Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2020, 12(1): 28-32
(http://www.derpharmachemica.com/archive.html)

Antibacterial Effect of Anatolian Ethanolic Propolis Extracts on Clinical Strains of *Helicobacter pylori*

Sevgi KOLAYLI [1], M. Ali Ayvaz Halil RAKICI[2], Nimet BALTAS [3], Muhammed IMRAN [4*], Inam IQBAL [4], Şengül KARAOGLU [3]

[1]*Faculty of Science, Karadeniz Technical University, Trabzon, Turkey.*
[2]*Faculty of Medicine,RTE University, Rize, Turkey.*
[3]*Faculty of Science, RTE University, Rize, Turkey.*
[4]*Lecturer, General Studies department, Jubail Industrial College, Saudi Arabia.*
[*]***Corresponding author:*** *Muhammad Imran, Lecturer, General Studies Department, Jubail Industrial College, 8244 Al Huwailat, Road No. 6, Al Jubai-35718, Saudi Arabia, Tel: 00966543255149, Email: Imran_m@jic.edu.sa*

ABSTRACT

In this screening study we are reporting antibacterial effect of ethanol extract of Anatolian propolis on Helicobacter pylori. For this five different strains of Helicobacter pylori (HP1, HP2, HP5, HP9 and HP13) were isolated by endoscopic indications and fifteen different ethanolic extracts were used to evaluate their antimicrobial activities. J99 strains of the bacteria was also obtained from Refik Saydam Institutes and the results were compared with them. Inhibition effects of the samples on Helicobacter pylori urease were also investigated. All propolis extracts showed antimicrobial effects against the H. pylori at different zone diameters, ranged from 12 to 47 mm. All samples also showed inhibition for J99 bacteria urease enzyme at different concentration, IC_{50} were changed from 0.26 to 1.53 mg/mL. In conclusion, ethanolic propolis extracts have highly effective potential against the bacteria, but types of propolis is a key factor in it.

Keywords: Propolis, Antimicrobial, *Helicobacter pylori*.

INTRODUCTION

Helicobacter pylori (HP)-infection is one of the most important causative agent for developing gastric and duodenal ulcers, furthermore, it is considered to be a risk factor for gastric cancer and mucosa-associated lymphoid tissue (MALT) [1,2]. This bacteria was discovered in 1983 by Marshall *et al.* who were awarded by the Noble Prize in 2005. Its discovery was infect a milestone in understanding the pathophysiology of gastric ulcers. It is a small curved, s- or spiral-shaped, microaerophilic, gram-negative bacterium of 2-4 µm length. *H. pylori* is a urease-producing bacterium growing in the digestive tract with the tendency to attack the stomach lining. Statistics revealed that It could be found in about two-third of the world's population. The prevalence of *H. pylori* infection remains high that is > 50% [3]. The bacteria is found in wide areas of the world, e.g. in Middle and South America, wide areas of Africa, Middle East, Russia, China and India. The lowest incidences were reported in Scandinavian countries, Australia and North America [1,2]. The transmission of *H. pylori* infection is considered to occur through an oro-orale or by fecal-oral route[1]. *H. pylori* primarily colonizes in the stomach, but evidence shows occasional or persistent colonization on the other sites, e.g. oro-nasal cavity, gall bladder, liver, peritoneum and large intestine [2]. If the infection is treated, it causes atrophic and metaplastic changes in the stomach and is one of the most important causes of chronic gastritis, gastric and duodenal ulcers, gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma [2].

