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Original Article

Comparative Study of the Antioxidant, Antibacterial, Antidiabetic Analysis and Chemical Compounds of Two Different Geographical Regions Propolis for Production of Functional Chewing Gum

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ABSTRACT

Apis mellifera L. is a honeybee species responsible for gathering propolis, a resinous material from tree cracks. This study assessed the antibacterial activity by agar well diffusion, antioxidant activity by Trolox equivalent antioxidant capacity assay (TEAC), and α-glucosidase inhibitory activity of north (Mazandaran) and south (Khuzestan) propolis of Iran. The ethanolic extract of propolis (EEP) of both samples demonstrates the highest antibacterial activity against gram-positive bacteria *Streptococcus mutans*, *Streptococcus sanguinis*, and Khuzestan EEP was more effective against assessed bacteria than that of Mazandaran. The results of antioxidant activity indicated that higher EEP concentration had more antioxidant activity (31.2 mg/ml of Khuzestan EEP and Mazandaran EEP showed 16.71±0.05 µmol/L and 16.35±0.19 µmol/L Trolox antioxidant activity, respectively). Both EEPs inhibited the α-glucosidase enzyme. EEP chemical compounds have been investigated by GC-MS. Khuzestan and Mazandaran EEP demonstrated 49 and 29 volatile compounds. Chewing gums were prepared with different Khuzestan propolis concentrations, and sensory evaluation of basic properties (smell, texture, appearance, taste, bubble size) was carried out, with results demonstrating that Khuzestan propolis can be used as a functional food and can be formulated into chewing gums as an antibacterial agent.

INTRODUCTION

Apis mellifera L. is a species of honeybee responsible for gathering and transforming propolis into resinous material, this transformation occurs after bees collect these resinous substances from cracks and barks of trees and then change them into propolis by their secretory enzymes (β-glycosidase of their saliva) to finally add them to wax [1,2].

Bees use propolis not only for sealing purposes but also for protecting their hive from intruders and microorganisms [3]. Many researchers have indicated that propolis compositions differ from one hive to the next, depending on the collected resin's geographical and botanical origins [1,3,4]. Thus, there are more than 850 different compounds in propolis but on average each contains 80-100 different ones. They are especially rich in bioactive constituents such as phenolics and their esters, terpene and terpenoids, aromatic aldehyde, alcohol, protein, fatty acid, ketones, steroids, lignans, sugars, vitamins, minerals, and enzymes [5,6].

Poplars and other trees like birches, oaks, and pine trees are important propolis botanical resources [7]. There is a vast range of propolis with different colors and types available around the world, as a case in point, there is popular red propolis found in mangrove parts of northeast Brazil, its intense color natures from oxidation of the constituents collected by bees [8].

It has been reported that propolis can positively affect dental diseases. Hence, it has been used in many dental medicines and products [9]. According to surveys, propolis demonstrate higher inhibitory activity against Gram-positive bacteria than Gram-negative ones. That is due to the outer membrane species-specific structure of Gram-negative bacteria and its hydrolytic enzymes in breaking down the propolis active components [10]. *S. mutans* and *S.*

sanguinis are considered a leading factor in causing dental caries. *S. aureus* is one of the bacteria causing root canal infections. [11,12]. *P. aeruginosa* causes oral infections in patients with clinical conditions, including apical periodontitis, pulp necrosis, and pulpitis [13]. Some Gram-negative bacteria such as *E. coli* cell wall endotoxin released due to multiplication or cell death causes many biological reactions such as penetrating toward dentinal tubules and causing root canal infections [14]. Accordingly, propolis has been implemented in folk medicine extensively [15]. Free radical scavenging, anti-bacterial, anti-inflammatory, and anti-proliferative activities are the prominent Propolis and its constituent's biological activities [1,15]. The presence of different classes of volatile compounds in propolis chemical patterns such as aldehydes, ketones, alcohols, esters, terpenes, acids, etc. is the reason for the complexity of propolis composition [16]. Bees enhance the chemical composition of propolis during gathering from various plant sources by incorporating plant pollen. The types of pollen present in propolis provide insights into the regional flora visited by the bees. For instance, the pollen reveals that the plant species belong to the Fabaceae, Lamiaceae, or Asteraceae families [17]. The propolis organoleptic character and its consumer appreciation is one of the most essential qualitative indicators, highly depending on its volatile fraction [18]. It has been considered superfood recently due to the importance of natural preservative application into foods as an alternative to chemical ones [19]. However, propolis has a high concentration of phenolic compounds, making it bitter, astringe, and smelling tangy [9]. It has been widely used in various food product formulations with no perceptible change in their sensory properties. To illustrate, 5% propolis extract was applied to cheese and other dairy products, 1.5-2 % propolis extract into minced meat, up to 8% propolis extract in chicken breast, and 16% propolis extract in Trout fish fillet, all with no change in sensory properties and even in some cases it increased the general acceptance as well [6]. The total free radical scavenging activity of pure substances solutions, aqueous mixtures, and beverages can be assessed by forming the ABTS [2,29-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] cation radical, known as the foundation of one of the spectrophotometric methods [20].

Restraining carbohydrate digestion and eventually glucose absorption can be achieved by hindering α-glucosidase in the small intestine and α-amylase in the pancreas, which ultimately leads to the decrease of postprandial blood glucose after having a mixed carbohydrate diet [20].

Chewing gum is considered a suitable drug delivery agent because of countless merits such as medicine intake without water, high rate of acceptance among children because of its taste and flavor, streamlining the treatment process, prevention of motion sickness and nausea, making it an appropriate way for systemic delivery [22].

Despite the accomplishment of the vast range of worldwide propolis studies, there is little information about Iranian propolis [23]. In this study, the antibacterial activity, and the chemical composition of two different propolis extracts from the north (Mazandaran) and south (Khuzestan) of Iran were investigated. In addition, a propolis chewing gum sensory evaluation was also performed on eight different concentrations of Khuzestan propolis to produce a functional food product.

