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RESEARCH ARTICLE

Effect of propolis in gastric disorders: inhibition studies on the growth of *Helicobacter pylori* and production of its urease

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Abstract

There is considerable interest in alternative approaches to inhibit *Helicobacter pylori* (*H. pylori*) and thus treat many stomach diseases. Propolis is a pharmaceutical mixture containing many natural bioactive substances. The aim of this study was to use propolis samples to treat *H. pylori*. The anti-*H. pylori* and anti-urease activities of 15 different ethanolic propolis extracts (EPEs) were tested. The total phenolic contents and total flavonoid contents of the EPE were also measured. The agar-well diffusion assay was carried out on *H. pylori* strain J99 and the inhibition zones were measured and compared with standards. All propolis extracts showed high inhibition of *H. pylori* J99, with inhibition diameters ranging from 31.0 to 47.0 mm. *Helicobacter pylori* urease inhibitory activity was measured using the phenol-hypochlorite assay; all EPEs showed significant inhibition against the enzyme, with inhibition concentrations (IC_{50}; mg/mL) ranging from 0.260 to 1.525 mg/mL. The degree of inhibition was related to the phenolic content of the EPE. In conclusion, propolis extract was found to be a good inhibitor that can be used in *H. pylori* treatment to improve human health.

Keywords

Helicobacter pylori, inhibition, propolis, urease

History

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Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative, flagellated, spiral-shaped, urease-producing bacterium that grows in the digestive tract and has a tendency to attack the stomach lining[1,2]. For acid acclimation of *H. pylori*, two types of enzymes, urease and carbonic anhydrase (CA), play a central role. They cooperatively function to maintain neutral pH in the bacterial cytoplasm and periplasm[3]. *Helicobacter pylori* and its urease are strongly associated with chronic gastritis, peptic ulcers, gastric cancer, gastric adenocarcinoma, urinary catheter encrustation mucosa-associated lymphoid tissue, lymphoma and primary gastric non-Hodgkin's lymphoma. Urease is an enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia[4,5]. Actually, urease activity is essential for buffering the acidic pH value in the stomach, nutrient acquisition and improving the ability of *H. pylori* to colonize the gastric epithelium[6]. Its inhibition is very important for the treatment of *H. pylori*-related diseases. Antibiotic therapy or a combination of two or three drugs has been widely used for the management of these infections. However, the commercially available urease inhibitors, such as phosphorodiamidates, hydroxamic acid derivatives and imidazoles, are toxic and have poor stability, features that prevent their clinical use[7,8]. In addition to this, one of the reasons for the failure of *H. pylori* eradication in many countries is the increasing antibiotic resistance[2]. The prevalence of antibiotic-resistant *H. pylori* strains and the side effects associated with the present chemotherapeutic approach driven the search for alternatives to currently available anti-*H. pylori* drugs, especially regarding safe and effective non-antibiotic agents[9]. Thus, the search for novel *H. pylori* urease inhibitors in natural products such as plant extracts[10], honey[11,12] and propolis, with improved stability and low toxicity, is mandatory to improve the quality of life of human with *H. pylori* infection.

Honey, pollen and propolis are bee products that have been used in traditional and complementary medicine since ancient times[13]. Propolis is a resinous mixture used to protect hives from many diseases and threats. The composition of propolis depends mainly on the floral source; it contains nearly 40–50% resins, 30% wax, 5–10% essential oils and 3–10% phenolic substances, such as phenolic acids, flavonoids, tannins and so on[14,15]. Propolis contains a number of bioactive substances such as phenolic compounds and essential oils. Flavonoids are the major phenolics in propolis and are present as free or alkyl and phenyl esters. Caffeic acid phenethyl ester (CAPE) and chrysin are also present in all propolis samples at different concentrations, and are responsible for the many antioxidant, antimicrobial, antiinflammatory and anticancer properties of propolis[16]. It has been reported that Brazilian red and green propolis contain some specific flavonoids such as artepillin C, inocembrin, daidzein and biochanin[17,18].

