Phytochemical Composition of Iraqi Propolis and its effect on some Microorganism

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Abstract

The study was aimed at antibacterial activities of propolis against (gram+ve), (gram-ve) bacteria. Propolis extract was obtained by 70% ethanol and serial dilutions of 10.96, 5.48, 2.74, 1.37, 0.68, 0.34, 0.17, 0.085, 0.043, and 0.0215 mg/ml prepared using disc diffusion method and MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration). The chemical composition of the propolis was also investigated using thin layer chromatography (TLC) and Bioautography analysis of the TLC plates identified fractions with inhibitory activity. Ethanol extract of propolis showed more activity against Gram-positives *(S. aureus , S. epidermidis , B. subtilis , B. cereus*) than Gram-negatives *(E.coli , S. enteritidis , K. pneumoniae*, *P.vulgaris*). Thin layer chromatography screening revealed the presence of bactericidal flavonol galangin, kaempferol, naringenin, apigenin and caffeic acid in propolis. The total flavonoid and phenolic contents were 23.87% and 31.25 %, respectively, The findings suggest that propolis is a very effective antimicrobial agent may be due to high levels of phenolic and flavonoid compounds.

المكونات الكيميائية لصمغ النحل العراقي وتأثيره في بعض الجراثيم

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الخلاصة

كان هدف الدراسة معرفة الفعالية المضادة لصمغ النحل ضد البكتيريا الموجبة والسالبة لصبغة كرام، حضر مستخلص صمغ النحل 70% إيثانول وبالتخافيف العشرية 10.96، 5.48، 2.74، 1.37، 0.68، 0.34، 0.17، 0.085، 0.043 و0.0215 ملغم/ مل باستخدام طريقة انتشار القرص وMIC (الحد الأدنى من التركيز المثبط) وMBC (الحد الأدنى من التركيز المبيد للجراثيم). كما تم التحقق من التركيب الكيميائي لصمغ النحل باستخدام (TLC) طريقة استشراب الطبقة الرقيقة والتحليل الحيوي الذاتي Bioautography لتحديد العناصر ذات الفعالية المثبطة للجراثيم. أظهر مستخلص صمغ النحل بالايثانول فعالية عالية ضد الجراثيم الموجبة لصبغة كرام *(S. aureus , S. epidermidis , B. subtilis*, *B. cereus*) أكثر من الجراثيم السالبة (*E.coli , S. enteritidis , K. pneumoniae*, *P.vulgaris*). وكشف فحص استشراب الطبقة الرقيقة عن وجود المركبات المبيدة للبكتريا flavonol, galangin, kaempferol, naringenin, apigenin and caffeic acid في صمغ النحل. والمحتوى الكلي من الفلافونويد والفينول كان 23.87٪ و31.25 ٪ على التوالي، وتشير النتائج إلى أن صمغ النحل له فعالية عالية جدا ضد الأحياء المجهرية وقد يعود السبب إلى المستويات العالية من مركبات الفينول والفلافونويد.

Introduction

Propolis is a natural hive product with a complex chemical composition, consisting of mixture of balsams (resins), beeswaxes, oils, and pollen. Propolis has been used in folk medicine for many years, and there is substantial evidence indicating that propolis has antimicrobial, anti-inflammatory, antioxidant, immunomodulatory- properties etc.(1,2). Raw propolis is rich in biochemical constituents, including mostly a mixture of 50% resin (polyphenolic fraction), 30% wax, 10% essential oils, 5% pollen and 5% various organic and mineral compounds (3). Chemical composition of propolis is very complex and it has been identified more than 200 compounds. Its biological activities depend on a large number of polyphenols (resin), mainly flavonoids (flavonoid aglycones), aromatic acids, phenolic acid esters (caffeates and ferulates), triterpens, diterpenic acids and lignanes (2,3). Flavonoids, as one of the main group of polyphenolic compounds in propolis are aglycones (without sugar component) because bees during collecting propolis mix it with enzymes (ß-glucosidases) of hypopharingeal glands. These lipophilic flavonoids are chemically derived in subgroups of flavones, flavanones, flavonols, dihydroflavonols, isoflavones and chalcones (4,5).These groups of compounds are reported to have bactericidal (6,7),fungicidal and antiviral (8), antiprotozoal (6), antioxidant (9), anti-inflammatory (10) activities. The medicinal and antimicrobial properties of propolis have been widely reported and have a long history (11) due to the increasing rate of antibiotic resistances by most bacteria. Therefore, the antibacterial activity of propolis, a product from honey bee, which has been reported to act against *Escherichia coli, Salmonella enteritidis , S. aureus, S.epidermidis, Bacillus subtilis, Bacillus cereus, Klebsiella pneumoniae, Proteus vulgaris* among others, is reported in this study for the possible use of propolis for the treatment and control of many infection and to analyze its chemical compositions.