MATERIALS AND METHODS

Propolis Collection

Propolis samples were collected from the Mazandaran (36°23′N, 52°11′E) located in the north and Khuzestan (31° 19' 5.9772" N, 48° 40' 14.2320" E) located in the south of Iran by the Apiculture Industry Development and Advocacy Fund (Tehran, Iran). Hand-collected propolis was milled into small amounts (10 g) and eventually stored at -18 ℃ for further use.

Preparation of Propolis Ethanolic Extracts

100 ml of 80% ethanol was dissolved with 22.5 g of ground propolis and the solution was kept for 24 hours at room temperature with magnetic stirring to obtain propolis ethanol extract (EEP). Then, the remained wax was removed with Whatman No. 1 filter paper. The filtered EEP was then placed at 35 ℃ to evaporate the residual ethanol to obtain a gummy mass. Ultimately, it was stored at 4 ℃ for further experiments [4].

Antimicrobial Activity Assay

Antimicrobial activities were performed using agar well diffusion assay against two Gram-negative bacteria strains [*E. coli* (PTCC 1399) and *P.*

aeruginosa (PTCC 1430)] and three Gram-positive bacteria strains [*S. aureus* (PTCC 1431), *S. mutans* (PTCC 1683) and *S. sanguinis* (PTCC 1449)]. All the bacteria were provided by the Persian Type Culture Collection (PTCC, Tehran, Iran) from the Iranian Research Organization for Science and Technology (IROST). Stock bacteria were cultured overnight at 37℃ in the BHI (Brain Heart Infusion) broth medium. The antimicrobial activity assay was performed using BHI broth and 0.75% agar medium. The agar well diffusion was carried out by adding (10^8 CFU m/L) of each culture into the mixture of agar and BHI broth medium. After a few seconds of slowly homogenizing, the mixture was conveyed into Petri dishes to solidify before making 5 mm diameter wells using a sterilized cork borer. Fifty microliters of the sample concentration made out of 500 mg/ml of propolis extract were added to each well. Finally, each plate was incubated at 37 ℃ for 24 h. The inhibition zone was measured by the diameter of each well surrounding [24].

Minimum Inhibitory Concentration Determination (MIC)

The MIC, or Minimum Inhibitory Concentration, is the smallest amount of an antimicrobial substance in mg/L that stops the visible growth of a microorganism within a set time frame and under specific laboratory conditions. As previously described, to investigate the MIC, the antimicrobial activity assay was performed by Mistry *et al*., but by adding nine different concentrations of propolis extract (7.81 mg/ml - 1500 mg/ml) to each well and incubating them at 37 ℃ for 24 h. MIC was specified as the lowest concentration prohibiting visible growth, or in other words, the last plate that shows no microbial growth is considered the MIC or minimum inhibitory concentration [25].

Determination of Minimum Bactericidal Concentration (MBC)

All the plates employed to determine MIC, which showed the least inhibition zone, were subcultured by streaking method into BHI broth and agar medium and then incubated at 37 ℃ for 24 h. The lowest concentration without bacterial growth was decided as the MBC value [26].

Antioxidant Activity Assay

Antioxidant activity was measured using the Trolox equivalent antioxidant capacity method. This method is hinged on the decolorization of ABTS radical cation assay. 11.5 mg ABTS was dissolved in 3ml deionized water. Then, 2 mg potassium persulfate was added to the mixture and was kept to stand for 12-16 h in a dark place to produce ABTS radical cation ($ABTS^{\circ+}$). A diluted $ABTS^{\circ+}$ solution with PBS (phosphate buffer saline, pH:7.4) was prepared to gain an absorbance of 0.70 (±0.02) at 734 nm at room temperature. The absorbance of each EEP sample added to $ABTS^{\circ+}$ is readable with an ELISA microplate reader 6 min after initial mixing at 734 nm. (Table 1). The inhibition percentage of every EEP concentration was calculated according to the formula below [20]:

$$\text{Inhibition \%} = (Abs_{control}\text{-}Abs_{sample}/Abs_{control}) \times 100$$

α-glucosidase Inhibitory Activity

This assay was followed as described by Ganiyu *et al*. The process was carried out spectrophotometrically using *ρ*-nitrophenyl-*α*-D-glucoside (pNPG) as substrate. This enzyme is known to liberate D-glucose from pNPG. The assay began by adding 20 µL of different EEP concentrations, 100 µL phosphate buffer saline (100 m mol/L, pH 6.9), 50 µL pre-incubated pNPG (5 m mol/L), and 50 µL α-glucosidase (1.0 U/mL) to 96 well plates. Then, the plate was stored at 37 ℃ for 15 min. Finally, the reaction stopped by adding 50µl sodium carbonate (0.2 mol/L) to each cell, and the absorbance reading was recorded at 405nm. Control absorbance was recorded with buffer, enzyme, and substrate in the plate. Blank absorbance was measured, with enzyme solution substituted with buffer solution. This assay was carried out thrice. The α-glucosidase inhibitory activity was determined as inhibition percentage by the following formula: [27]

$$\text{Inhibition \%} = (Abs_{control} - Abs_{sample}/ Abs_{control}) \times 100$$

Gas Chromatography-mass Spectrometry Analysis (GC-MS)

In this assay, we used a 6890 Agilent gas chromatograph with a 5273-mass spectrometer selective detector (MSD) facilitated with an HP-5 column and 30 m length. Samples were diluted by methanol and after filtration, 1µL of each was injected into the GC-MS port at 250 ℃ in split-less mode. Helium with a 1ml/min flow rate is used as a carrier gas.

Table 1 Antioxidant activity of Mazandaran and Khuzestan Propolis.

