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Physicochemical and antibacterial assessment of Iranian Propolis

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ABSTRACT

Background: Propolis is one of the useful bee colony products that have been used in traditional medicine for centuries. In this study, the physicochemical characters and their antibacterial effect of Iranian Propolis collected from Qazvin province was assessed.

Methods: In this study, Thin Layer Chromatography and Vacuum Liquid Chromatography to detect different compounds of the extract have been used. In the initial evaluation of Propolis extract, it was found that the extract includes variable compounds with different polarity. Therefore, the initial classification of extract with different polarity solvents was essential. Finally, 0.1 gr hydro alcoholic Propolis was injected to the HPLC by ultrasound. The antibacterial effect of Iranian ethanol extract Propolis was measured using a microdilution method against *Pseudomonas aeruginosa: P. aeruginosa* and *Staphylococcus aureus: S.aureus* standard strains and the minimum bactericidal and inhibitory concentration were defined.

Results: Primary analysis of the ethanol extract by analytical Thin Layer Chromatography, demonstrated the presence of flavonoid and phenol in it. minimum inhibitory concentration and minimum bactericidal concentration for *Staphylococcus aureus: S.aureus* standard strain was 2.5mg/ml. The same procedure was done for *Pseudomonas aeruginosa: P. aeruginosa* standard strain and the minimum inhibitory concentration and minimum bactericidal concentration were 50mg/ml of Propolis extracts.

Conclusion: According to the results, the alcoholic extract of propolis from Qazvin province of Iran provides significant antimicrobial activity. Its powerful activity may be due to high total phenolic and flavonoid contents.

Keywords: Iranian propolis, Antibacterial activity, Phenolic compounds, Flavonoid compound

BACKGROUND:

Propolis is one useful honeybee colony product that is produced by bees and is a dense yellow-brown resin-like material which is soluble in ethanol much more than water [1]. Ingredients of propolis, include different types of trees,plant resin, wax, pollen, and volatile oils that honeybee workerscollectin the basket. The chemical analysis proved that it is made by 300 different compounds [2]. Using biochemical analysis, it was found thata variety of compounds such as alcohols, aldehydes, flavonoids, amino acids, Kalkvn, acetone, fatty acids, and ethers, have been formed. Any of these compounds have a high value in the pharmaceutical industry. Propolis was first recognized by the ancient Egyptians, they named it Propolis, and they made some changes to it for use, as a matter of sealing, polishing, and disinfection inside the hives and wax cells inside the hive as well as embalming the dead carcasses [3].

Propolis has many uses in ancient culture, as local anesthetics, anti-inflammatory, (for the treatment of mouth inflammatory disease), strengthening the immune system,treatment of burns, acne, abrasions, swelling, skin rashes, sores, boils, warts, strains, throat, and head pains. In recent decades, many researchers in different countries have studied the antibacterial properties of propolis on pathogenic microorganisms and, in some cases, have proven that it was more effective than synthetic antibiotics [4,5,6,7]. In many parts of the world, it is recommended that Propolis be chewed for some hours to treat mouth or throat inflammation. For internal use, 10 drops of its alcoholic extract can be eaten with one spoon of honey. For curing face acne, skin blisters, itching and inflammation, warts, scratching, injuries, and this kind of pain, the Propolis is used as salve. Alcoholic extract of Propolis is effective against Streptococcus mutans, therefore, it can be used to remove teeth plaques [8]. The application of Propolis against vancomycin-resistant Enterococcus and methicillin-resistant Staphylococcus has demonstrated its high efficacy in treating the infections caused by the mentioned bacteria [8].

Currently, *Methicillin resistant Staphylococcus aureus: MRSA* is one of the serious problems in medical societies. *Pseudomonas aeruginosa: P. aeruginosa* is a gram-negative and opportunistic environmental bacillus that is capable of infecting humans and is one of the most significant nosocomial infection agents, especially in burns and accidental wounds. It also causes bed sores in spinal cord injury patients. In this study, the physicochemical and two microbial species inhibitory effects of alcoholic extract of Iranian Propolis collected of Qazvin province were examined.

METHODS:

Providing the Propolis extract:

After collecting the Propolis from Qazvin beekeepers, extraction was done by cold method. Then it was crushed into 2 mm particles, and 30 gr of the crushed Propolis was added to 300 cc ethanol 96%. Then it was kept at room temperature for 20 days, and every day it was put in the shaker incubator for some hours (37°c, 150 rpm). After 20 days, the obtained suspension was

filtered by Watman 41 filter. The filtered solution was placed in Rotary and concentrated in 45°c. Eventually, 7.5 dry purred Propolis was obtained, and it was dissolved in 15 cc ethanol 96%, and the final concentration of stocked solution was 500 mg/ml [9].