For these reasons, propolis is a good pharmaceutical agent and propolis extracts have been used for different purposes such as

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throat sprays, cosmetics and toothpaste to boost the immune system[19,20]. The antioxidant and anticancer activities of propolis samples cited in the literature make propolis attractive for scientific enquiry and screening as a novel therapeutic natural agent[14,15,19,20]. Taking into account the circumstantial literature, we endeavored to undertake an *in vitro* assessment of ethanolic propolis extracts (EPEs) to determine their effect on *H. pylori* growth inhibition and urease inhibition. Our previous study was showed that EPEs exhibit a good inhibitory effect on *Jack Bean* urease[21]. This study aimed to investigate the inhibitory effect of EPEs both the growth of *H. pylori* and the production of its urease.

Materials and methods

Chemicals

Bovine serum albumin, Folin-Ciocalteu phenol reagent, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, urea, acetohydroxamic acid (AA), ethylenediaminetetraacetic acid (EDTA), disodium salt dihydrate, lithium chloride (LiCl), phenol, sodium nitroprusside, sodium hydroxide (NaOH), sodium hypochlorite (NaOCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$) and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of propolis extracts

Raw propolis samples were obtained from experienced beekeepers in 2015 from different areas of Turkey. Raw propolis samples were frozen at −20 °C than ground into powder. For extraction, 5 g of the powdered propolis was placed with 50 mL of 70% ethanol in a glass flask and stirred in an ultrasonic bath (Heidolph Promax 2020, Schwabach, Germany) at room temperature for 12 h. The suspension was filtered and centrifuged at 10 000*g* for 15 min, than the supernatant was evaporated. The residue was resolved in a minimal volume of 70% ethanol.

Total phenolic content

The total content of phenolic compounds in the ethanolic extracts of propolis samples was determined using the Folin-Ciocalteu method[22]. Briefly, 680 µL of distilled water, 20 µL of propolis extract, 400 µL of 0.2 N Folin reagent and 400 µL of Na_2CO_3 (7.5%) were added in a test tube. After 2 h of incubation at room temperature, the absorbance was measured at 740 nm. The result was expressed as mg of gallic acid equivalents/g sample.

Total flavonoid content

The total flavonoid concentration was measured using a spectrometric assay. Briefly, 0.5 mL of the sample, 0.1 mL of 10% $Al(NO_3)_3$ and 0.1 mL of 1 M $NH_4{\cdot}CH_3COO$ were added to a test tube and incubated at room temperature for 40 min. The absorbance was then measured against a blank at 415 nm. Quercetin was used for the standard calibration curve ($R^2 = 0.998$). The total flavonoid concentration was expressed as mg of quercetin equivalents/g sample[23].

Antimicrobial activity assessment

Laboratory strain of *H. pylori* (J99) was used as an indicator for the detection of the antimicrobial activity of propolis extracts. *Helicobacter pylori* J99 was purchased from the Health Public Institute of Refik Saydam (Ankara, Turkey). All propolis extracts were diluted in ethanol (70%) and tested at a final concentration of 75 µg/mL.

Agar-well diffusion method

Simple susceptibility screening using the agar-well diffusion method as adapted earlier was performed[11]. *Helicobacter pylori* J99 was stored at −80 °C in Brucella broth (BB; pH 7.0 ± 0.2, Merck) with 10% fetal calf serum supplemented with 20% glycerol. Brucella agar (BA) with 7% horse blood and supplement (DENT) was used for *H. pylori* culture. *Helicobacter pylori*-selective supplement (DENT) containing vancomycin (10 mg/L), trimethroprim lactate (5 mg/L), cefsulodin (5 mg/L) and amphotericin B (5 mg/L), purchased from Oxoid (Hampshire, UK)[11]. Then, the *H. pylori* suspension was diluted to approximately 10^6 colony forming unit (cfu)/mL. This was 'flood-inoculated' onto the surface of all specific agar plates and then dried. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 80 µL of the propolis extracts were delivered into the wells. *H. pylori* cultures were incubated under microaerophilic (5% O_2, 10% CO_2 and 85% N_2) conditions at 37 °C for up to 7 days. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Each test was performed in triplicate. Amoxicillin was used as the standard drug. Ethanol (70%) was used as the control solvent[11].