Sample		Concentration (mg/ml)	Antioxidant activity percentage	Trolox antioxidant capacity (µM)
Mazandaran	1	31.2	88.21 ± 0.95 a	16.35±0.19 a
	2	15.6	89.30 ± 0.22 a	16.57±0.04 a
	3	7.8	82.76 ± 1.43 b	15.26±0.28 b
	4	3.9	71.52 ± 1.54 c	13.08±0.39 c
	5	1.95	42.09 ± 0.22 d	7.09±0.45 d
	6	0.975	32.72 ± 1.54 e	5.22±0.31 e
	7	0.4875	11.71 ± 5.88 f	1±0.17 f
Khuzestan	1	31.2	90.04 ± 0.29 a	16.71±0.05 a
	2	15.6	88.97 ± 0.46 a	16.5±0.09 a
	3	7.8	89.67 ± 0.36 a	16.64±0.07 a
	4	3.9	89.38 ± 0.22 a	16.58±0.45 a
	5	1.95	69.33 ± 1.73 b	12.56±0.34 b
	6	0.975	46.91 ± 1.20 c	8.06±0.24 c
	7	0.4875	19.72 ± 1.1 d	2.61±0.23 d

Value is Mean ± SD. Means with different superscript letters within a column are significantly different at P<0.05

Scanning the mass spectra were in the range m/z 39–800 amu at 1-s intervals. The initial oven temperature was 50 ℃ (5 min) and was ramped at 5℃/min to 280℃, held for 15 min. The source temperature was set at 250 ℃. The mass detector was run with electronic impact (EI) mode with 70 eV. The comparison of mass spectra with the retention index (RI) records of Wiley and the National Institute of Standards Mass Spectral Library helped to discern the volatile components [28].

Production of Chewing gum

Using Khuzestan propolis, gum was identified as the most effective sample in the analysis. The Propolis sample was freeze-dried into powder to enhance its combination with other ingredients. Six different concentrations of propolis and a control sample were made. The chewing gum production process was carried out using a modified method suggested by Hovart *et al*. 2012. All ingredients included sweeteners containing mannitol, xylitol, and acesulfame potassium 6% (0.3), glycerin and lecithin 1.5% (0.3), mint flavor 3% (1), and propolis 5% (0.5). After melting the gum base, 30% is added to the dough. After blending for a few minutes and resting the gum for cooling, was removed from the kneader and cut into pieces [29].

Sensory Evaluation

Seven panelists were selected and trained to identify and quantify the sensory characteristics of propolis chewing gum. A 5-point hedonic scale test was performed as a sensory evaluation of propolis chewing gum in a quiet, well-lighted, and odor-free room. A cup of warm water was provided for each panelist to drink before the start of the sensory evaluation. Smell, taste, texture, appearance, and sweetness were graded with scores from 1 to 5, with 1 being disliked very much, 2=disliked slightly, 3=neither liked nor disliked, 4=liked slightly, 5=liked very much [29].

Statistical Analysis

Statistical analysis was performed with SPSS statistical software version 16.0 (SPSS, Chicago, IL, USA). The results were reported as mean ± standard deviation (SD). For data analysis, the variance analysis was determined by one-way ANOVA and differences among samples were determined by Duncan post hoc multiple comparisons. *A P*-value less than 0.05 was considered a significant difference. All the experiments were done thrice, except for antimicrobial activity done in five replications.

RESULTS

Antioxidant Activity Assay

The antioxidant activity of two propolis ethanolic extracts is demonstrated in (Table 1.) The results show that the antioxidant activity increased due to the increase in propolis concentration. The Khuzestan's propolis at the highest concentration (31.2 mg/mL) and the lowest concentration (0.4875 mg/mL) showed more antioxidant activity (90.04±0.29%) and (19.72±1.1%) respectively, and Mazandaran's propolis demonstrated an antioxidant activity of (88.21±0.95%) and (11.71±5.88%) at

highest and lowest concentrations, respectively. It is worth mentioning that Khuzestan's and Mazandaran's propolis concentrations were the same in this test. Furthermore, Trolox antioxidant capacity for Khuzestan is 16.71±0.05 µmol/L in the highest concentration and 2.61±0.23 µmol/L in the lowest concentration and Trolox antioxidant capacity for Mazandaran is 16.35±0.19 µmol/L in the highest concentration and 1±0.17 µmol/L in the lowest concentration. It was concluded that Khuzestan's propolis showed more antioxidant activity compared to that of Mazandaran. Investigations show that the high antioxidant activity of both Mazandaran and Khuzestan propolis owes to its richness in flavonoids, the reason being the samples prepared using 80% ethanol. The antioxidant assay demonstrates a vast environmental impact on Khuzestan and Mazandaran propolis compounds, followed by their free radical scavenging properties. Another point to consider is that the north and south of Iran are ecologically different, with various botanical resources. Therefore, some of the antioxidant compounds might be extracted by bees.

Antimicrobial Test

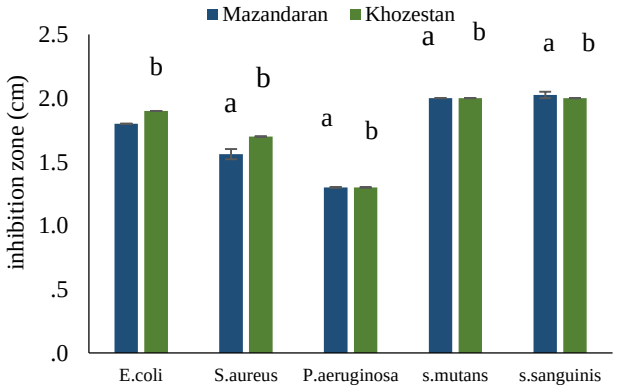

Fig. 1 Diameter of tested bacteria inhibition zones (cm) on Mazandaran and Khuzestan's Ethanolic Extracts of propolis

This essay investigated antibacterial assay including inhibition zone, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). The results of the inhibition zone assay are illustrated in Fig. 1. Both propolis samples showed antibacterial activity against all bacteria and their antimicrobial activity varies according to their region. In these two samples of propolis, there is strong antibacterial activity against gram-positive bacteria strains. However, the interesting fact is that they were effective against a gram-negative bacterium, *E. coli* (Fig. 1). Khuzestan's propolis had a more significant inhibition zone on *S. aureus* (17±0.0 mm) and *E. coli* (19±0.0 mm) but a smaller one on *S. sanguinis* (20±0.0 mm) compared to Mazandaran's sample (20.25±0.25 mm). They both showed the same antimicrobial effect on *S.mutans* (20±0.0 mm) and *P.aeruginosa* (13±0.0 mm).