Preliminary extract's compound assessment

A Thin-layer Chromatography (TLC) 3×10 aluminum sheet/foil (silica gel 60 GF 254) was used for a basic assessment of ether petroleum, n-hexane, and dichloromethane compartments. For this purpose, spotting was done by capillary tube in 2 centimeters distant from the edge of the sheet/foil. When the spot dried, the sheet/foil was put in the acetone (5%) - chloroform (95%) solvent system containing tank (for ether petroleum, n- hexane parts). When the mobile phase reached the last 1 cm away from the edge of the sheet/foil, it was taken out of the tank and dried on the TLC plate heater. The forming of the bond in the so-called sheet/foil was investigated by Ultraviolet-cabinet **(**UV-cabinet) in the 254nm and 366 nm wavelength. After checking the bonds, dichloromethane part was chosen for more analysis [10].

Vacuum liquid chromatography (VLC)

The extract contained a variety of compounds with different polarities. Therefore, not only the primary classification of compounds by different solvents with different polarities was essential, as well as purification of all compounds by one same solvent system was impossible, while more specific purification of them by one type of the chromatography methods was vital. In the present study, Vacuum Liquid Chromatography (VLC) was used for the basic isolation of the constituents of the dichloromethane part of the extract.

A filter chromatography column was used in this study. The so-called column is attached to a glass connector, and the connector is joined to the decanter from the top and a vacuum pump from its side. The absorbent or filler substance is type D silica gel, which is poured to the column up to the proper height in the way that there would be no vacant space between the silica gel. The dichloromethane portion (10gr) was completely mixed with 4grs of silica gel and poured on the top of the column of silica gel. A filtering paper was placed on top of all these substances. By turning the vacuum pump on all the mobile phases were added to the column, respectively, without any pauses. The mobile phases were respectively contained different proportions of acetone, chloroform, methanol due to polarity augmentation.

The volume of all the phases were 150 ml. After finishing each phase, it was poured in a separate balloon by opening the decanter valve, so eventually, after the chromatography, the dichloromethane extract was divided into seven parts which were concentrated in the low temperature and vacuity by vacuum evaporating device (rotary operator). 0.1 gr of Propolis' hydroalcoholic extract was dissolved in 50 ml methanol for High Performance Liquid Chromatography (HPLC). The solution was sonicated by ultrasound (p=100W, f=40 kHz) for 20 minutes and consequently filtered by 0.45-micron filter .20 micro liter of the solution was injected to the HPLC apparatus. The mobile phase includes methanol/phosphoric acid 0.4% (40/60), which was injected into the column (C18 column, 5 µm. 4.6 × 250 mm) with 0.8ml/min

flow. The column temperature was adjusted on 26°c and its absorption of the apparatus was adjusted on 280nm [11].

Determination of antibacterial activity.

Bacterial strains

The standard bacterial strains used in this study were *Staphylococcus aureus: S.aureus* ATCC25923, *Pseudomonas aeruginosa: P. aeruginosa* ATCC 27853 and *methicillin-resistant Staphylococcus aureus: MRSA.*

Antibacterial Assays

MIC and MBC values of the Propolis extract were determined by broth microdilution method according to CLSI protocol. Briefly, the propolis extract serially diluted twofold in a 96-well microplate. First, 100 μL double serial dilution (1,2,8, 16,32,64,128,256, 512 mg/ml) of Propolis extract was prepared in Muller Hinton broth tubes. Then to each tube 5×10^5 CFU / ml per milliliters of broth from microbial suspension of *Staphylococcus aureus: S.aureus* and *Pseudomonas aeruginosa: P. aeruginosa* equals 0.5 McFarland was added. Finally, after 18-24 hours -incubation at 37 ° C, turbidity was read and recorded. The last dilution that microbial opacity was visible was recorded as the minimum microbial concentration. To determine the minimum bactericidal concentration (MBC), 5 μg/mL removed from the first and second dilution tubes with no growth observed and cultured on the Müller Hinton agar medium. The absence of further growth on the culture medium was indicated MBC. Tetracycline and ceftriaxone were used as positive controls for *Staphylococcus aureus: S.aureus* and *Pseudomonas aeruginosa: P. aeruginosa,* respectively. It should be noted that an ethanol 96% containing test tube was used as a control in all steps of the examination [12,13].

Ethics committee

All steps of the present study conformed to the Research Council of Qazvin University of Medical Sciences. The study protocol was approved by the Committee of Ethics; the code of ethics, as well as the necessary licenses to conduct research were obtained from the relevant university.

Statistical analysis

Antimicrobial data were analyzed using descriptive and analytical statistics. Significant differences were detected by one-way ANOVA with Tukey's post-test using SPSS16 software. (P value<0.05)

RESULTS:

The primary analysis of ethanolic extract by analytical TLC confirms the existence of some phenolic and flavonoid combinations. Hexane, methanol, and dichloromethane solvents were used for separation, purification, and recognition of these chemicals in ethanolic extract and

divided the extract into three parts (Figure 1). Some previously mentioned combinations were purified by preparative TLC. Hydrogen-1 Proton nuclear magnetic resonance (H-NMR) and Carbon-13 nuclear magnetic resonance (C-NMR) were used for their accurate molecular recognition. The outcomes of Propolis sample injection and HPLC standards are demonstrated in (Table 1, Figure 2). Propolis ingredients depend on the region bees are kept in and as well as the variety of regional plants [14].