H. pylori growth medium

Helicobacter pylori J99 was cultured in duplicate on BA (Oxoid, Hampshire, USA) medium with 7% (v/v) horse blood plus DENT supplement 0.4% (v/v) and incubated in a microaerobic environment at 37 °C for 5–7 days (GasPak, Oxoid). The bacterial suspensions, containing all colonies grown on BA medium, were prepared in BB and adjusted to 2.0 McFarland standard (containing 1×10^7 to 1×10^8 cfu/mL), prepared from a 72-h-old subculture of a blood agar plate.

The liquid urea broth medium was used for urease production (Merck, Darmstadt, Germany), containing peptone, glucose, NaCl, KH_2PO_4 and phenol red indicator. The broth was sterilized by autoclaving. Then, the urea solution filtered (40%, 30 mL) was added to the urea base broth medium (6%, v/v), under sterile conditions. The medium was distributed to sterile screw cap bottles, inoculated 1 mL ($1 \times 10^{7\text{-}8}$ cfu/mL) *H. pylori* suspension and incubated at 37 °C at 5–7 days with gentle shaking. *Helicobacter pylori* is an organism that can be easily identified by this test because of its very high endogenous urease activity[24,25].

Preparation of *H. pylori* urease

Briefly, the broth cultures (200 mL) were subjected to centrifugation (5000*g*, +4 °C) and the recovered bacterial mass was washed twice using phosphate-buffered saline (0.1 M, pH 7.4, PBS) and then stored at −80 °C until use. Subsequently, the *H. pylori* pellet was thawed to ambient (room) temperature, then mixed with 15 mL of PBS and protease inhibitors and sonicated for 90 s in an ice bath (Branson Sonifier 250, with an output of 40). Following centrifugation (10 000*g*, 15 min, 4 °C), the supernatant (crude urease extract) was desalted and further concentrated using a centrifugal filter device Amicon ultrafiltration membrane (10 000 MWCO) at 4 °C. One unit of urease activity was described as the amount of enzyme that released 1 µM of ammonium/min at 25 °C[10]. A calibration curve was prepared with ammonium chloride solution (R^2: 0.997). The protein concentration of the enzyme solution (mg/mL) was determined using the Lowry method[26] with bovine serum albumin as the standard (R^2: 0.998). The urease enzyme solution of *H. pylori* was prepared at 2 U/mg protein, to use in inhibition studies.

Urease inhibition assay

The reaction mixture comprising 500 µL of buffer (100 mM urea, 0.01 M K_2HPO_4, 1 mM EDTA and 0.01 M LiCl, pH 8.2), 200 µL of urease enzyme solution and propolis extract (200 µL, only those extracts that exhibited activity against *H. pylori*) were subjected to incubation for 15 min (30 °C) in test tubes. Then, a phenol reagent (500 µL, 1% w/v phenol and 0.005% w/v sodium nitroprusside) and an alkali reagent (600 µL, 0.5% w/v sodium hydroxide and 0.1% v/v NaOCl) were added to each tube and the absorbance at 625 nm was measured after 50 min using a UV/VIS spectrophotometer (1601UV-Shimadzu, Australia). Urease activity was determined by measuring the amount of ammonia released during the reaction. The production of the ammonia was measured using the phenol-hypochlorite method[27]. The standard used was AA and the IC_{50} values (the concentration that inhibited the hydrolysis of 50% of the substrate) were determined from the dose–response curve.

Statistical analysis

The data were calculated in the form of the arithmetical mean and standard deviation. SPSS 13.00 for Windows was used for the statistical analysis of the data (SPSS Inc., Chicago, IL). The significance of the results was based on the Kruskal–Wallis test and Pearson correlations; differences were considered significant at $p < 0.05$.

Results and discussion

This study represents the first effort made to investigate the effect of EPE, a natural bee product employed as a treatment for gastric diseases, on *H. pylori* growth *in vitro* and the production of its urease, which are of vital importance to human health. A common inhibitor of this microorganism and its urease will be important for the treatment of gastric ulcers and related diseases. There results regarding the inhibition zones of the bacteria and the inhibition of the enzyme (IC_{50}) by EPEs were given in Table 1.