Gastric urease activity is very important for the bacteria to have a suitable pH in the stomach. The enzyme hydrolyzes urea to carbon dioxide and ammonia. Inhibition of the extracellular enzyme secreted by the bacteria is of vital importance for *H. pylori* treatment. It was reported in many studies that honey and propolis extracts have a good potential to inhibit the urease [4,5]. There are invasive and non-invasive tests for detection of *Helicobacter pylori*. This treatment of *Helicobacter pylori* infection is the eradication therapy based on different antibiotic regimens.
Propolis, or bee glue, are important bee products, collected from hives, contains many bioactive compounds [6]. It was reported to eliminate bacteria, viruses and fungi, to possess local anesthetic effect, anti-ulcer and anti-inflammatory effects, to lower blood pressure and to stimulate the immune system. Propolis is a resinous material that bees use to seal small cracks and gaps in the hive which has a long history of medicinal uses, dating back to 350 B.C. with many other health benefits. In the last ten years, interest in propolis extract has immensely increased to use as complementary and alternative medicine. The compositions and biological active features of propolis samples are depend floral sources and farms where it was collected. In spite of all, it is well known that propolis are good pharmaceutical agents that has anti-inflammatory, [7-9] anti-oxidant, [10-12] antibiotic, [13] anti-parasitic [14] and anti-tumor properties [15].

Due to the problem of growing microbial resistance worldwide towards the antibiotics and its side effects [2] herbal therapy is the most important way to be use as of alternate medicines. Despite the reported studies including ours, does not completely eradicate *Helicobacter pylori* bacterium. But it's important to note that even after the treatment with traditional antibiotic eradication therapies, complete cure from *Helicobacter pylori* was unsuccessful and results are seen with the need of 2nd or even 3rd line of therapies (after anti-microbial assessment tests). Furthermore, many patients have allergies or intolerances against different antibiotics, especially penicillin.

For these reasons, in this study, we aimed to show beneficial effect of Anatolian propolis samples on *Helicobacter pylori* clinical strains. The study is depend only a screening strategy with ethanolic propolis extract on clinic strains of *Helicobacter pylori.*

MATERIAL AND METHODS

Before starting the work, we acquired ethical consent from RTEU Medical School (155/2018/104). After that the *Helicobacter pylori* clinical strains were isolated from biopsy specimens, which were obtained from patients undergoing gastroscopy because of different indications at the RTEU Medical School. *Helicobacter pylori* was detected by invasive tests and conventional diagnosis (urease test, culture, gram staining and microscopic examination) methods were used for *H. pylori* identification and isolation. Brucella agar (BA) with 7% human blood and supplement DENT (Oxoid, Hampshire, England) was used for *H. pylori* culture.

Clinical isolates of *H. pylori* were encoded as «HP». *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. Urease test (CLO) was done on biopsy specimen - rapid detection (sensitivity 79-100%, specificity 92-100%). A total of 5 different strains (HP1, HP2, HP5, HP9, HP13) were isolated and identified. *H. pylori* J99 standard strain was used as control that supplied from Refik Saydam Instituted of Ankara. Some properties of the biopsies samples were given in Table 1**.**

Table 1: Some properties of *H. pylori* samples obtained biopsies

Biopsy specimen	Direct staining Microscopy	Biopsy urease test	Cultural growth	Culture urease test	Oxidase activity	Catalase activity
HP1, HP2, HP5, HP9, HP13	Gr (-) Spiral bacteria	+	+	+	+	-
HP-J99	Gr (-) Spiral bacteria	+	+	+	+	-

Preparation of ethanolic propolis extracts (EPES)

Fifteen different raw propolis samples were obtained from experienced beekeepers of different areas in Anatolian part of Turkey by a propolis supplier company Bee'O (BEE&YOU)® Propolis (SBS Bilimsel Bio Çözümler (Istanbul, Turkey) (Table 2). The raw propolis samples were frozen at -20°C then grounded into the powder. For extraction, 5 g of the powdered propolis was placed with 50 mL of 70% ethanol and stirred in an ultrasonicator bath at room temperature for 12 h. The suspension was filtered and centrifuged at 10,000 g for 15 min, then the supernatant was evaporated. The residue was resolved in a minimal volume of 10mL in 70% ethanol.