Material and Methods

1. Extraction of propolis: Propolis were collected from honeybee hives during the months of December, 2008 to January, 2009 from Baghdad apiary site and were kept until processed. The whole sample of propolis (30g) was frozen, ground and homogenized prior to beginning extraction. The methods of (12,13) were used. During extraction, propolis was ground to a fine powder and 2 g (dry weight) was mixed with 25 ml of 70% (v/v) ethanol and shaken in volumetric flask for 30 min. After extraction, the mixture was centrifuged and the supernatant was evaporated to produce the ethanolic extract of propolis (EEP) which was prepared at 1% with 70% (v/v) and the filtrate diluted to 100 ml with 70% ethanol in a volumetric flask.

2. Microorganisms: The microorganisms were used in this study to test antimicrobial activity of propolis. *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Bacillus cereus, Salmonella enteritidis, Escherichia coli, Klebsiella pneumoniae, and proteus vulgaris*, All microorganisms were provided by College of Veterinary Medicine, Baghdad University Medicine and Microbiology Department.

3. Preparation of inoculums: All bacteria were cultured for 8 h at 37º C in a liquid medium (brain heart infusion) and used as inoculums. The turbidity of the suspension was adjusted to the McFarland 0.5 turbidity standard.

4. Antimicrobial activity of propolis: The disc diffusion method was used as described (14). Briefly, a small single well isolated colony grown in Muller Hinton Broth (MHB, Merck) medium at 37°C for 24 h. Final inoculum bacterial number were adjusted to 10^8 cfu mL-1 with reference to the Mc Farland turbidometry. A sterile cotton swab with the adjusted suspension was used to evenly spread the entire surface of the Mueller- Hinton agar (Biotec Lab Ltd, UK) plates to obtain uniform inoculums. The plates were dried

for 2 - 4 min. Ten concentrations of ethanolic Propolis extracts (10.96, 5.48, 2.74, 1.37, 0.68, 0.34, 0.17, 0.085, 0.043 and 0.0215 mg/ml) were prepared. The sterile filter paper discs (6 mm diameter) were saturated through adding 50 µL of different concentrations of extracts. Then, impregnated disc were applied to the surface of inoculated plates with sterile forceps, ensuring complete contact of disc with agar. The plates were incubated at 37°C for 18 h. and examined for zones of complete inhibition to the nearest mm. As positive controls, discs containing different concentrations of seven antibiotics including nafcillin 1µg, colistin 10µg, doxycycline 30µg, novobiocin 30µg, carbenicillin 100µg, methicillin 5µg and oxacillin 1µg were used. All these synthetic antibiotics were produced by Difco. Resistance and sensitivity to ethanolic Propolis extracts was measured by the method of (15). When the antibiotic agent was 16 mm or higher, it was recorded as sensitive and resistant when less than 16 mm.

5. MIC and MBC determination: MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) of ethanolic extracts of Propolis was determined against *S. aureus*, *S. epidermidis*, *B. subtilis*, *B. cereus*, *S. enteritidis*, *E. coli*, *K. pneumoniae* and *p. vulgaris*. MIC was determined by macro broth dilution assay method (16). In the tube dilution assay, standard bacterial suspension was added to tubes containing 1 mL MHB (Muller Hinton Broth) and different concentrations of extracts (10.96, 5.48, 2.74, 1.37, 0.68, 0.34, 0.17, 0.085, 0.043 and 0.0215 mg/ml). The tubes were incubated at 37°C for 24 h. The first tube in the above series with no sign of visible growth was reported as the MIC. MBC was determined by culturing one standard loop of the tubes showing no apparent growth on MHA and subsequent incubation at 37°C for 24 h. The least concentration that was inhibited no colony formation on agar assumed as MBC for these extracts.