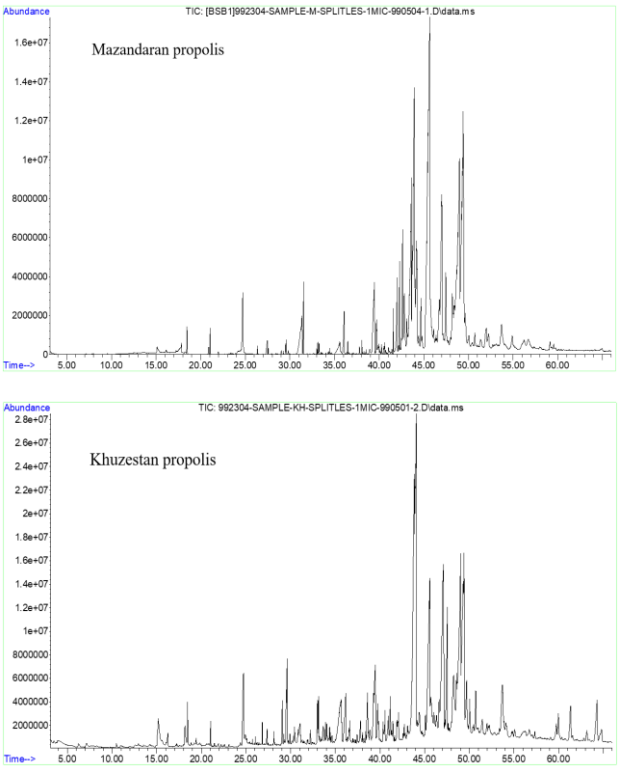

Fig. 2 GC_MS chromatogram of Mazandaran and Khuzestan propolis samples

Later, minimum inhibitory concentration (MIC) was investigated, the results are shown in (Table 2.) Both samples showed the same MIC against *E. coli* (7.81 mg/mL), but Mazandaran's sample had a higher MIC (15.62 mg/mL) against *P.aeruginosa* and *S.aureus* compared to Khuzestan's (7.81 mg/mL). The MBC for Mazandaran and Khuzestan's propolis was an extract concentration higher than 1500 mg/mL. In total, both Iranian EEPs were effective against *E. coli* (highest anti-bacterial activity), *S. aureus*, and *P. aeruginosa* (lowest anti-bacterial activity).

α-glucosidase Inhibitory Assay

In the α-glucosidase inhibitory assay, each propolis sample demonstrated an α-glucosidase inhibitory effect (Table 3), while Khuzestan's sample displayed lower IC50 (0.066±0.002 gm/L). Hence, Khuzestan's propolis had more α-glucosidase inhibition ability and it is inferred, that the more

antioxidant activity the propolis has, the more anti-diabetic properties it has.

GC-MS Analysis

In this study, the volatile contents of propolis samples from the north and south of Iran were specified (Table 4). Table 5 provides the average value percentage of volatile component content of the propolis samples. Among the 68 volatile chemicals found in examined propolis samples, 57 with unlike chemical classifications were detected, including aromatic acid, aliphatic acid, alcohol, fatty acid, ester, terpene, pyran, and some other volatile compounds such as 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one,3-O-Benzyl-d-glucose,

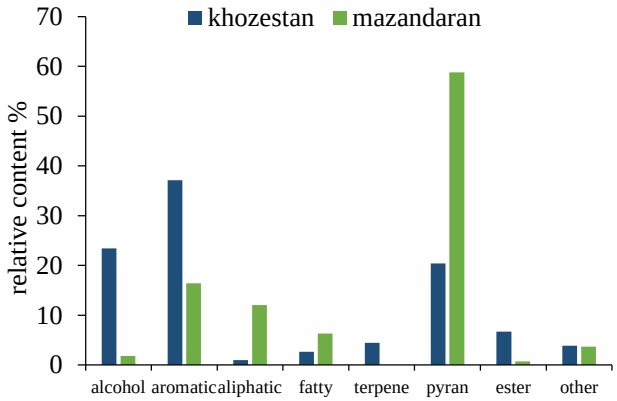

Fig. 3 Volatile compound concentration of Mazandaran and Khuzestan propolis samples

Benzofuran, 2,3-dihydro, to name a few. It is worth mentioning that seven similar volatile compounds out of the total volatile compounds were detected in both samples. The chromatogram of the mentioned samples is also depicted in figure 2. Alcohols, pyrans, aromatic, and terpene chemicals predominate in the composition of all investigated volatile components of samples. (Fig 3).

Propolis of Khuzestan, located in the south of Iran shows that there are 49 volatile compounds with various chemical classifications, such as alcohols 23.43%, aromatics 37.16%, acids 1.01%, fatty acids 2.65%, terpenes 4.49%, pyran 20.43%, esters 6.73%, and others 3.87%. The ample component was aromatics, accounting for about 37% of the total GC-MS Chromatogram area, and then 2-Propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-, (E)- 27.42%, chrysin 13.69%, 4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl 10.01% and 4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)- 9.03%. Propolis of Mazandaran, located in the north of Iran, demonstrates a total of 29 volatile compounds with different chemical classifications, such as alcohols 1.85%, aromatics 16.41%, acids 12.06%, fatty acids 6.3%, pyran 58.82%, esters 0.74%, and others 3.68%. Pyran was the most plentiful compound, covering about 59% of the total GC-MS area, followed by 4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S) 31.70%, chrysin 17.07% and 2-Propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-, (E) 15.63%.