Total phenolic contents

Total phenolic contents of the samples were also measured by the procedure of Folin-Ciocalteu assay [16]. In the assay, 680 µL distilled water, 20 µL propolis extract, 400 µL of 0.5 N Folin-Ciocalteu reagent and 400 µL Na_2CO_3 (10 %) were added in a test tube. After 2 hours of incubation at room temperature, the absorbance was measured at 760 nm. The results were expressed as mg of Gallic acid equivalents (GAE) per g propolis. All experiments were studied at triplicates (Table 2).

Table 2: The location of the raw propolis samples

Propolis Number	Obtained Area of Turkey	Total Phenolic Contents (mgGA/g)
P1	Ardahan	118±4,50
P2	Artvin	86.40±1.05
P3	Kars	130.50±3.10
P4	Van	142.10±4.08
P5	Ardahan	98.56±2.44
P6	Ankara	64.30±2.10
P7	Kayseri	76.40±1.16
P8	Çankırı	86.40±3.10
P9	Çorum	123.10±7.10
P10	Sinop	116.10±5.03
P11	Ereğli	169.80±4.30
P12	Balıkesir	186.10±2.23
P13	Zonguldak	155.10±4.44
P14	Erzurum	129.10±6.10
P15	Trabzon	114.04±3.10

Antimicrobial activity assessment

Agar well diffusion method was used for antimicrobial activity assessment of the propolis extracts. For antimicrobial activity tests, a suspension of *H. pylori* fresh culture (106 colony forming units, cfu/ml) in 3 mL BB was prepared. Antimicrobial activity was evaluated by measuring the zone of inhibition against *H. pylori*. Ethanol (70%) was used as the control solvent. The results were compared also with frequently used drug of

amoxicillin as positive controls. 70 µL extracts were used and all determinations were made in triplicate and the results were expressed in terms of zone diameters as mm; <6 mm, inactive, 7-10 mm very low activity, 11-15 mm low activity, 16-20 mm average activity, <21 high activity. 70% ethanol also used as solvent black (Figure 1).

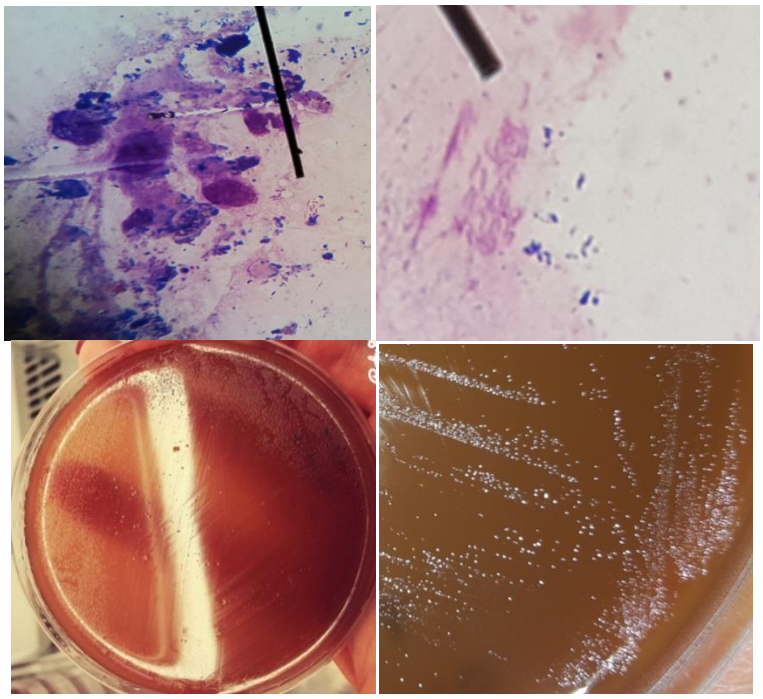

Figure 1: *H. pylori* microscopic appearance of Gram stain preparations in direct biopsy samples and macroscopic appearance in culture medium.