6. Thin layer chromatography analysis: The preliminary analysis was conducted with TLC according to the method developed by (17). The TLC plate used was Silica gel 60 F254 coated in aluminum. The EEP sample was dissolved in 96% Ethyl alcohol and the aliquots were applied to the plates with a micropipette. Two mobile phases were used, containing different concentrations of toluene, ethylacetate and formic acid: (5:4:1, V/V/V) and (3.6: 1.2: 1.5, V/V/V) (18).The mobile phase used for the TLC in the present sample was: Toluene: Ethyl-acetate: Formic acid (5:4:1V/V/V) because of its best resolution. The TLC chamber was saturated with the mobile phase at least 1 hour before analysis. The developed plates were air dried and heated for 10 minute at 110 Co to facilitate the development of spots. The phenolic acids and flavonoids were visualized under long (366 nm) and short (254 nm) UV lights before and after spraying with reagents 1% (W/V) methanolic solution of diphenylboric acid aminoethyl ester followed by 5% (V/V) ethanolic solution of polyethylene glycol 4000. For qualitative determination of flavonoid aglycones in PEEs, standard solutions of galangin, chrysin, kaempferol, rhamnetin, apigenin quercetin and naringenin were used as 0.05% (W/V) solution in 70% ethanol. The position of the spots on the TLC plate was expressed as the retention factor (Rf), the distance the components traveled divided by the distance the solvent traveled from the base(19).

7. The Bioautography: Bioautography was carried out after airing the TLC plates for over 8 hours. The plates were covered with 20 mL of sterile Mueller-Hinton agar at 45ºC inoculated with the saline suspension of *S. aureus*, and *E.coli* then incubated for 24 hs at 37ºC. After this period each plate was covered with 5 mL of a 1% aqueous solution of 2,3,5 triphenyltetrazolium chloride and incubated for up to 24 hours at 37ºC. Inhibition zones were visualised as clear areas against a red coloured background. Preparative TLC plates with a thickness of 1 mm were prepared using the same

stationary and mobile phases as above, with the objective of isolating the components of propolis that inhibited the growth of *S. aureus*, and *E.coli*.

8. Estimation of total flavonoids content: The measurements were carried out UV-Visible spectrophotometer. The content of flavonoids was determined by two independent colorimetric methods for deterimination of flavones, flavonols and isoflavones and for determination of flavanones. (20)

- **Aluminum Chloride Colorimetric Method:** This was modified from the procedure reported by (20). Quercetin was used to make the calibration curve. 10 mg of quercetin was dissolved in 80% ethanol and then diluted to 25, 50 and 100 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with spectrophotometer (Kyoto, Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 mL of ethanol extracts or 10 flavonoid standard solutions (100ppm) were reacted with aluminum chloride for determination of flavonoid content as described above.
- **2,4-Dinitrophenylhydrazine Colorimetric Method:** (±)-Naringenin was used as the reference standard. 20 mgrs of (±)-naringenin was dissolved in methanol and then diluted to 500,1000 and 2000 µg/mL. (1) milliliter of each of the diluted standard solutions was separately reacted with 2 mL of 1% 2,4-dinitrophenylhydrazine reagent and 2 mL of methanol at 50°C for 50 min. After cooling to room temperature, the reaction mixture was mixed with 5 mL of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then, 1 mL of the mixture was taken, mixed with 5 mL of methanol and centrifuged at 1,000 x g for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 mL. The absorbance of the supernatant was measured at 495 nm. The ethanol extracts of propolis and 15 flavonoid standard solutions (1000 ppm) were similarly reacted with 2,4-dinitrophenylhydrazine for determination of flavonoid content as described above.

Table (1) Systematic names of the 10 flavonoid standards used in the present study

Flavonoids	Systematic name
Flavones	
chrysin	5,7-dihydroxyflavone
apigenin	4’,5,7 -trihydroxyflavone
luteolin	3’,4’,5,7 –tetrahydroxyflavone
Flavonols	
quercetin	3,3’,4’,5,7-pentahydroxyflavone
myricetin	3,3’,4’,5,5’,7-hexahydroxyflavone
kaempferol	3,4’,5,7-tetrahydroxyflavone
quercitrin	3,3’,4’,5,7-pentahydroxyflavone-3-Lrhamnopyranoside
galangin	3,5,7-trihydroxyflavone
Flavanones	
naringenin	4’,5,7-trihydroxyflavanone
Isoflavones	
daidzein	4’,7-dihydroxyisoflavone

9. Estimation of total phenolics content: Total phenolic content the Folin-Ciocalteu method (21) was used to determine the quantities of total phenolics. A calibration curve was built using standard aqueous solutions of phenol containing between 2 and 24 ug /mL. One mL of each solution was added to 250 uL of sodium carbonate-tartarate buffer and 25 uL of the Folin–Ciocalteau reagent in a test tube, homogenized and allowed to

react for 30 minutes at a temperature of 20 °C. Absorbance was measured at 700 nm on a spectrophotometer and the calibration curve calculated by the minimal squares method. The dry extracts of propolis were dissolved in absolute alcohol to a concentration of 20% (w/v), one mL of this ethanolic solution was further diluted in 1000 mL of distilled water and homogenized. One mL of this final solution was prepared and analyzed in the same way as the standards. The results are given as a percentage of the dry extract in weight. This method depend on oxidation-reduction reaction in alkaline conditions, where the phenolate ion was oxidized while Folin's reagent was reduced, turning the solution blue.