Propolis Chewing Gum Sensory Evaluation

Combining the perfect propolis concentration for its health effects and chewing gum merits to produce propolis chewing gum as a functional food is the main reason behind this study. As shown in Table 6, there was no significant difference between the appearance, taste, smell, and texture of control chewing gum with 0.5% to 4% propolis. In contrast, the sweetness of chewing gum with 3% propolis demonstrated a higher score than 4% and 5% propolis. Therefore, chewing gum containing 3% propolis was selected as the best formulation.

Table 2 The minimum inhibitory concentration of Mazandaran and Khuzestan's ethanolic extract of propolis.

Sample	*S. aureus*	*P. aeruginosa*	*E. coli*
Khuzestan	7.81 ± 0.00 b	7.81 ± 0.00 b	7.81 ± 0.00 a
Mazandaran	15.62 ± 0.00 a	15.62 ± 0.00 a	7.81± 0.00 a

Value is Mean ± SD. Means with different superscript letters within a column are significantly different at P<0.05

Table 3 α-glucosidase inhibition activity of Mazandaran and Khuzestan propolis.

Propolis	IC50
Khuzestan	0.066 ± 0.002 a
Mazandaran	2.160 ± 0.036 b

Value is Mean ± SD. Means with different superscript letters within a column are significantly different at P<0.05

Table 4 Identified compounds of Khuzestan and Mazandaran propolis samples. (The peak numbers in the table are given according to the retention time only to the major peaks).

Khuzestan			Mazandaran		
Peak	Compound	RT (min)	Peak	Compound	RT(min)
1	2-Furanmethanol	6.25	1	Phenylethyl Alcohol	15.11
2	Butanoic acid, 2-ethyl-, methyl ester	7.12	2	Ethyl hydrogen succinate	17.83
3	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	10.48	3	Benzofuran, 2,3-dihydro	18.45
4	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)	10.96	4	2-Propen-1-ol, 3-phenyl	20.90
5	3-O-Benzyl-d-glucose	12.94	5	4-Acetoxy-3-methoxystyrene	21.05
6	Phenylethyl Alcohol	15.19	6	Benzene, 1-(bromomethyl)-3-nitro	24.71
7	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	16.26	7	Cyclohexane, 1,3-dichloro-, trans	26.35
8	1,2-Benzenediol	18.16	8	4-Pentenoic acid, 5-phenyl	27.46
9	Benzofuran, 2,3-dihydro	18.46	9	5-Hepten-2-one, 7-phenyl	27.56
10	2,6-Octadien-1-ol, 3,7-dimethyl	19.41	10	3,6-Dimethyl-4H-furo[3,2-c]pyran-4-one	29.49
11	2-Methoxy-4-vinylphenol	21.04	11	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,8a-octahydro-α,α,4a,8-tetramethyl-, [2R-(2α,4aα,8aβ)]	29.56
12	Benzenepropanoic acid	21.92	12	5-Phenylpenta-2,4-diecoic acid	31.35
13	2-Hydroxy-5-methylbenzaldehyde	24.70	13	7-Acetyl-2-hydroxy-2-methyl-5-isopropyl bicyclo[4.3.0]nonane	31.52
14	Benzyl alcohol, α-isobutyl-2,4,5-trimethyl	25.70	14	2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro-α,α,4a,8-tetramethyl-, [2R-(2α,4aβ,8β)]	33.12
15	2,5-di-tert-Butyl-1,4-benzoquinone	26.03	15	10-Propyl-10H-acridin-9-one	35.57
16	Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol, α,α,6,8-tetramethyl-, stereoisomer	26.82	16	n-Hexadecanoic acid	36.07
17	4-Pentenoic acid, 5-phenyl	27.36	17	Hexadecanoic acid, ethyl ester	36.46
18	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-α,α,4a,8-tetramethyl-, (2R-cis)	29.06	18	9-Octadecenoic acid, (E)	39.45
19	2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene-, [2R-(2α,4aα,8aβ)	29.53	19	Ethyl Oleate	39.69
20	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,8a-octahydro-α,α,4a,8-tetramethyl-, [2R-(2α,4aα,8aβ)]	29.59	20	5-Pregnene-3-acetoxy	41.56
21	2-Propenoic acid, 3-(4-methoxyphenyl)-, (E)	31.05	21	Pimaric acid	41.99
22	Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol, α,α,6,8-tetramethyl-, stereoisomer	33.01	22	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-, (4bS-trans)	42.27
23	2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-naphthalen-2-yl)-prop-2-en-1-ol	33.64	23	2-Propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-, (E)	43.93
24	5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3,6-dimethyl-α-methylene-2-oxo-, methyl ester	33.88	24	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1α,4aα,10)	44.19

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25	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	34.03	25	Palustric acid	44.65
26	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester	34.36	26	4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)	45.63
27	3-Hydroxy-4-methoxycinnamic acid	35.65	27	4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl	47
28	n-Hexadecanoic acid	36.15	28	3-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one	47.45
29	β-Guaiene	36.60	29	Chrysin	49.01
30	1-Heptatriacotanol	37.82			
31	2,6,10-Dodecatrien-1-ol, 12-acetoxy-2,6,10-trimethyl-, (E,E,E)-	38.61			
32	cis-Vaccenic acid	39.26			
33	Osthole	39.46			
34	Retinal, 9-cis	39.74			
35	n-Propyl 9-hexadecenoate	40.35			
36	Acetate, [6-(acetyloxy)-5,5,8a-trimethyl-2-methyleneperhydro-1-naphthalenyl]methyl ester	40.56			
37	6,9,12,15-Docosatetraenoic acid, methyl ester	40.94			
38	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)	41.16			
39	2-Propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-, (E)-	44.06			
40	4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)-	45.58			
41	4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl	47.12			
42	7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one	47.53			
43	Chrysin	49.03			
44	Androst-5-en-3-one, 19-acetoxy-4,4-dimethyl-, oxime (lepedine)	53.70			
45	α-Amyrin	59.98			
46	Lupeol	61.34			

Table 5 volatile compound concentration percentage of Khuzestan and Mazandaran propolis samples