Anti-urease activity test

H. pylori urease was produced in urease liquid medium and prepared. One unit of urease activity was described as the amount of enzyme that released 1mM of ammonium/min at 25°C [17]. The urease enzyme solution of *H. pylori* was prepared at 2 U/mg protein, to be use in inhibition studies. 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 [18]. Thiourea was used as standard and the IC50 values (the concentration that inhibited the hydrolysis of 50% of the substrate) were measured from the dose-response curve.

Statistical analysis

The statistical analyses were performed on SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) software. The results are given as mean values and standard deviation. Differences between the groups were analyzed using the independent samples t-test.

RESULTS

The antibacterial activities of the fifteen different propolis extracts were tested against clinical strains of *Helicobacter pylori* is given in Table 3. Inhibition zones were ranging 12 and 47 mm on the all six strains of bacteria, *H. pylori.* All propolis samples were showed high antimicrobial activity against to *H. pylori* strains. But some samples were showed a excellent activity against some of them.

DISCUSSION

It is also observed that clinical specimens are different strains, and susceptibility to propolis were different from each other. It was observed that the most sensitive strains toward EPEs were HP1, HP5, J99 and HP13 respectively, while the most resistant strains were HP9 and HP2. Samples of the P1, P2 and P12 were the most effective propolis against to all *H. pylori* strains. The results showed that ethanolic propolis extracts could be used as an effective agent in the treatment of *Helicobacter pylori*, but the propolis compositions or contents is important. In this study, the content of propolis was not determined, since the purpose was screening and basic studies. But the results showed that the propolis composition may be important for the treatment. Our previous study as parallel this study was performed on *H. pylori* strain J99, ethanolic propolis extracts were observed to have inhibition diameters ranging from 31.0 to 47.0 mm [4,5] It has been reported that propolis inhibits the growth of many infectious bacteria as well as *H. pylori* [19]. 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 [20]. Similar results were obtained in this study too, furthermore clinical *H. pylori* strains were also found to be highly sensitive to propolis extracts. In conclusion, propolis extract was found to be a good inhibitor which can be used in *H. pylori* treatment to improve human health. It was earlier reported that inhibitory effect of 22 propolis extracts against to ten *H. pylori* strains isolated from the gastric mucosa and all of the extracts were active on the tested strains [18]. In addition to that, propolis is being used in the treatment of oral mucositis, e.g. seen in cancer patients, as well as in the treatment of ulcerative colitis by reducing bacterial translocation, which involves mucosal inflammatory response [21] Gastric ulcers are defined as damage involving the lamina muscularis mucosae mainly induced by *H. pylori* or non-steroidal anti-inflammatory drugs as well. Studies about the gastro protective effects of ethanolic extract of propolis against ethanol-induced gastric ulcers in rats revealed that propolis extract prevented the occurrence of gastric ulcerations [21]

As mentioned in the results part, the anti-proliferative and cytotoxic effects of Cuban propolis extract were investigated in a study that induce mitochondrial dysfunction and lactate dehydrogenases (LDH) release, what indicated cell necrosis associate with reactive oxygen species production and decreased cell migration [22]. Propolis contains many antioxidant compounds such as caffeic acid, rutin and caffeic acid phenyl ester, etc. [6]

In the present studies, we have also determined inhibition effects of the samples on urease activities that is the enzyme released from the bacterial strains as extracellular product. At the beginning, all strains showed urease active, then we studied inhibition effects of the propolis extracts. The results regarding the inhibition of the urease enzyme (IC50) by the samples were given in Table 3.