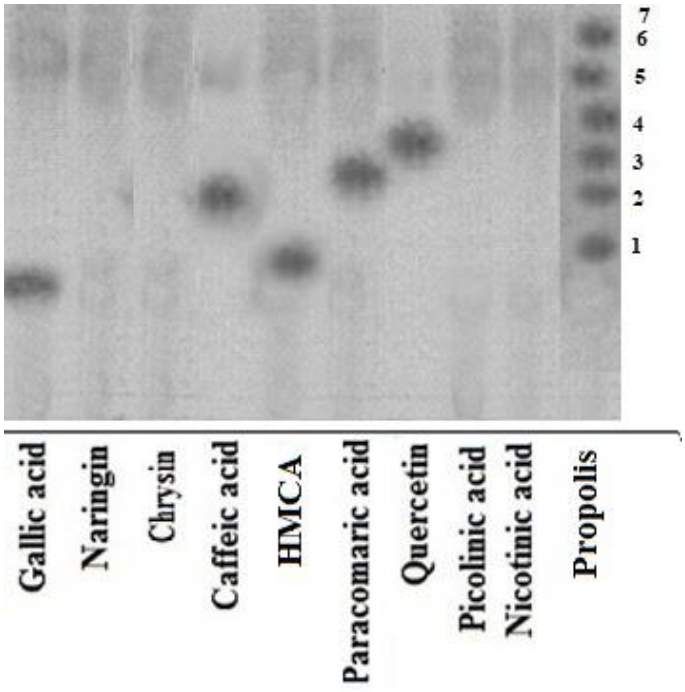
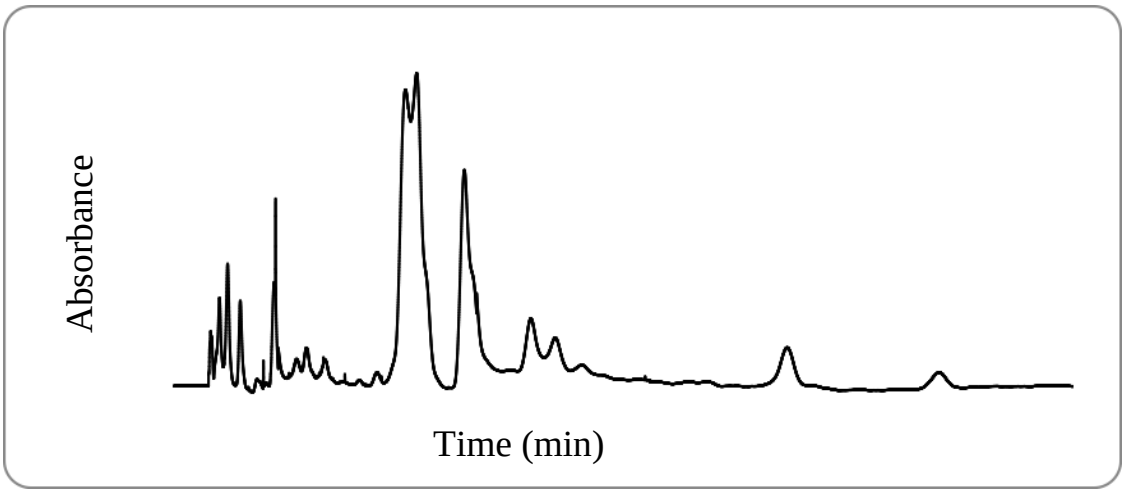


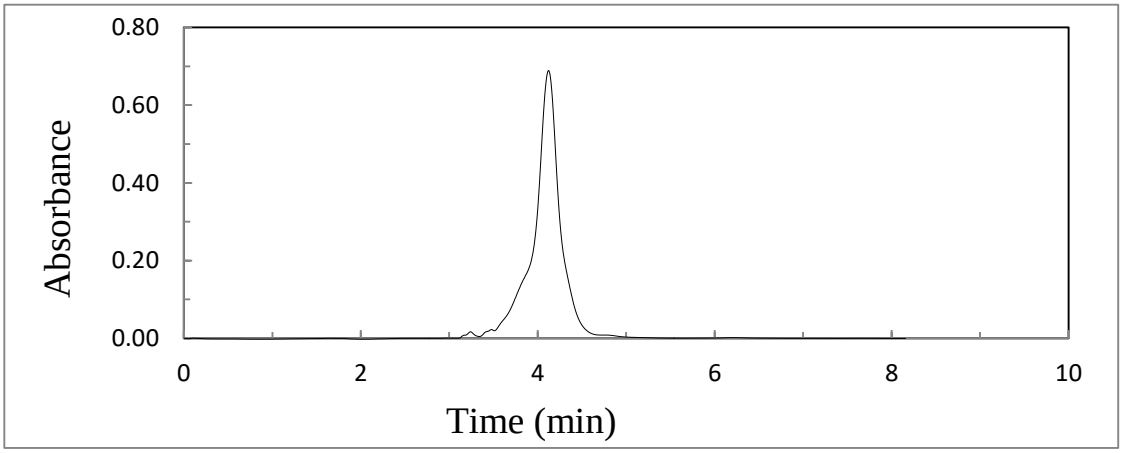
Figure1. The Propolis compounds with TLC. Gallic acid, Naringin, Chrysin, Caffeic acid, HMCA:4-hydroxy-3-methoxy cinnamic acid, Paracomaric acid, Quercetin, Picolinic acid, nicotinic acid.