Compound	Khuzestan	Mazandaran
Alcohol		
2-Furanmethanol	0.17	-
Phenylethyl Alcohol	2	0.28
2,6-Octadien-1-ol, 3,7-dimethyl	0.1	-
Benzyl alcohol, α-isobutyl-2,4,5-trimethyl	0.04	-
1-Heptatriacotanol	0.61	-
2,6,10-Dodecatrien-1-ol, 12-acetoxy-2,6,10-trimethyl-, (E,E,E)-	1.47	-
4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)-	9.03	-
4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl	10.01	-
2-Propen-1-ol, 3-phenyl	-	0.1
2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-, (4bS-trans)	-	1.47
Aromatic Acid		
1,2-Benzenediol	0.71	-
2-Methoxy-4-vinylphenol	0.28	-
Benzenepropanoic acid	0.08	-
Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol, α,α,6,8-tetramethyl-, stereoisomer	0.25	-
4-Pentenoic acid, 5-phenyl	0.33	0.46
2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-α,α,4a,8-tetramethyl-, (2R-cis)	0.76	0.15
2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro-α,α,4a,8-tetramethyl-, [2R-(2α,4aβ,8β)]	-	0.17
2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene-, [2R-(2α,4aα,8aβ)	1.12	-
2-Naphthalenemethanol, 1,2,3,4,4a,5,6,8a-octahydro-α,α,4a,8-tetramethyl-, [2R-(2α,4aα,8aβ)]	0.94	-
Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol, α,α,6,8-tetramethyl-, stereoisomer	0.85	-
2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-naphthalen-2-yl)-prop-2-en-1-ol	0.37	-
2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	0.34	-
3-Hydroxy-4-methoxycinnamic acid	3.71	-
2-Propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-, (E)-	27.42	15.63
Aliphatic Acid		
5-Phenylpenta-2,4-diecoic acid	-	2.88
Pimaric acid	-	1.89
1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1α,4aα,10)	-	5.67
Palustric acid	-	1.62
2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)	1.01	-
Fatty Acid		
Ethyl hydrogen succinate	-	0.43
n-Hexadecanoic acid	1.81	1.33
Hexadecanoic acid, ethyl ester	-	0.14
9-Octadecenoic acid, (E)	-	3.68
Ethyl Oleate	-	0.72
cis-Vaccenic acid	0.84	-
Terpene		
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.05	-
β-Guaiene	0.72	-
Retinal, 9-cis	1.15	-
α-Amyrin	1.17	-
Lupeol	1.4	-
Pyran		
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	0.49	-
Osthole	2.88	-
7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one	3.37	-

3-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one	-	2.41
3,6-Dimethyl-4H-furo[3,2-c] pyran-4-one	-	0.1
4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)	-	31.70
4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl	-	7.54
chrysin	13.69	17.07
Ester		
Butanoic acid, 2-ethyl-, methyl ester	0.24	-
2-Propenoic acid, 3-(4-methoxyphenyl)-, (E)	1.15	-
5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3,6-dimethyl-α-methylene-2-oxo-, methyl ester	0.23	-
2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester	0.26	-
n-Propyl 9-hexadecenoate	0.38	-
Acetate, [6-(acetyloxy)-5,5,8a-trimethyl-2-methyleneperhydro-1- naphthalenyl] methyl ester	0.62	-
6,9,12,15-Docosatetraenoic acid, methyl ester	0.82	-
Androst-5-en-3-one, 19-acetoxy-4,4-dimethyl-, oxime (lepedine)	3.3	-
4-Acetoxy-3-methoxystyrene	-	0.27
5-Pregnene-3-acetoxy	-	0.47
Other		
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	0.1	-
3-O-Benzyl-d-glucose	0.16	-
Benzofuran, 2,3-dihydro	0.76	0.39
2-Hydroxy-5-methylbenzaldehyde	2.77	-
2,5-di-tert-Butyl-1,4-benzoquinone	0.8	-
Benzene, 1-(bromomethyl)-3-nitro	-	0.88
Cyclohexane, 1,3-dichloro-, trans	-	0.09
5-Hepten-2-one, 7-phenyl	-	0.07
7-Acetyl-2-hydroxy-2-methyl-5-isopropyl bicyclo[4.3.0]nonane	-	1.21
10-Propyl-10H-acridin-9-one	-	1.04

* The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.

Table 6 Chewing gum sensory evaluation

Sample	Appearance	Smell	Taste	Texture	Sweetness
control	4.57 ± 0.53 a	4.85 ± 0.37 a	3.85 ± 0.89 a	4.42 ± 0.78 a	4.00 ± 0.57 a
0.5%	3.71 ± 1.38 a	4.14 ± 1.21 a	4.00 ± 1.15 a	3.42 ± 0.97 a	2.57 ± 0.97 b
1%	3.14 ± 0.89 a	3.57 ± 1.27 a	3.14 ± 1.34 a	3.28 ± 0.75 a	2.85 ± 1.21 b
2%	3.14 ± 1.34 a	3.85 ± 0.89 a	3.42 ± 1.27 a	3.71 ± 1.38 a	2.57 ± 1.27 b
3%	3.28 ± 1.11 a	4.28 ± 0.75 a	3.42 ± 1.27 a	3.85 ± 0.37 a	2.57 ± 0.78 b
4%	3.28 ± 1.60 a	4.14 ± 1.5 a	4.28 ± 0.75 a	3.71 ± 1.11 a	2.14 ± 1.06 b
5%	1.71 ± 0.75 b	3.42 ± 1.51 a	3.14 ± 1.57 a	2.00 ± 0.81 b	1.71 ± 0.75 c

Value is Mean ± SD. Means with different superscript letters within a column are significantly different at $P<0.05$

DISCUSSION

Antioxidant Activity Assay

The antioxidant capacity of a chemical may contribute to avoiding the oxidative stress-related disorders that are brought on by an imbalance between the production and neutralization of free radicals in the body. These illnesses include cancer, diabetes, heart disease, rheumatoid arthritis, and cardiovascular diseases. In recent years, there has been a rise in the pursuit of a novel drug based on natural substances that could be utilized as a chemigraphy agent [30]. Flavonoids are potential pharmacologically active components in propolis and various phenolic compounds able to scavenge free radicals. It has been proposed that propolis containing high amounts of phenolic compounds and other non-flavonoid free radical scavengers such as enzymes and antioxidant vitamins demonstrates more potent antioxidant activity. Not to mention, geographic area, as well as ecology, have an enormous impact on the amount of phenolic and flavonoid compounds of propolis [31]. As previously mentioned, the propolis antioxidant

activity is influenced by their phenolic content. This is not solely determined by the types of plants in the area, but also by various other factors, including the age and condition of the beehives, the strength of the bee colony, and the method used to gather the samples [32].

