters in the lines represent statistically significant differences for each sample (P<0.05).
CUPRAC, cupric ion reducing antioxidant capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TE, Trolox equivalent.

mg TE/g for GP-LA, and 70.4±1.0 mg TE/g and 124.2±14.0 mg TE/g for GP-EtOH. The antioxidant activity of green propolis was higher than that of red propolis, which is consistent with the results of TPC analysis.

During the initial phases of the test, the ethanolic extracts had higher antioxidant activity than the LA extracts, which is similar to the results obtained by TPC. However, in the intestinal phase, the LA extracts exhibited greater antioxidant activity than the ethanolic extracts. The antioxidant activity of LA extracts showed an increasing trend from the initial phase to the intestinal phase, whereas that of ethanolic extracts showed a decrease in the stomach phase and an increase in the intestinal phase.

The results of DPPH assay showed a variation in the total antioxidant capacity during digestion: between 10.9±2.0 mg TE/g and 40.1±2.5 mg TE/g for RP-LA, 1.4±0.5 mg TE/g and 19.5±0.5 mg TE/g for RP-EtOH, 9.2±0.1 mg TE/g and 58.4±2.0 mg TE/g for GP-LA, and 7.6±0.1 mg TE/g and 19.5±0.1 mg TE/g for GP-EtOH. Compared with CUPRAC assay, no significant difference was observed in the antioxidant activity between red and green propolis in the DPPH assay. In CUPRAC assay, the initial antioxidant activity of RP-EtOH (40.2±3.6 mg TE/g) was significantly higher (P<0.05) than that of red propolis extracted with LA. In the case of green propolis, ethanolic extracts exhibited a higher initial antioxidant capacity than LA extracts based on DPPH analysis. However, no significant differences were observed by CUPRAC assay (P>0.05). The results of CUPRAC assay indicated that green propolis extracts (LA or EtOH) had a notably higher antioxidant capacity than red propolis extracts. As opposed to CUPRAC assay, no significant difference was found between the antioxidant activities of red and green propolis in DPPH assay. The total antioxidant capacity of red and GP-LA significantly increased after the completion of the gastric and intestinal digestion phases. In contrast to LA extracts, the RP-EtOH samples showed a notably lower total antioxidant capacity during the gastric (25.8±2.3 mg TE/g) and intestinal (28.3±0.4 mg TE/g) digestion phases compared with the undigested control group. However, the results of CUPRAC assay showed that the total antioxidant capacity of GP-EtOH decreased during gastric digestion (70.4±1.0 mg TE/g) and then significantly increased during intestinal digestion.

***Phenolic profiles of propolis samples exposed to in vitro gastrointestinal digestion*:** On the basis of the initial and digested samples, HPLC-DAD analysis was performed to determine the major phenolic compounds of green and red propolis samples. The phenolic profiles of red and green propolis samples are shown in Table 4 and 5, respectively. Up to 22 individual phenolic compounds were identified in the samples. Pinocembrin, galangin, and kaempferol were the major phenolic compounds found in red propolis extracts, whereas pinocembrin, caffeic acid, ferulic acid, *p*-coumaric acid, and rosmarinic acid were the major phenolic compounds found in green propolis extracts. Protocatechuic acid and vanillin were only detected in the ethanolic extracts of red propolis samples.

In the red propolis samples, the accessibility of LA extracts in the intestinal phase was higher than that of ethanolic extracts, with some exceptions (Table 4). In fact, kaempferol was more accessible in ethanolic extraction than in LA extraction. The intestinal phase of hesperidin and naringenin was only accessible through ethanolic extraction, whereas that of galangin was only accessible through LA extraction.

Except for 2,3,4-trihydroxybenzoic acid, quercetin, and kaempferol, other components were more accessible in the intestinal phase in green propolis samples extracted with EtOH than in red propolis samples (Table 5). Moreover, hydroxycinnamic acids, including chlorogenic acid, cryptochlorogenic acid, caffeic acid, and cynarin, were only detected in green propolis samples.