Table 1: The outcomes of Propolis sample with TLC.

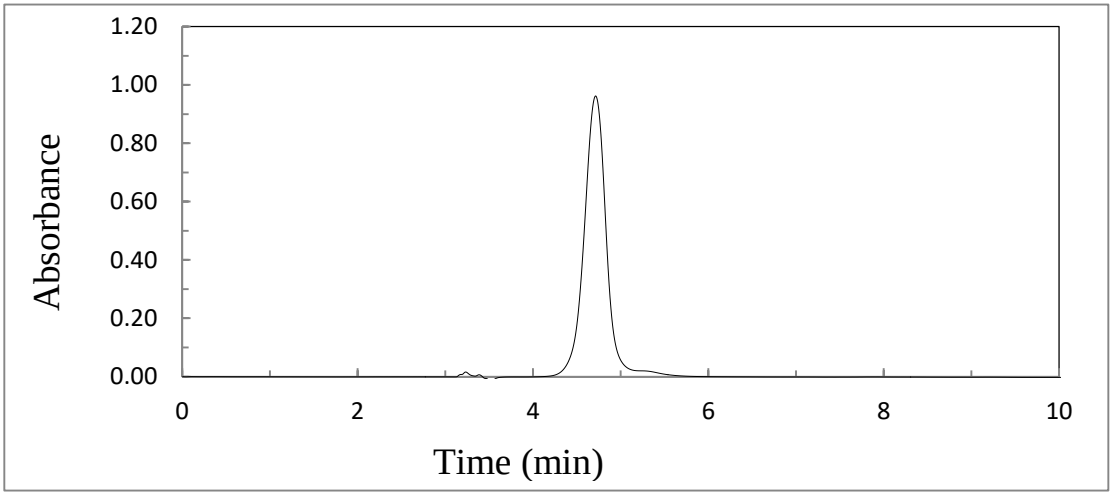
List	Compounds	The No. of spots in the Propolis sample image1	Retention Factor
1	Gallic acid	2	0.4
2	Naringin	5	0.5
3	Chrysin	3	0.6
4	Caffeic acid	1	0.7
5	4-hydroxy-3-methoxy cinnamic acid	3	0.8
6	Paracomaric acid	4	-
7	Quercetin	0	-



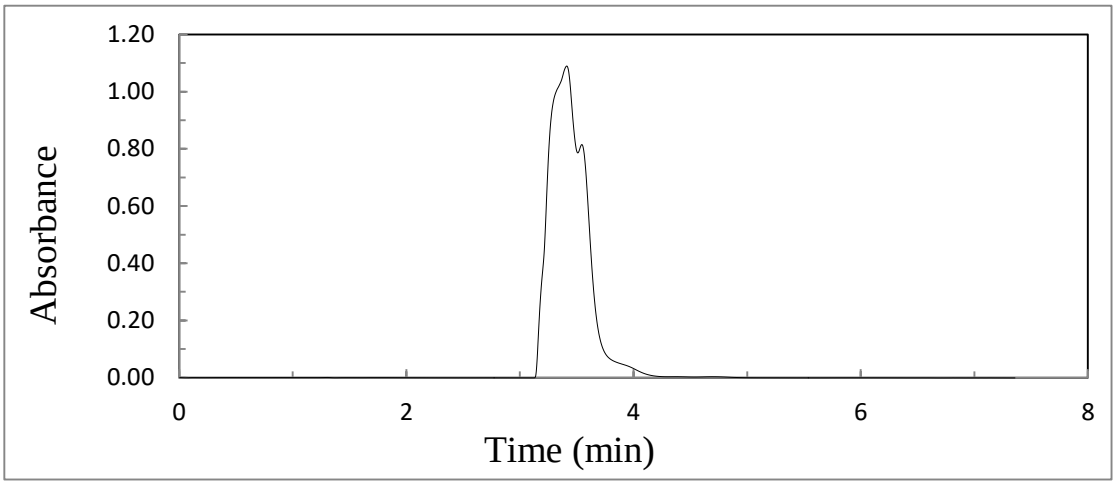
A: Iranian Propolis HPLC peak



B: Caffeic acid HPLC peak



C: Coumaric acid HPLC peak



D: Gallic acid HPLC peak

Figure 2. HPLC chromatogram of propolis has been investigated in Qazvin province samples of Iran. A: Ethanolic extract of Propolis and B: Caffeic acid, C: Coumaric acid, D: Gallic acid that verified from their retention time in propolis extract.