The original plants contain compounds that directly influence extremely versatile propolis composition. As mentioned before the phenolic compounds, including the flavonoids, cinnamic acid derivatives, esters, and some terpenes are considered the main bioactive components of propolis [33]. The study by Jung *et al*., 2014 showed that propolis is a source of polyphenols, antioxidants, radical scavenging, and chelating properties despite different extraction methods and conditions. Flavonoids are characterized as an antioxidant agent and the most active radical scavenger among the extracts was the extract prepared using 80% ethanol and the extraction method did not affect the antioxidant activity [34]. Based on our investigation, it can be inferred that the notable antioxidant potency found in both Mazandaran and Khuzestan propolis can be attributed to their high content of flavonoids, particularly in samples prepared using 80% ethanol. The antioxidant assessment underscores the significant environmental influence on the composition of propolis from Khuzestan and Mazandaran, evident in their efficacy in scavenging free radicals. Furthermore, it is essential to acknowledge the ecological diversity between the northern and southern regions of Iran, which results in distinct botanical resources.

Antimicrobial Test

Propolis is well-recognized among the recently investigated natural products for antimicrobial activity [33]. Bispo Junior *et al*. in 2012 verified that ethanolic propolis extract showed antimicrobial activity against both gram-positive and gram-negative strains. This study analyzed species including *Pseudomonas aeruginosa* (*P. aeruginosa*), *S. aureus*, *Klebsiella pneumoniae*, and *E. coli* that were susceptible to ethyl acetate fractions (which acts as a solvent to prepare propolis ethanolic extract) with best antibacterial activity [35]. Righi *et al*., 2011 verified that red propolis methanolic extract showed inhibitory activity in all tested bacteria. The MIC was 256 μg/mL for *P.aeruginosa*, *Bacillus subtilis*, and *Candida albicans*, 512 μg/mL for *E. coli*, and 512 μg/mL for *Streptococcus pyogenes*. In addition, the ethanolic extract propolis showed larger inhibition zones (27.25±0.25 mm) for *S. mutans* and (19.33±0.94 mm) for *Streptococcus sanguinis* [33].

α-glucosidase Inhibitory Assay

Millions of people have been affected by a chronic metabolic disease called type 2 diabetes mellitus [36]. Recent reports suggest that because of people's lifestyles, the number of diabetic patients particularly type 2 diabetes mellitus, would reach 366 million in 2030, amounting to 9% of global mortality. Patients with type 2 diabetes mellitus are prone to retinopathy, impaired wound healing, neuropathy, and nephropathy, which are considered long-term complications [37]. Chronically elevated blood glucose concentrations or hyperglycemia is characteristic of type 2 diabetes mellitus, which results in multi-organ malfunction. The increase in blood glucose levels is caused by the release of absorbable monosaccharides by α-glucosidases enzymes. Sucrase and maltase are two α-glucosidases enzymes secreted from the brush-border surface of intestinal cells responsible for catalyzing the final stage of the carbohydrate digesting process. One of the efficient diabetes treatments is controlling blood glucose, and many anti-diabetic therapies are based on decreasing the blood glucose level. The basis of anti-diabetic drugs is α-glucosidase inhibitors such as acarbose. This inhibitor mechanism reduces postprandial hyperglycemia by slowing carbohydrate digestion followed by D-glucose distribution in the intestines. Thus, they have been one of the therapeutic approaches for diabetes mellitus since the early 1990s [36,38,39]. The IC50 value is the enzymatic inhibition effectiveness calculated in various extracts. The lower the value, the higher the enzymatic inhibition of the extract [37]. In the α-glucosidase inhibitory assay, each propolis sample demonstrated an α-glucosidase inhibitory effect (as indicated in Table 3), while Khuzestan's sample exhibited a lower IC50 value (0.066±0.002 gm/L). Consequently, Khuzestan's propolis displayed greater α-glucosidase inhibition ability. Therefore, it can be inferred that propolis with higher antioxidant activity also tends to possess substantial anti-diabetic properties. Phenolic compounds with the ability to bind with proteins can inhibit the activities of carbohydrate-hydrolyzing enzymes. Zhang *et al*., 2015 investigated the α-glucosidase inhibitory

activity of aqueous and different ethanolic propolis extracts (25%, 50%, 75%, 95%, 100%). Consequently, the 25% ethanolic extract and the aqueous extract had higher α-glucosidase inhibitory activity than the others. Besides, these two extracts had higher total phenolic compounds. Thus, it can be concluded that the more available total phenolic compounds are in an extract, the more α-glucosidase inhibitory activity results, probably because phenolic compounds in different propolis extracts have different bound modes with α-glucosidase [37].

GC-MS Analysis

Silici *et al*., 2005 reported that 9-octadecanoic acid, hexadecenoic acid, benzoic acid, 3- hydroxy- 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, benzyl benzoate, benzene ethanol, β-eudesmol, β-bisabolol, glycerin, 2-nonadecanone, 2-propen-1-one, 4H-1-benzopyrane-4-on, 2-propenoic acid, heneicosane, and eicosane were presented in all of the samples of the Turkish propolis [16]. Iranian Propolis showed the presence of 9-octadecanoic acid, hexadecenoic acid, 3- hydroxy- 4-methoxy cinnamic acid, 2-propen-1-one, 4H-1-benzopyrane-4-on, and 2-propenoic acid. Cheng *et al*., 2013 studied Chinese propolis that 78 compounds out of 99 different volatile compounds belonged to unlike chemical classes, including acids (6), esters (8), alcohols (10), terpenes (31), olefins (3), and aromatics (20). other volatile compounds such as 3-methyl-2-butenal and benzaldehyde were classified into other chemical groups [28]. Iranian propolis demonstrates 68 volatile compounds, including alcohol (10), aromatic acid (14), aliphatic acid (5), fatty acid (6), terpene (5), pyran (8), ester (10), and others (10).