Anticancer effects of LA and ethanolic propolis extracts

Treatment with RP-LA for 24 h remarkably reduced the viability of A549 and Caco-2 cells in a dose-dependent manner (Fig. 2). When these cells were subjected to the lowest (750 ppm) and highest (1,750 ppm) RP-LA concentrations, approximately 28% to 85% and 40% to 90% reductions in the cell survival rate (P<0.0001) were ob-

Table 4. Phenolic profiles of the initial, gastric, and intestinal phases of red propolis extracts after *in vitro* gastrointestinal digestion

Compound	Phenolic profiles (mg/100 g)					
	Red propolis-lactic acid solution (10%)			Red propolis-ethanolic solution (10%)		
	Initial	Gastric phase	Intestinal phase	Initial	Gastric phase	Intestinal phase
Gallic acid	23.4±0.0[c]	32.9±0.1[b]	135.4±4.1[a]	14.8±0.0[d]	19.5±0.0[cd]	37.6±0.0[b]
Protocatechuic acid	ND	ND	ND	11.8±0.0[a]	3.0±0.0[c]	5.5±0.0[b]
2,3,4-Trihydroxybenzoic acid	ND	ND	ND	ND	ND	ND
o-Coumaric acid	10.5±0.0[ab]	8.8±0.1[b]	13.7±2.4[a]	10.1±0.0[ab]	6.7±0.1[b]	6.9±0.0[b]
trans-Cinnamic acid	12.5±0.0[c]	6.4±0.3[d]	33.5±1.1[a]	23.5±0.0[b]	29.8±3.1[a]	9.3±0.0[cd]
Hesperedin	45.7±0.0[a]	38.8±0.0[b]	ND	24.8±0.0[c]	24.5±0.9[c]	16.1±0.0[d]
Vanilin	ND	ND	ND	287.5±0.0[a]	116.8±1.6[c]	149.7±0.0[b]
Pinocembrin	120.9±0.0[b]	188.4±5.8[a]	60.8±27.9[c]	130.4±0.0[b]	33.9±2.9[c]	35.6±0.0[c]
Naringenin	195.9±0.0[a]	88.3±0.0[c]	ND	130.5±0.0[b]	27.6±0.7[d]	26.9±0.0[d]
Taxifolin	112.3±0.0[a]	54.6±2.5[c]	11.3±2.9[cd]	85.6±0.0[b]	16.3±1.0[c]	6.9±0.0[d]
Galangin	564.1±0.0[b]	667.6±22.2[a]	166.4±10.2[d]	459.2±0.0[c]	ND	ND
Chlorogenic acid	ND	ND	ND	ND	ND	ND
Cryptochlorogenic acid	ND	ND	ND	ND	ND	ND
Caffeic acid	ND	ND	ND	ND	ND	ND
Cynarin	ND	ND	ND	ND	ND	ND
Ferulic acid	2.3±0.0[a]	ND	ND	2.7±0.0[a]	ND	ND
Sinapic acid	1.3±0.0[a]	ND	ND	1.1±0.0[a]	ND	ND
p-Coumaric acid	18.6±0.0[bc]	29.3±2.2[a]	21.5±3.0[b]	13.7±0.0[c]	20.5±1.8[b]	20.0±0.0[bc]
Rosmarinic acid	9.0±0.0[a]	6.7±0.0[b]	6.1±1.1[bc]	4.7±0.0[cd]	3.2±0.4[d]	ND
Apigenin	ND	ND	ND	ND	ND	ND
Quercetin	ND	ND	ND	ND	ND	ND
Kaempferol	841.7±0.0[a]	342.1±15.4[c]	48.7±0.4[e]	707.9±0.0[b]	104.5±12.5[d]	64.3±0.0[e]

Values are presented as mean±SD of three independent samples.
Different letters in the rows represent statistically significant differences (P<0.05).
ND, not determined.

served, respectively, compared with those in control cells. The IC_{50} value of RP-LA was 1,143 and 736.8 ppm for A549 and Caco-2 cells, respectively. In line with our expectations, RP-EtOH exposure did not exhibit a cytotoxic effect in A549 and Caco-2 cells compared with RP-LA exposure. This is consistent with the finding that LA as a solvent is more effective than EtOH for the extraction or separation of some bioactive compounds of red propolis (Table 4), which would be responsible for the cytotoxic effects. Furthermore, the relative percentage of cell survival in A549 cells treated with RP-EtOH at the two highest concentrations (1,500 and 1,750 ppm) was significantly higher than that in cells exposed to LA or EtOH alone. The fact that cell viability did not change significantly in A549 and Caco-2 cells exposed to LA or EtOH alone at the same experimental concentrations (750 – 1,750 ppm) suggested that cell death was triggered by propolis exposure.