All of the EPE actively inhibited *H. pylori* J99 urease with a wide inhibitory range, i.e. from 0.26 to 1.53 mg/mL. Similar to our results, a number of natural compounds, rich in polyphenolic agents, such as caffeic acid, [23,24] as well as chestnut and oak honeys [25] and honey fractions [20] have been reported to inhibit *H. pylori* urease. 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 [20]. Similar results were obtained in our study and clinical *H. pylori* strains were also found to be highly sensitive to propolis extracts. The lowest IC50 value is belong P11 propolis samples, that the sample showed was the widest antimicrobial zone than the others. The highest IC50 value was found against P9 sample, that sample sowed lower inhibition effect than the others also. Different IC50 values of urease inhibitions were proved that compositions of propolis extracts were important factor, to use of the sample in treatment of *H. pylori*, as well as other apitherapeutic applications. Total phenolic contents of propolis extracts are also important factor to determine, and the values were depended on floral sources of the raw propolis [5,26]. Since mostly polyphenols are responsible for biological activities of plants, the higher the total phenolic contents cause the higher antimicrobial activities. For these reasons, in recent years, vivo and vitro investigations have focused to polyphenol-rich extracts such as bee products as well as propolis [27]

Table 3: Inhibition of ethanolic propolis extracts on *H. pylori* strains

	Inhibition Zone Diameter values (mm)					
	J99	HP1	HP2	HP5	HP9	HP13
P1	38	>30	>30	>30	>30	>30
P2	39	>30	>30	>30	>30	40
P3	39	>30	>30	>30	15	38
P4	36	>30	>30	>30	25	36
P5	37	>30	>30	>30	25	>30
P6	40	40	>30	>30	10	>30
P7	31	40	>30	>30	15	>30
P8	32	40	>30	>30	12	>30
P9	32	40	15	>30	10	>30
P10	35	40	12	>30	12	>30
P11	47	42	16	>30	15	>30
P12	45	36	30	>30	30	>30
P13	43	33	12	>30	12	>30
P14	41	38	ND	>30	12	>30
P15	45	45	ND	>30	ND	>30
Amoxicillin	>40	>40	>40	>40	>40	>40
70% Ethanol	10	8	10	9	9	8

CONCLUSION

In conclusion, both antibacterial studies as well as urease inhibitions activities indicated that ethanolic extracts of propolis could be used an effective agent for the treatment of gastric bacteria (*H. pylori*). Liquid/extract/tincture or tablet propolis can be used for gastric ulcer. However, additional microbiological and etc. studies are needed before a potential clinical utility of these natural products is guaranteed. In addition, further works are required to understand which molecules are responsible for anti-bacterial activity in propolis extracts.

DECLARATIONS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Thank you, propolis provider company of Bee'O(BEE&YOU)® Propolis from Istanbul, Turkey