Ezzat *et al*., 2019 described Egyptian propolis with 42 compounds, and fatty acid and alcohol are the predominant components. Hence, the most biologically active components encompass hexadecenoic acid with free radical scavenging activity, and hexadecenoic acid-ethyl ester anti-inflammatory activity [40]. Iranian propolis showed the presence of hexadecenoic acid in both samples and hexadecenoic ethyl esters in the Mazandaran sample. Additionally, in another study Propolis from Osmaniye, Turkey is notable for its high content of unsaturated fatty acids (PUFA and MUFA) and its UFA/SFA ratio, along with its FRAP values and phenolic compounds like pinocembrin, pinobanksin, and p-coumaric acid, indicating numerous health benefits. The geographic origin greatly influences the biochemical properties of propolis samples due to variations in environmental conditions, bee species, plant coverage, flower types, and seasonal changes [32].

Ramnath *et al*., 2015 reported that the chemical composition of Indian propolis includes significant amounts of carboxylic acids and their derivatives, such as hexadecenoic acid, cis-vaccenic acid, and ethyl oleate. Additionally, it contains terpenoids like α-amyrin and lupeol, known for their aromatic properties and use in herbal medicine as antibacterial agents and for other pharmaceutical properties. Indian propolis is also rich in flavonoids, such as chrysin, which contribute to its antioxidant and antimicrobial activities, as well as its overall pharmacological benefits [41]. All these constituents are also present in the studied Iranian propolis samples.

Mohamed *et al*., 2020 reported that the Malaysian stingless bee propolis demonstrated 28 compounds with the predominance of sesquiterpenes. Cyclohexane such as 1-ethenyl-1-methyl-2,4-bis (1-methyl ethenyl)-, [1S-(1.alpha,2.beta,4.beta.)] presented in this type of propolis is reported to have high anti-inflammatory properties, and its richness in triterpenoids such as α-amyrin, and β-amyrin is associated with anti-cancer properties [42]. The Cyclohexane, 1,3-dichloro-, trans is presented in the Mazandaran sample, and the Khuzestan sample shows the presence of α-amyrin and other terpens. Although it can be inferred that this propolis has these therapeutic activities, further investigation through other chromatography methods such as HPLC and LC-MS is needed to prove this claim.

The physicochemical characterization of each propolis sample enabled the identification of each sample based on its geographical origin. This origin is linked to various pollen sources and distinct sensory characteristics. Dias *et al*., 2012 studied the physicochemical properties of various Portuguese propolis samples. The study categorized propolis into two groups: Hot Land and Cold Land. Samples from Hot Land showed high pH values, a higher total amount of soluble substances, greater moisture percentage, and higher conductivity. Conversely, samples from Cold Land had higher values of ash and wax and were rich in phenolic and flavonoid compounds [43].

Chewing Gum Sensory Evaluation

Many studies have been conducted on chewing gum's health effects, demonstrating numerous benefits. Thus, medicated chewing gum (MCG) can be a convenient systematic drug delivery agent because it of be administered, is waterless, and conveys a pleasant taste [22,29].

Chewing gums are mainly made up of gum base, sweetener, and flavor. Therefore, different ingredients play different roles in chewing gum's sensory features. Although little flavor is added to a gum base in comparison to other ingredients, it plays a paramount role in sensory features as well as texture because generally, it impacts gum base softness [29]. Recent studies suggest that propolis toothpaste and mouth rinse have anti-bacterial activity against pathogens of gingivitis, acting as a preventative since propolis limits the formation of dental plaque by slowing down the formation of calcium phosphate precipitation. Propolis has therapeutic agents as well. The propolis anti-bacterial effects originate from its flavonoid, phenolic acids, and esters [44].

CONCLUSION

This study confirms that both propolis samples were most effective against all gram-positive bacteria strains (*S. mutans* was the most vulnerable bacteria against EEP). Interestingly, both EEPs showed good inhibitory activity against *E. coli,* even though it is a gram-negative bacteria strain. Keeping this in mind, Khuzestan EEP demonstrates more antibacterial activity than Mazandaran EEP. Both EEPs inhibited the α-glucosidase enzyme, but Khuzestan EEP was more effective in inhibiting the enzyme as this sample had a lower IC50 range at α-glucosidase inhibition in comparison to Mazandaran EEP. As previously discussed, both EEPs scavenged the free radicals in antioxidant activity assay and the results confirmed the fact that Khuzestan EEP had the highest range of free radical scavenging compared to that of Mazandaran. As anticipated, both tests showed the commonplace pattern of the 'Poplar' propolis. They carry high concentrations of pinocembrin, chrysin, caffeic acid, 3- hydroxy-4-methoxy cinnamic acid, fatty acid, etc. Last but not least, sensory evaluation of Khuzestan's different propolis demonstrated that the most desirable propolis concentration was chewing gum with a 3% propolis concentration. It could be good news for food companies since using high concentrations of propolis as a functional food led to numerous health effects. Further studies can be conducted to discern the propolis type based on plant bases. Considering its countless health effects, Khuzestan propolis can be used as an essential compound in a dietary supplement, shelf-life extender, and functional food.

Ethical Statements and Declarations

The data that support the findings of this study are available from the corresponding author.

The authors declare that they do not have any conflict of interest.

This study does not involve any human or animal testing.

Written informed consent was obtained from all study participants.

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All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Negin Assaie Ardakani. The first draft of the manuscript was written by Negin Assaie Ardakani, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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