As shown in Table 4, galangin was only accessible through LA extraction after *in vitro* digestion. As a major difference between LA and ethanolic extraction among both types of propolis, galangin could play a key role in the remarkable cytotoxicity effect exhibited by LA extracts. Indeed, galangin, a flavonol found in bee products (e.g., propolis and honey) and some plants (e.g., *Zingiber officinale* Roscoe, *Helichrysum aureonitens*, *Alpinia officinarum*, *Alnus pendula*, *Plantago major*, and *Scutellaria galericulata*), is known for its anticancer effects against certain types of cancer, including human lung and colorectal cancers (Patel et al., 2012; Singh et al., 2022).

The cytotoxicity of GP-LA and GP-EtOH against A549 and Caco-2 cells is shown in Fig. 3. Upon exposure to GP-LA and GP-EtOH, the viability of A549 and Caco-2 cells decreased in a dose-dependent manner. However, treatment with LA and EtOH alone did not significantly affect the viability of A549 and Caco-2 cells across different concentrations. Notably, the most significant reduction in the cell survival rate (approximately 62%, P< 0.0001) was observed in Caco-2 cells treated with GP-EtOH compared with that in untreated control cells. These findings suggest that green propolis extracts were more effective in reducing the viability of Caco-2 cells compared with A549 cells.

DISCUSSION

The findings of the present study indicate that LA is a more effective solvent for propolis extraction than EtOH because it retains or even enhances phenolic compounds during digestion. In particular, the green propolis samples extracted with LA exhibited the most notable in-

Table 5. Phenolic profiles of the initial, gastric, and intestinal phases of green propolis extracts after *in vitro* gastrointestinal digestion

Compound	Phenolic profiles (mg/100 g)					
	Green propolis-lactic acid solution (10%)			Green propolis-ethanolic solution (10%)		
	Initial	Gastric phase	Intestinal phase	Initial	Gastric phase	Intestinal phase
Gallic acid	26.1±0.0[b]	36.7±0.0[b]	167.3±30.5[a]	12.1±0.0[b]	4.4±0.2[c]	48.1±36.3[b]
Protocatechuic acid	ND	ND	ND	9.8±0.0[a]	4.4±0.1[a]	36.8±23.4[a]
2,3,4-Trihydroxybenzoic acid	70.7±0[a]	68.0±0.0[b]	3.6±0.2[e]	7.4±0.0[c]	6.0±0.0[d]	ND
o-Coumaric acid	ND	ND	ND	ND	ND	ND
trans-Cinnamic acid	ND	ND	ND	ND	ND	ND
Hesperedin	39.7±0.0[d]	23.1±0.0[e]	9.8±0.1[f]	62.1±0.0[b]	44.2±0.3[c]	209.0±0.5[a]
Vanilin	ND	ND	ND	ND	ND	ND
Pinocembrin	459.6±0.0[a]	167.9±0.0[d]	153.3±36.1[d]	379.6±0.0[b]	121.8±7.0[d]	240.3±14.4[c]
Naringenin	ND	ND	ND	ND	ND	ND
Taxifolin	ND	ND	ND	ND	ND	ND
Galangin	ND	ND	ND	ND	ND	ND
Chlorogenic acid	33.8±0.0[b]	18.2±0.0[c]	43.4±0.7[b]	21.8±0.0[c]	17.6±0.4[c]	150.8±6.8[a]
Cryptochlorogenic acid	6.2±0.0[b]	5.5±0.0[bc]	5.2±1.4[bc]	4.4±0.0[bc]	3.0±0.0[c]	13.2±0.9[a]
Caffeic acid	599.6±0.0[b]	588.2±0.0[c]	428.1±2.3[d]	305.6±0.0[e]	305.7±1.7[e]	819.3±6.4[a]
Cynarin	196.3±0.0[c]	164.3±0.0[c]	264.0±2.1[b]	116.3±0.0[d]	101.8±5.8[d]	1,006.5±28.8[a]
Ferulic acid	1,241.8±0.0[b]	1,175.5±0.0[b]	1,026.5±24.7[b]	1,041.8±0.0[b]	749.0±2.2[c]	2,869.9±147.5[a]
Sinapic acid	ND	294.0±0.0[a]	271.8±2.0[b]	ND	169.3±8.4[c]	ND
p-Coumaric acid	1,989.6±0.0[b]	1,759.3±0.0[c]	2,014.3±14.8[b]	1,589.6±0.0[d]	1,231.0±5.2[e]	6,118.7±62.1[a]
Rosmarinic acid	1,735.1±0.0[b]	1,588.2±0.0[b]	1,492.2±6.8[b]	1,535.1±0.0[b]	1,062.3±8.9[c]	4,679.5±205.6[a]
Apigenin	93.2±0.0[a]	13.8±0.0[c]	ND	73.4±0.0[b]	ND	ND
Quercetin	873.0±0.0[a]	ND	53.2±4.3[c]	519.0±0.0[b]	3.7±2.7[e]	19.4±3.6[d]
Kaempferol	905.4±0.0[a]	ND	37.7±10.2[c]	464.6±0.0[b]	0.9±1.3[e]	12.8±5.8[d]