In this research, we compared the Propolis which was bought from Sigma Co. with the one which was collected from Qazvin province.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Staphylococcus aureus: S.aureus* standard strain (ATCC25923) was found between 0.5-5 mg/ml. When repeating the experiment, MIC and MBC for standard *Staphylococcus aureus: S.aureus* was 2.5mg/ml. The same procedures were done for *Pseudomonas aeruginosa:* P. aeruginosa standard strain (American Type Culture Collection) (ATCc27853) and the MIC and MBC were 50mg/ml of Propolis extract (Table 1). It should be noted that an ethanol 96% containing test tube was used as a control in all steps of the examination. The results are shown in (Table 2).

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for the Propolis ethanolic extract on *Staphylococcus aureus: S.aureus* standard strain and *Pseudomonas aeruginosa: P. aeruginosa* standard strain.

Bacterial strains	Ethanolic extract of Propolis concentration	
	MIC mg/mL	MBC mg/mL
Staphylococcus aureus	2.5	2.5
Pseudomonas aeruginosa	50	50

DISCUSSION

The physicochemical characteristics and the antimicrobial effects of the ethanolic extract of Propolis on two important bacterial species were assessed in this research. The results of this

study point to the antimicrobial activity of ethanolic extract of Iranian Propolis that is provided from Qazvin province. Studies have shown that Propolis has a significant antibacterial, anti-protozoon, anti-viral and antifungal effects [15,16,17,18]. Although, some reports have identified, despite having a great inhibitory influence on the growth of Gram-positive bacteria, it has little effect on Gram-negative bacteria [17].

In the present study, the phenolic compounds of Propolis were almost like those determined in Touzani S et al. and Alday E et al. research, but with different concentrations. Therefore, bioactive composites of Propolis specimens, especially phenolic and flavonoid components gathered from different regions of the world were similar [19,20].

The results of Santos et al. study demonstrated ethanolic extracts of geopropolis samples had antibacterial properties against *Pseudomonas aeruginosa: P. aeruginosa* (MIC = MBC = 250 μg/mL) and *methicillin-resistant Staphylococcus aureus: MRSA* (MIC and MBC >1000, μg/mL) [21]. The results of the study were positive. However, not as strong as our results.

Santos et al. investigated the antibacterial, antioxidant, and antiproliferative properties of Propolis extract samples from Brazil, and found that they were different. They found that the antimicrobial activity of Propolis varied depending on Propolis sample, dosage of Propolis, and the extraction solvents for all tested Propolis samples. The results revealed that genopropolis samples from Brazil were a
potential source of novel bioactive compounds [21].

In another study done by University of Toronto in 2010, the MIC and MBC of *Staphylococcus aureus: S.aureus* and *Escherichia coli's: E coli's* was estimated 0.08-1.2 μg/mL , 0.1-2.5 μg/mL and 0.6-5 μg/mL, 2.5-6.2 μg/mL, respectively [22]. Their findings were lower than our results.

Other studies demonstrate, the antimicrobial activity of 19 Propolis extracts geared up in different regions in the Basque Country, and was evaluated against some bacterial isolates using the agar-well diffusion method. Propolis extracts had highly sensitive antimicrobial action against Grams-positive bacteria at a concentration of 20% *(Staphylococcus aureus: S.aureus, Streptococcus mutans: S. mutans , Candida albicans: C. albicans* and *Saccharomyces cerevisae: S. cerevisiae*) with a minimal inhibitory concentration (MIC) ranging from 0.5 to 1.5 mg/ml, with a moderate effect against *Streptococcus pyogenes: S. pyogenes* (MIC from 17 to 26 mg/ml). This study showed activity against Gram-negative bacteria [*Salmonella enterica: S. enterica (*MIC from 0.6 to 1.4 mg/ml)] and less activity against *Helicobacter Pylori: H. Pylori* (MIC up to a concentration of 6 mg / ml 14), while the bacteria *Escherichia coli: E. coli* was resistant [23]. Their findings were similar to our results.

In other studies, the chemical and botanical characterization of Chilean Propolis samples was determined by reverse-phase HPLC and High-Performance Liquid Chromatography–mass spectrometry (HPLC-MS) and evaluated their biological activity against the cariogenic bacteria Streptococcus mutans and Streptococcus sobrinus. The characterization found evidence of the presence of quercetin, myricetin, kaempferol, rutine, pinocembrin, coumaric acid, caffeic acid, and caffeic acid phenethyl ester, that have already been described in Propolis with conventional HPLC. All Propolis samples inhibited the *Streptococcus mutans: S. mutans* growth, with a wide spectrum of action (MIC 0.90 to 8.22 μg mL (-1)) [24].