- Statistical analysis: The data were analyzed using one-way ANOVA. When appropriate, independent student t-test was used. P value less than 0.05 was considered statistically significant. All data were expressed as mean ± standard error of the mean (M ±SEM).(22)

Results

The results indicate that ethanolic extracts of propolis has highly antibacterial activities against Gram-positive compared with the Gram-negative bacteria. The highest inhibition zone (20.0 ± 0.12 mm 18.0 ± 0.11 mm, 16.0 ± 0.12 mm, 15.0 ± 0.10 mm) was recorded from propolis at the concentration of 10.96 mg/ml followed by 5.48 mg/ml against *S. aureus, B. subtilis, S.epidermidis, B. cereus* respectively in disc diffusion method (Fig. 1). On the contrary higher inhibition zone (16 ± 0.11 mm, 14.0 ± 0.12 mm, 12.0 ± 0.11 mm, 11.0 ± 0.11 mm,) was recorded from propolis at the concentration of 10.96 mg/ml followed by lower to lowest concentrations against *E. coli, S. enteritidis, K. pneumoniae, p.vulgaris* respectively (Fig. 2). The minimum inhibitory concentration of propolis against *S. aureus* and *E. coli* are presented in (Tables 2 and 3). Growth and no growth tubes were identified comparing to the turbidity of the positive control. The negative growth was observed in propolis at concentrations of 0.68 to 10.96 mg/ml for *S. aureus, B. subtilis* and 1.37 to 10.96 mg/ml for *S.epidermidis , B. cereus* while growth was observed at concentrations of 0.34 mg/ml followed by lower concentrations. (Table 2). In case of *E. coli , S. enteritidis , K. pneumoniae, p.vulgaris* negative growth was observed in propolis at the concentration of 2.74 to 10.96 mg/ml while growth was observed at lower concentrations. (Table 3). The MBC of propolis against *S. aureus, B. subtilis ,S.epidermidis , B. cereus* and *E. coli , S. enteritidis , K. pneumoniae, p.vulgaris* grown in TSA plate are presented in Table 2. TSA plates (spot plate) streaked from no growth (negative growth) tubes showed no colonies while streaked from positive tubes showed colonies of Gram- positives and Gram-negatives bacteria. The result of MBC value of the propolis against Gram- positives and Gram-negatives bacteria was exactly similar compared to MIC value. Ethanolic extracts of propolis was more effective than all antibacterial standard doxycycline, carbenicillin, and novobiocin on *S. aureus, S. epidermidis , B. cereus, B. subtilis, E.coli , S. enteritidis, K. pneumoniae* and *P.vulgaris* strains, with significant differences ($P < 0.01$) (Table 4). All of the tested bacteria were resistant to oxacillin and most of them presented resistance to nafcillin and methicillin.