Values are presented as mean±SD of three independent samples.
Different letters in the rows represent statistically significant differences (P<0.05).
ND, not determined.

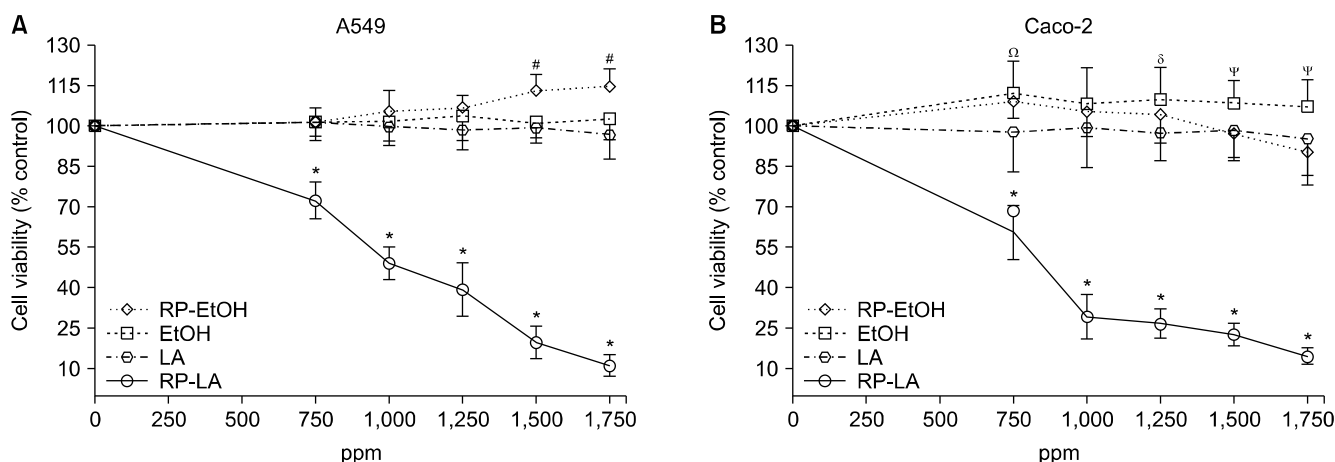

Fig. 2. *In vitro* cytotoxic effect of RP-LA on the survival of human lung (A549) and colon (Caco-2) cancer cells in a dose-dependent manner. Following treatment with increasing concentrations of RP-LA, RP-EtOH, LA, and EtOH (750–1,750 ppm) for 24 h, the relative viability of A549 (A) and Caco-2 (B) cells was assessed by MTT assay. The values are reported as mean±SD of four separate experiments, each with three replicates. Data were analyzed by two-way ANOVA. A two-way treatment-dose interaction was noted for the viability of A549 and Caco-2 cells (P<0.0001). Multiple pairwise comparisons were made using Tukey's post hoc test to determine differences among individual groups. Statistically significant differences between groups at the same treatment concentrations were depicted as follows: *P<0.0001: RP-LA vs RP-EtOH, LA, and EtOH; $^{\#}P$<0.0001: RP-EtOH vs EtOH; $^{\Omega}P$=0.0061: RP-EtOH vs LA; $^{\delta}P$=0.0058: LA vs EtOH; $^{\Psi}P$<0.05: EtOH vs LA and RP-EtOH. LA, lactic acid; EtOH, ethanol; RP-LA, red propolis LA extract; RP-EtOH, red propolis-ethanolic extract.