In our study, the values of MIC and MBC display anti-bacterial property against Gram-positive *cocci, including Staphylococcus aureus: S.*aureus of extract propolis and also lower antibacterial effectiveness against Gram negative Bacilli *including Pseudomonas aeruginosa: P. aeruginosa* similar to of AL-Ani I, studies results[19,25].

Recent studies proved the antiviral effects of Propolis against replication of herpes simplex virus type 1 and type 2 [25].

In addition, another examination demonstrates that Propolis can also be used to treat heart failure because flavonoids extraction from Propolis exerts an effective role in cardiac protection [26].

In other research, the antibacterial activity of Propolis (30% in methyl cellulose), curcumin (2.5mg/mL of methyl cellulose), 2% chlorhexidine gel (CHX), 2% metronidazole gel (MZ) and a mixture of 2% CHX and 2% MZ against *Enterococcus faecalis: E. faecalis* were compared in vitro. The result showed that all tested agents demonstrated significant reduction of viable bacteria at the time periods [27].

Antimicrobial activity of Propolis may be due to its direct action on microorganisms or indirectly by stimulating the immune system and killing more microorganisms. Propolis may have synergistic effects with antimicrobial drugs [25].

In vivo and in vitro methods were demonstrated that Propolis can activate macrophages and increase their antimicrobial activity and may stimulate antibody production [28].

Propolis has potential anti-microbial compounds such as; cinnamic, carboxylic acids, aliphatic acid ester, alcohol, ether, esters, Tepene, ketone, hydrocarbon phenolic, and it has antibacterial properties too [29].

In addition, synergy between these compounds is unique compared to alone against microorganisms, and indicates the antibacterial effect of Propolis. Data confirms that the effect of Propolis against pathogens is higher than its components [30, 19].

Raheem et al. with micro calorimetric and electron microscopic studies were shown that the Propolis inhibits bacterial growth by preventing cell division, thus as a result of which is prevention of the formation pseudo-multicellular streptococci. In addition, Propolis disorganized the cytoplasm, the cytoplasmic membrane, and the cell wall, and caused a partial bacteriolysis and inhibited protein synthesis. It was declared that the mechanism of action of Propolis on the bacterial cell is complex and has not any relation to the mode of action of usual antibiotics [31].

As Bosalec and collogues reported, all of the Propolis taken from different parts of the world have antibacterial property as some parts of this ability depend on bees themselves, although different plants are growing in various places around the world also has effects on the amount of antibacterial property [32]. Due to Kayaoglu's research on the antibacterial ability of the Propolis, studies show it is driven from the flavonoid, cyclic acids, and esters compounds in it [29].

These days, bacterial resistance is one of the serious problems in medical societies, and there are only a few medical centers that have not spared a great amount of money for confronting this phenomenon.

The bacterial strains which were sensitive to the routine antibiotics now are killed by the most potent new antibiotics, and the human gets weaker toward them. Therefore, it seems that

the clue to solve the problem must be searched in nature as natural substances have less harmful effects.

Propolis is one of the useful products of honeybees that has great pharmacological properties such as anti-microbial and anti-bacterial effects [25].

Propolis is used globally to cure different kinds of disease or prevent them [14, 25, 33], and it is made in the shape of tablets, toothpaste, and chewing gum. Propolis is rich in more than 300 various chemical compounds, including polyphenols, flavonoids, phenol acids, phenol aldehyde, ketones, terpens, sterols, vitamins, amino acids, and etc. [2,33]. Annual commercial production of this substance is between 1,800 and 2,400 tonnes.Unfortunately, Iran has no portion in this amount [34]. In addition, the researcher in Iran are not abundant, and therefore Iran is still not able to use it as a vital substance in treating and preventing numerous diseases. Considering the results of this research about Propolis antibacterial property and the result of plenty of the previous investigations in the various other properties of it we hope that we become able to reach the goal of using this vital substance in our country.

CONCLUSION

It may be concluded that the ethanolic extract of Iranian Propolis that is provided from the Qazvin province has significant antimicrobial activity. The Iranian Propolis TLC analysis results confirmed the presence of Gallic acid, Naringin, Chrysin, Caffeic acid, 4-hydroxy-3-methoxy cinnamic acid, and Paracomaric acid. The strong antimicrobial activity may be due to high total phenolic and flavonoid contents. Propolis is one of the useful products that have numerous pharmacological materials against microbial strain, which makes it useful for the prevention and treatment of many infectious diseases. Further research is required for clarification of its chief chemical compositions and antibacterial properties.

Limitation: In science, there are significant differences in antibacterial activities between in vitro and in vivo status. We must understand carefully that inhibition of bacterial growth in culture is not identical to the way Propolis might behave in animal models and human subjects, which is something that needs to be tested.