All EPEs inhibited the growth of *H. pylori* at different extract concentrations, with inhibition zones ranging from 31 to 47 mm (Table 1). The P11 extract (Eregli propolis) was distinguished from the others, showing the highest anti-*H. pylori* activity (having the highest inhibition zone) at a lower concentration compared to the other propolis samples (Table 1). A significant positive correlation between the total phenolic content (TPC) and anti-*H. pylori* activity of the EPE extracts (R^2: 0.861, $p < 0.01$, Table 2) was found.

Helicobacter pylori is an anaerobic microorganism that survives in an acidic environment with the help of the enzyme urease, a nickel-metallo enzyme, causing numerous gastric disorders. The main defense against this bacterium is to prevent it from adhering to the gastric mucosa by urease inhibition; urease inhibitors are particularly important in the treatment of gastric ulcers[11]. Propolis is a good pharmaceutical agent that has antibiotic, antitumor, antioxidant and anti-inflammatory properties. It has been reported that propolis inhibits the growth of many infectious bacteria as well as *H. pylori*[9]. In recent years, *in vivo* and *in vitro* investigations have indicated that polyphenol-rich extracts such as bee products have substantial inhibitory effects[12].

All of the EPEs inhibited *H. pylori* urease with a wide inhibitory range, that is from 0.260 to 1.525 mg/mL (Table 1). P11 (Eregli propolis) showed the strongest inhibitory effect (Table 1). Similarly, the P11 was distinguished from the other samples as it showed the greatest degree of urease inhibition. Similar to our results, a number of natural compounds, rich in polyphenolic agents, such as caffeic acid[28,29], rutin[30], as well as chestnut and oak honeys[13] and honey fractions[11] have been reported to inhibit *H. pylori* urease.

In this study, although the floral properties of the propolis samples were not taken into account, their phenolic contents were measured. The total phenolic and total flavonoids contents were measured in the 15 propolis samples, which also showed different amounts phenolic substances. The results of the phenolic contents and total flavonoids of the EPE are summarized in Table 1. The TPC of the EPE samples ranged from 23.503 to 236.136 mg GAE/g (Table 1). It was interesting that all propolis samples had significantly different TPC, and the highest phenolic contents were found in the P11 and P12, which were the Eregli and the Balikesir propolis samples, respectively. It has been reported that the TPC found in Chinese propolis ranged from 43 to 302 mg GAE/g[31]. The TPC was reported to be between 115 and 210 mg GAE/g in Turkish propolis of different origins[32]. A slightly narrower range of the TPC was reported in Polish propolis

Table 1. *Helicobacter pylori* growth inhibition, secreted urease, phenolic and flavonoid contents of EPEs.*

Samples	Location	Total phenolic contents mg GAE/g	Total flavonoid contents mg Q/g	Inhibition of *H. pylori* urease IC_{50} (mg/mL)	Inhibition zone (mm)
P1	Ardahan	81.396 ± 0.235^{e}	7.451 ± 0.021^{c}	0.880 ± 0.019^{b}	38.0^{e}
P2	Artvin	68.218 ± 0.338^{c}	5.602 ± 0.014^{b}	0.904 ± 0.024^{a}	39.0^{f}
P3	Kars	113.770 ± 0.371^{i}	8.103 ± 0.039^{c}	0.810 ± 0.051^{a}	39.0^{f}
P4	Van	117.558 ± 0.265^{j}	10.564 ± 0.024^{e}	0.780 ± 0.013^{a}	36.0^{d}
P5	Ardahan	103.350 ± 0.761^{g}	4.560 ± 0.019^{a}	1.295 ± 0.049^{a}	37.0^{d}
P6	Ankara	108.769 ± 0.452^{h}	9.605 ± 0.013^{d}	0.882 ± 0.027^{a}	40.0^{g}
P7	Ankara	23.503 ± 0.314^{a}	4.106 ± 0.009^{a}	1.100 ± 0.046^{a}	31.0^{a}
P8	Çankırı	92.127 ± 0.230^{f}	4.058 ± 0.017^{a}	1.220 ± 0.041^{a}	32.0^{a}
P9	Çorum	74.628 ± 0.393^{d}	3.882 ± 0.011^{a}	1.525 ± 0.078^{a}	32.0^{b}
P10	Sinop	66.323 ± 0.422^{b}	6.111 ± 0.021^{b}	1.182 ± 0.020^{a}	35.0^{c}
P11	Eregli	236.136 ± 1.083^{o}	12.453 ± 0.027^{f}	0.260 ± 0.023^{a}	47.0^{j}
P12	Balıkesir	223.087 ± 0.506^{n}	11.904 ± 0.031^{f}	0.400 ± 0.040^{a}	45.0^{i}
P13	Zonguldak	204.89 ± 0.478^{l}	9.086 ± 0.013^{d}	0.650 ± 0.037^{a}	43.0^{h}
P14	Erzurum	212.484 ± 0.761^{m}	12.453 ± 0.010^{f}	0.512 ± 0.019^{a}	41.0^{g}
P15	Trabzon	188.602 ± 0.286^{k}	7.909 ± 0.009^{cf}	0.681 ± 0.033^{a}	45.0^{i}
AA	–	–	–	24.610 ± 0.186	–
Amoxicillin	–	–	–	–	40.0
70% Ethanol	–	–	–	–	10.0