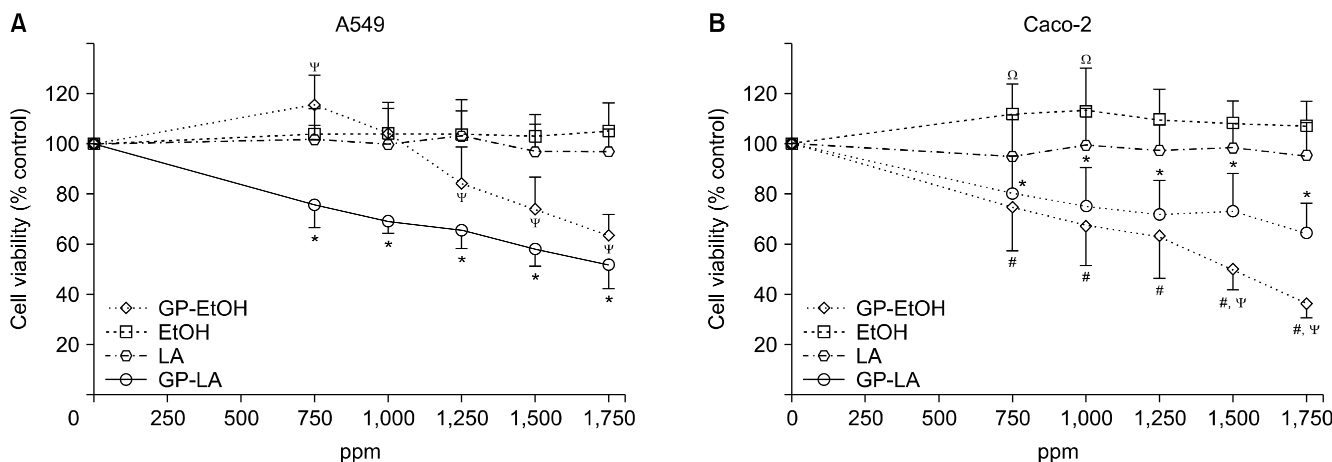

Fig. 3. *In vitro* cytotoxic effect of GP-LA on the survival of human lung (A549) and colon (Caco-2) cancer cells in a dose-dependent manner. Following treatment with increasing concentrations of GP-LA, GP-EtOH, LA, and EtOH (750–1,750 ppm) for 24 h, the relative viability of A549 (A) and Caco-2 (B) cells was assessed by MTT assay. The values are reported as mean±SD of four separate experiments, each with three replicates. Data were analyzed by two-way ANOVA. A two-way treatment-dose interaction was noted for the viability of A549 and Caco-2 cells ($P<0.0001$). Multiple pairwise comparisons were made using Tukey's post hoc test to determine differences among individual groups. Statistically significant differences between groups at the same treatment concentrations were depicted as follows: $^{*}P<0.0001$: GP-LA vs GP-EtOH, LA, and EtOH; $^{\#}P<0.0001$: GP-EtOH vs EtOH; $^{\Omega}P=0.0061$: GP-EtOH vs LA; $^{\delta}P=0.0058$: LA vs EtOH; $^{\Psi}P<0.05$: EtOH vs LA and GP-EtOH. LA, L-lactic acid; EtOH, ethanol; GP-LA, green propolis LA extract; GP-EtOH, green propolis EtOH extract.

crease in TPC when subjected to the GI digestion process, suggesting its potential superiority in terms of maintaining phenolic stability and bioavailability. Depending on the extracting medium and the type of propolis, the TPC varied between 30.5±4.1 mg GAE/g propolis and 75.4±7.9 mg GAE/g propolis in the initial extracts, 27.5±0.9 mg GAE/g propolis and 146.3±7.4 mg GAE/g propolis for the gastric phase, and between 31.4±1.3 mg GAE/g propolis and 173.3±25.0 mg GAE/g propolis for the intestinal phase.