List of Abbreviations: ATCC, American Type Culture Collection; CHX, Chlorhexidine; C-NMR, Carbon-13 nuclear magnetic resonance; DMSO, ; H-NMR,Hydrogen-1 Proton nuclear magnetic resonance; HPLC, High Performance Liquid Chromatography; HPLC-MS, High Performance Liquid Chromatography–mass spectrometry; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MRSA, Methicillin-resistant Staphylococcus aureus; MZ, Metronidazole; TLC, Thin-layer Chromatography; UV, Ultraviolet; VLC, Vacuum liquid chromatography.

Author Contributions: Nematollah Gheibi designed the research. Nematollah Gheibi and Fatemeh Samieerad provided test opinions and supported the research. Nematollah Gheibi and Fatemeh Samieerad conducted the research. Nematollah Gheibi and Fatemeh Samieerad performed clinical analysis. Nematollah Gheibi and Fatemeh Samieerad performed statistical analyses. Nematollah Gheibi and Fatemeh Samieerad wrote the manuscript. Nematollah Gheibi

and Fatemeh Samieerad had primary responsibility for the final content. All authors read and approved the final version of the manuscript.

Conflict of interest: The authors report no conflict of interest.

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REFERENCES:

1. Pasupuleti VR, Sammugam L, Ramesh N, Gan SH: Honey, propolis, and royal jelly: a comprehensive review of their biological actions and health benefits. Oxid Med Cell Longev. 2017.
2. Wolska K, Gorska A, Antosik K, Lugowska K:Immunomodulatory effects of Propolis and its components on basic immune cell functions. Indian J Pharm Sci2019,81(4):575-588.
3. Przybyłek I, Karpiński TM. Antibacterial properties of propolis. Molecules 2019, (11):2047.
4. Münstedt K. Bee products and the treatment of blister-like lesions around the mouth, skin and genitalia caused by herpes viruses—A systematic review. Complement Ther Med. 2019, 43:81-84.
5. Al-Hariri M. Immune's-boosting agent: Immunomodulation potentials of propolis. J Family Community Med. 2019, (1):57.
6. Chaa S, Boufadi MY, Keddari S, Benchaib AH, Soubhye J, Van Antwerpen P, Riazi A. Chemical composition of propolis extract and its effects on epirubicin-induced hepatotoxicity in rats. Rev bras farmacogn. 2019, 29(3):294-300.
7. Kasote DM, Pawar MV, Gundu SS, Bhatia R, Nandre VS, Jagtap SD, Mahajan SG, Kulkarni MV. Chemical profiling, antioxidant, and antimicrobial activities of Indian stingless bees propolis samples. J Apic Res. 2019, 58(4):617-625.
8. Czyżewski A, Kierzkowska M, Majewska A, Młynarczyk G. The in vitro antimicrobial efficacy of Polish propolis and five plant extracts against selected bacteria and fungi. Og Nauk Zdr. 2019,25(2): 107–111.
9. Kazemi F, Divsalar A, Saboury AA. Structural analysis of the interaction between free, glycated and fructated hemoglobin with propolis nanoparticles: A spectroscopic study. Int J Biol Macromol 2018,109:1329-1337.
10. Amanzadeh Y, Khanavi M, Khatamsaz M, Rajabi A, SADAT ES. High-performance thin-layer chromatographic fingerprints of flavonoids and phenol carboxylic acids for standardization of Iranian species of the Genus Crataegus L. Iran J Pharm Sci 2007:143-152.