*Amc (10 µg): amoxicillin, EtOH. Seventy percent ethanol was used as control for antimicrobial studies. All propolis extracts were tested at 75.00 µg/mL final concentration in anti-*H. pylori* inhibition test. AA was used standard for urease inhibition and its IC_{50} value is given as µg/mL. Mean ± standard deviation; different letters in the same lines are significantly different at the 5% level ($p < 0.05$).

Table 2. Correlations between study parameters.

	Total phenolic contents	Total flavonoids	Inhibition of *H. pylori* urease	Inhibition zone (mm)
Total phenolic contents				
Pearson correlation	1	0.810*	−0.292	0.861*
Sig. (two-tailed)		0.000	0.052	0.000
N	45	45	45	45
Total flavonoids				
Pearson correlation	0.810*	1	-0.214	0.771*
Sig. (two-tailed)	0.000		0.158	0.000
N	45	45	45	45
Inhibition of H. pylori urease				
Pearson correlation	−0.292	−0.214	1	−0.210
Sig. (two-tailed)	0.052	0.158		0.166
N	45	45	45	45
Inhibition zone (mm)				
Pearson correlation	0.861*	0.771*	−0.210	1
Sig. (two-tailed)	0.000	0.000	0.166	
N	45	45	45	45

*Correlation is significant at the 0.01 level (two-tailed).

samples, that is between 150 and 190 mg GAE/g[32]. Therefore, the Eregli and Balikesir propolis were distinguished from the other propolis samples. It has been reported that the propolis from both of these areas could be derived from chestnut flora[12], and chestnut honey and pollen samples have high levels of phenolic substances[13]. The total flavonoid content of the propolis samples ranged from 3.882 to 12.453 mg Q/g sample (Table 1). A significant positive correlation was found between the total phenolic and total flavonoid contents in the samples (R^2: 0.810, $p < 0.01$, Table 2). Many studies have reported that propolis samples contain both high total phenolics and high total flavonoids[19,31–33].

Conclusion

The nuisance of *H. pylori* resistance to antibiotics is on the increase, constituting a global public health problem. Although the combination of a proton pump inhibitor with clarithromycin and amoxicillin is still very effective in the treatment of *H. pylori* infection and remains the treatment of choice, it would be prudent to preempt the resistance problem by searching for alternatives, as resistance to clarithromycin is increasingly reported[34]. Consequently, propolis extract exhibits good potential regarding the inhibition of *H. pylori* and its urease activity. Considering that some efficient urease inhibitors such as AA cannot be developed into therapeutic agents because of their toxicity and rapid metabolic degradation, propolis, being a natural product, has potential as a very safe resource to suppress and control *H. pylori*-associated gastro-duodenal diseases. In conclusion, we think that regular consumption of propolis extract, which is rich in phenolic compounds, can contribute to a reduction in several forms of *H. pylori*-associated disease.

Declaration of interest

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