Although some studies have found that the number of phenolic compounds in red propolis is higher than that in green propolis (Saito et al., 2021), other studies have reported no significant difference in the levels of phenolic compounds between green and red propolis (Andrade et al., 2017). Of note, the TPC can vary depending on the geographical location, plant sources, and bee species responsible for collecting the propolis. Moreover, the biological activity of propolis is not only determined by its TPC composition, but also by the composition of its phenolic compounds and other bioactive components. Compared with EtOH extraction, LA extraction was not superior during the initial phase of extraction for green and red propolis. By contrast, LA extraction outperformed EtOH extraction in the intestinal phase for green and red propolis. In terms of bioaccessibility, LA was more effective than EtOH according to the results. Polyphenols are highly bioaccessible in the intestinal tract because their aglycone forms, as well as their ester, glycoside, and polymer forms, which can be hydrolyzed by intestinal enzymes or colonic microflora, are easily absorbed, thereby facilitating their absorption (Manach et al., 2004). This highlights a value-added propolis when extracted by LA by improving its intestinal bioaccessibility.

In the case of green propolis, ethanolic extracts exhibited a higher initial antioxidant capacity than LA extracts based on DPPH analysis. However, no significant differences were observed by CUPRAC assay ($P>0.05$). The results of CUPRAC assay indicated that green propolis extracts (LA or EtOH) had a notably higher antioxidant capacity than red propolis extracts. As opposed to CUPRAC, no significant difference was found between the antioxidant activities of red and green propolis in the DPPH assay. The total antioxidant capacity of red and green propolis samples extracted with LA significantly increased after the completion of the gastric and intestinal digestion phases. In contrast to LA extracts, the RP-EtOH samples showed a notably lower total antioxidant capacity during the gastric (25.8±2.3 mg TE/g) and intestinal (28.3±0.4 mg TE/g) digestion phases compared with the undigested control group. However, the total antioxidant capacity of GP-EtOH decreased in the gastric digestion phase (70.4±1.0 mg TE/g) and then significantly increased in the intestinal digestion phase according to CUPRAC assay.

Several studies have shown that the choice of solvent plays a critical role in the dissolution and availability of phenolic compounds in propolis extracts. Machado et al. (2016) assessed the effects of supercritical extraction (supercritical carbon dioxide, SCO_2) and ethanolic extraction on different types of propolis, including green and red propolis. They focused on the role of solvents in the extraction of bioactive compounds and their subsequent bioaccessibility. Sun et al. (2015) found that most com-

monly used solvent for the extraction of phenolic compounds from propolis is EtOH, which is highly effective at solubilizing. Devequi-Nunes et al. (2018) demonstrated the importance of the extraction method in terms of the bioaccessibility of phenolic compounds extracted from green propolis using SCO_2 and EtOH as solvents. Furthermore, Woźniak et al. (2020) discovered that the solvent used in the extraction process affected the flavonoid and phenolic acid concentration in the propolis extracts, influencing their bioaccessibility and antioxidant properties. Based on these findings, selecting an appropriate solvent for the extraction of propolis is essential to maximize the bioaccessibility of phenolic compounds, thereby improving the antioxidant properties and health benefits of green and red propolis samples. In our previous study, LA was found to be a safe and more efficient solvent than EtOH, which is currently regarded as the most effective solvent for the extraction of propolis phenolics and their antioxidant activities (Atayoglu et al., 2023). In the present study, we also concluded that LA is an effective alternative to EtOH in the extraction of green and red propolis. However, further research is needed to validate these findings *in vivo* and to develop efficient delivery systems capable of protecting phenolic compounds during digestion.