REFERENCES

[1] M. D. Burkitt. *Helicobacter pylori*-induced gastric pathology: insights from in vivo and ex vivo models, Dis Model Mech. **2017,** 10(2), 89-104.
[2] T. L Testerman, J. Morris, Beyond the stomach: An updated view of *Helicobacter pylori* pathogenesis, diagnosis, and treatment, World J Gastroenterol **2014,** 20(36): 12781-12808, Published online **2014** Sep 28.
[3] JKY Hooi. Global Prevalence of *Helicobacter pylori* Infection: Systematic Review and Meta-Analysis, Gastroenterology **2017,** 153(2), 420-429.
[4] S. Kolayli. Evaluation of Anti-*Helicobacter pylori* Activity and Urease Inhibition by some Turkish Authrntic Honeys, J. of Food Science and Engineering **2017,** 67-73.
[5] N. Baltas. Effect of propolis in gastric disorders: inhibition studies on the growth of *Helicobacter pylori* and production of its urease, J Enzyme Inhib Med Chem., 31(sup2), 46-50, Epub **2016** May 27.
[6] V. Bankova, M. Popova, B. Trusheva., Plant sources of propolis: an update from a chemist's point of view, Natural Product Communications **2006,** 1023-1028.
[7] P. Michaluart. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation, Cancer Res. **1999,** 59(10), 2347-52.
[8] O. K. Mirzoeva, P. C. Calder., The effect of propolis and its components on eicosanoid production during the inflammatory response. Prostaglandins Leukot Essent Fatty Acids **1996,** 55(6), 441-9, PMID: 9014224.
[9] K. Frenkel. Inhibition of tumor promoter-mediated processes in mouse skin and bovine lens by caffeic acid phenethyl ester, Cancer Res. **1993**, 53(6), 1255-61.
[10] G. F Sud'ina. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties, FEBS Lett. **1993,** 329(1-2), 21-4.
[11] R. Bhimani. Inhibition of oxidative stress in HeLa cells by chemopreventive agents, Cancer Res. **1993,** 53(19), 4528-33.
[12] A. K. Jaiswal. Caffeic acid phenethyl ester stimulates human antioxidant response element-mediated expression of the NAD(P)H:quinone oxidoreductase (NQO1) gene, Cancer Res., **1997,** 57(3), 440-6.
[13] V. Campos. Antibacterial Activity of Propolis by Frieseomelitta varia. Ciência e Agrotecnologia **2011,** 35(6), 1043-1049
[14] A. Sena-Lopes. Chemical composition, immunostimulatory, cytotoxic and antiparasitic activities of the essential oil from Brazilian red propolis. PLoS One **2018,** 13(2): e0191797.
[15] H. Li. Caffeic acid phenethyl ester exhibiting distinctive binding interaction with human serum albumin implies the pharmacokinetic basis of propolis bioactive components, J. Pharm Biomed Anal., **2016,** 122, 21-8.
[16] V. L. Singleton, R. Orthofer, R. Lamuela-Raventós., Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, Methods in enzymology 299 C (1), 152-178.
[17] S. Brdanin. Antimicrobial Activity of Oregano (*Origanum vulgare L.*) and Basil (*Ocimum basilicum L.*) Extracts, Advanced Technologies **2015,** 5-10.
[18] M. W. Weatherburn., Phenol-hypochlorite reaction for determination of ammonia, Anal. Chem., **1967,** 39 (8), 971-4.
[19] S. G. Jadhav. Inhibition of growth of *Helicobacter pylori* and its urease by coumarin derivatives: molecular docking analysis, Journal of Pharmacy Research 7(8),705-711.
[20] F. Matongo, U. U. Nwodo., In vitro assessment of *Helicobacter pylori* ureases inhibition by honey fractions, Arch Med Res. **2014**, 45(7),540-6.
[21] L. M. Da Silva. Propolis and its potential to treat gastrointestinal disorders, Evidence-based Complementary and Alternative Medicine **2018**, (7),1-12.
[22] Y. Frion-Herrera. The cytotoxic effects of propolis on breast cancer cells involve Pİ3K/Akt and ERK 1/2 pathways, mitochondrial membrane ptoential, and reactive oxygen species generation, İnflammopharmacology **2018**, PMID: 29748880.
[23] A. Russo, R. Longo, A. Vanella., Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin, Fitoterapia, **2002**, 73 Suppl 1, S21-9.
[24] T. Masuda. Identification of a potent xanthine oxidase inhibitor from oxidation of caffeic acid, Free Radic Biol Med., **2014**, 69, 300-7.
[25] Z. Can. An investigation of Turkish honeys: their physico-chemical properties, antioxidant capacities and phenolic profiles, Food Chemistry **2015**, 180, 133-141.
[26] R. Aliyazıcıoglu. Properties of Phenolic Composition and Biological Activity of Propolis from Turkey, International Journal of Food Properties **2016,** 2, 277-287.
[27] C. E. Manyi-Loh, A. M. Clarke, R. N. Ndip., Detection of phytoconstituents in column fractions of n-hexane extract of Goldcrest honey exhibiting anti-*Helicobacter pylori* activity, Arch Med Res., **2012,** 43(3), 197-204.