11. Chelyn JL, Omar MH, Yousof M, Akmal NS, Ranggasamy R, Wasiman MI, Ismail Z. Analysis of flavone C-glycosides in the leaves of Clinacanthus nutans (Burm. f.) Lindau by HPTLC and HPLC-UV/DAD. The Sci World J 2014; 1–6.
12. Clinical and Laboratory Standards Institute (CLSI), 2009a. Methods for dilution antimicrobial susceptibility tests; approved standard eighth edition. CLSI document M07–A8 (ISBN1-56238- 689-1). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.
13. Zeighami H, Haghi F, Hajiahmadi F, Kashefiyeh M, Memariani M. Multi-drug-resistant enterotoxigenic and enterohemorrhagic Escherichia coli isolated from children with diarrhea. J Chemother. 2015, 27(3):152-155.
14. Machado BA, Silva RP, de Abreu Barreto G, Costa SS, da Silva DF, Brandao HN, da Rocha JL, Dellagostin OA, Henriques JA, Umsza-Guez MA, Padilha FF. Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil. PloS one 2016;11(1).
15. Anjum SI, Ullah A, Khan KA, Attaullah M, Khan H, Ali H, Bashir MA, Tahir M, Ansari MJ, Ghramh HA, Adgaba N. Composition and functional properties of propolis (bee glue): A review. Saudi J Biol Sci. 2019, (7):1695-1703. DOI: https://doi.org/10.1016/j.sjbs.2018.08.013
16. Iadnut A, Mamoon K, Thammasit P, Pawichai S, Tima S, Preechasuth K, Kaewkod T, Tragoolpua Y, Tragoolpua K. In Vitro antifungal and antivirulence activities of biologically synthesized ethanolic extract of Propolis-Loaded PLGA nanoparticles against candida albicans. Evid Based Complement Alternat Med. 2019.
17. Abdullah NA, Ja'afar F, Yasin HM, Taha H, Petalcorin MI, Mamit MH, Kusrini E, Usman A. Physicochemical analyses, antioxidant, antibacterial, and toxicity of propolis particles produced by stingless bee Heterotrigona itama found in Brunei Darussalam. Heliyon. 2019,;5(9):E02476..
18. Turcatto AP, Lourenço AP, De Jong D. Propolis consumption ramps up the immune response in honeybees infected with bacteria. Apidologie. 2018,;49(3):287-296.
19. Touzani S, Embaslat W, Imtara H, Kmail A, Kadan S, Zaid H, ElArabi I, Badiaa L, Saad B. In vitro evaluation of the potential use of Propolis as a multitarget terapeutic product: physicochemical properties, chemical composition, and immunomodulatory, antibacterial, and anticancer properties. Biomed Res Int. 2019.
20. Alday E, Navarro-Navarro M, Garibay-Escobar A, Robles-Zepeda R, Hernandez J, Velazquez C. Advances in pharmacological activities and chemical composition of Propolis produced in Americas. Beekeeping and Bee Conservation— Adv Res. 2016.
21. Santos TL, Queiroz RF, Sawaya AC, Lopez BG, Soares MB, Bezerra DP, Rodrigues AC, Paula Vf, Waldschmidt A. Melipona mondury produces a geopropolis with antioxidant, antibacterial and antiproliferative activities. An Acad Bras Cienc. 2017;89(3):2247-2259
22. AL-Ani I, Zimmermann S, Reichling J, Wink M. Antimicrobial activities of European Propolis collected from various geographic origins alone and in combination with antibiotics. Medicines. 2018,5(1):2.

23. Bonvehí JS, Gutiérrez AL. The antimicrobial effects of propolis collected in different regions in the Basque Country (Northern Spain). World J Microbiol Biotechnol. 201228(4):1351-1358.
24. Barrientos L, Herrera CL, Montenegro G, Ortega X, Veloz J, Alvear M, Cuevas A, Saavedra N, Salazar LA. Chemical and botanical characterization of Chilean Propolis and biological activity on cariogenic bacteria Streptococcus mutans and Streptococcus sobrinus. Braz J Microbiol. 2013,44(2):577-585.
25. Yildirim A, Duran GG, Duran N, Jenedi K, Bolgul BS, Miraloglu M, Muz M. Antiviral activity of hatay Propolis against replication of herpes simplex virus type 1 and type 2. Med Sci Monit: Med Sci Mon Int Med J Exp Clin Res. 2016, 22:422.
26. Sun GW, Qiu ZD, Wang WN, Sui X, Sui DJ. Flavonoids extraction from Propolis attenuates pathological cardiac hypertrophy through PI3K/AKT signaling pathway. Evid Based Complement Alternat Med. 2016,11.
27. Marickar RF, Geetha RV, Neelakantan P. Efficacy of contemporary and novel Intracanal medicaments against enterococcus faecalis. J Clin Pediatr Dent. 2014, 39(1):47-50.
28. De EV, Drago L. Propolis' antimicrobial activity: what's new? Le infezioni in medicina: rivista periodica di eziologia, epidemiologia, diagnostica, clinica e terapia delle patologie infettive. 2007, (1):7-15.
29. Kayaoglu G, Ömürlü H, Akca G, Gürel M, Gençay Ö, Sorkun K, Salih B. Antibacterial activity of Propolis versus conventional endodontic disinfectants against Enterococcus faecalis in infected dentinal tubules. J Endod. 2011, 37(3):376-381.
30. Farhadi F, Khameneh B, Iranshahi M, Iranshahy M. Antibacterial activity of flavonoids and their structure–activity relationship: An update review. Phytother Res. 2019, (1):13-40.
31. Raheem IA, Razek AA, Elgendy AA, Saleh NM, Shaaban MI, El-Hady FK. Design, evaluation and antimicrobial activity Of Egyptian Propolis-Loaded Nanoparticles: intrinsic role as a novel and naturally based root canal nanosealer. Int J Nanomedicine. 2019,14:8379.
32. Kocot J, Kiełczykowska M, Luchowska-Kocot D, Kurzepa J, Musik I. Antioxidant potential of Propolis, bee pollen, and royal jelly: Possible medical application. Oxid Med Cell Longev. 2018.
33. Sun GW, Qiu ZD, Wang WN, Sui X, Sui DJ. Flavonoids extraction from propolis attenuates pathological cardiac hypertrophy through PI3K/AKT signaling pathway. Evid Based Complement Alternat Med. 2016; 11.
34. Michael C. Australian propolis market and production potential. Agri Futures Australia. 2019, 19-022.