According to our findings, some phenolic compounds appear to be more readily accessible in LA extracts, whereas others seem to be more easily accessible in ethanolic extracts. One of the most important factors determining the potential health benefits of red and green propolis extracts is their bioaccessibility in the digestive tract. Several factors can affect the extraction efficacy of phenolic compounds and their subsequent bioavailability in the GI tract, including extraction solvents. Machado et al. (2016) examined the chemical composition and biological activity of propolis extracts obtained from brown, green, and red propolis collected from multiple Brazilian regions using supercritical and ethanolic extraction methods. Moreover, they investigated various compounds, including phenolics, flavonoids, artepillin C, and *p*-coumaric acid, and there *in vitro* antioxidant activity. They found that the solvent used during extraction may affect the phenolic profile and antioxidant properties of propolis extracts. Moise and Bobiş (2020) identified over 30 distinct types of phenolic compounds in red Brazilian propolis extracts. Therefore, red propolis has a wide range of phenolic compounds, which can affect its ability to be absorbed by the body and its potential health benefits. In another study, Zannou et al. (2024) used a natural deep eutectic solvent to assess the bioaccessibility of bitter melon leaf extracts. They found that different compounds had different levels of bioaccessibility, emphasizing the importance of investigating the effect that different solvents have on the bioavailability of phenolic compounds. It appears that the choice of solvents, including EtOH and LA, for the extraction of phenolic compounds from red and green propolis affects their bioavailability in the GI tract. To maximize the health benefits of propolis extracts, it is essential to understand how different solvents affect the bioavailability of phenolic compounds.

In the present study, GP-EtOH showed greater cytotoxicity on Caco-2 cells ($P<0.0001$) than LA extracts at the highest concentrations. Of note, certain bioactive polyphenols (i.e., chlorogenic acid, caffeic acid, cynarin, ferulic acid, *p*-coumaric acid, and rosmarinic acid) were identified in significantly higher bioaccessible quantities in the ethanolic extracts than in the GP-LA (Table 5). This could be the reason for the difference in the cytotoxic activity between the two extracts, especially since these compounds were not detected (or were present in much lower concentrations) in red propolis extracts, which excludes any controversy between our hypotheses. In addition, during the experiments, a dense particle structure was formed in the GP-LA extracts because of the high viscosity of LA. Furthermore, the dense particle structure did not disappear even after filtration, suggesting that nanosized precipitates were formed. This is thought to be the reason for the low bioavailability and low cytotoxic effect of GP-LA extracts on cancer cells compared with ethanolic extracts. This dense particle structure was not observed in RP-LA extracts.

Although ethanolic propolis extracts have been widely recognized as having anticancer properties, the use of LA-based propolis extracts provides a new perspective on the anticancer potential of propolis at lower concentrations. The shift from EtOH- to LA-based extracts highlights the importance of exploring alternative extraction methods to enhance the effectiveness of propolis in cancer treatment. Propolis extracts derived from LA have shown significant anticancer activity even at lower doses, offering an exciting avenue for further research and development in cancer therapeutics.

In conclusion, the TPC was higher in LA extracts than in ethanolic extracts during the intestinal phase, suggesting that LA extraction demonstrated greater efficacy in terms of bioavailability. The antioxidant activity of LA extracts increased from the initial phase to the intestinal phase. In the intestinal phase, the antioxidant activity of LA extracts was higher than that of ethanolic extracts. The phenolic compounds in propolis extracts were identified, and the changes in their content during *in vitro* digestion indicated that the bioaccessibility of phenolic compounds depended on the extraction medium. The administration of RP-LA extracts to A549 and Caco-2 cells significantly decreased their viability in a dosage-dependent manner; however, the administration of RP-EtOH did not exhibit a cytotoxic effect on these cells. To the best of our knowledge, this preliminary study is the

first to demonstrate the cytotoxic potential of LA propolis extracts compared with RP-EtOH propolis extracts on human lung and colon cancer. LA is a promising extraction medium for the preparation of propolis extracts with an enhanced cytotoxic effect against cancer cells. However, future studies are needed to determine the mechanisms behind the enhanced cytotoxicity of LA propolis extracts and substantiate their therapeutic potential as cancer treatment. Future studies should also focus on optimizing the LA extraction process to maximize the bioaccessibility of propolis extracts and the effectiveness of their therapeutic effects.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: CD, FDC, IP. Analysis and interpretation: CD, FDC, EC, EB, ATA, SU. Data collection: CD, FDC, EC, EB. Writing the article: CD, FDC. Critical revision of the article: all authors. Final approval of the article: all authors. Statistical analysis: CD, IP, FDC, EC. Overall responsibility: CD.

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