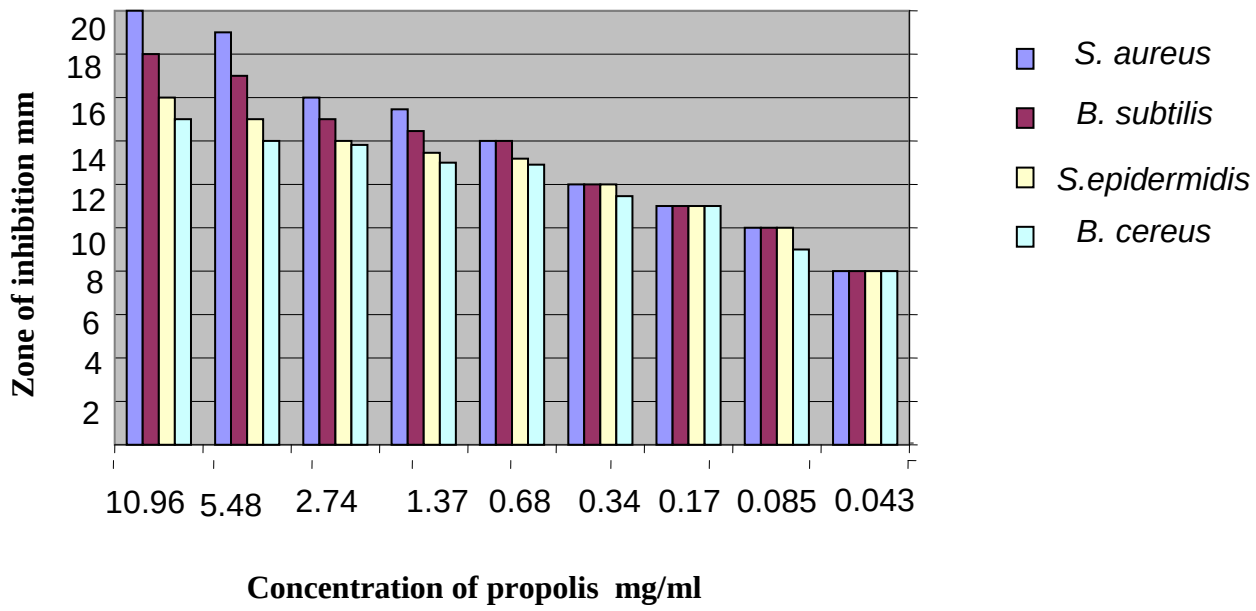

Fig. (1). Zone of inhibitory (mm) of propolis against Gram-positives.

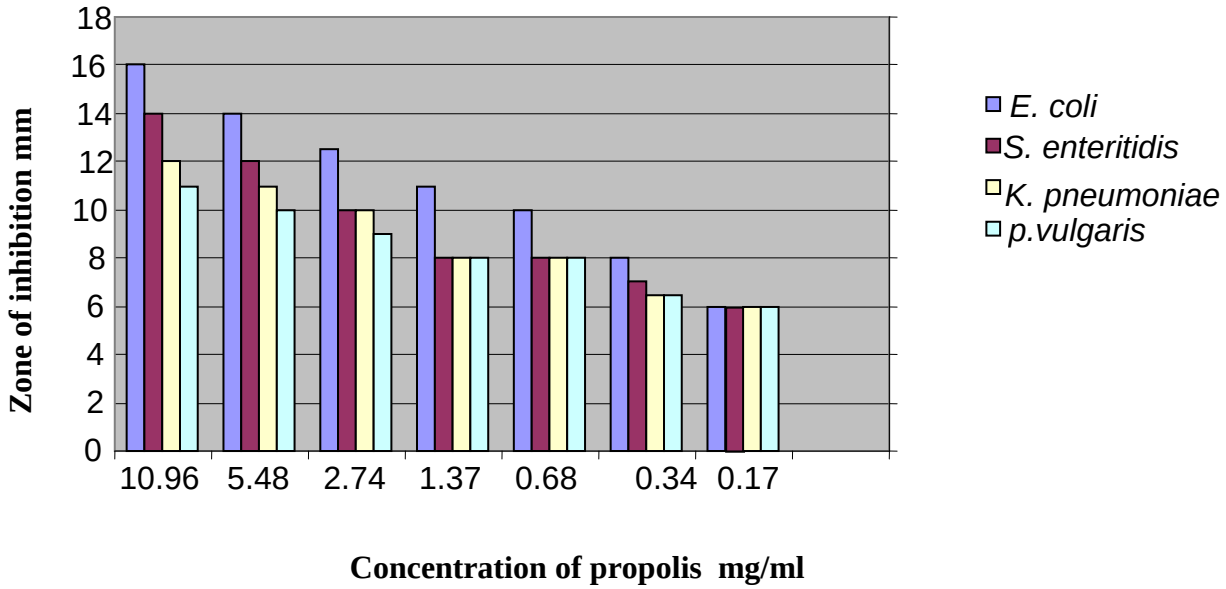

Fig. (2) Zone of inhibitory (mm) of propolis against Gram-negatives.

Table (2) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of propolis against Gram-positives

Dilution	Conc. (mg/ml) Propolis	MIC				MBC			
		S. aureus	*S. epidermidis*	*B. subtilis*	*B. cereus*	*S. aureus*	*S. epidermidis*	*B. subtilis*	*B. cereus*
1/2	10.96	-	-	-	-	-	-	-	-
1/4	5.48	-	-	-	-	-	-	-	-
1/8	2.74	-	+	-	-	-	+	-	-
1/16	1.37	-	+	-	+	+	+	-	+
1/32	0.68	+	+	+	+	+	+	+	+
1/64	0.34	+	+	+	+	+	+	+	+
1/128	0.17	+	+	+	+	+	+	+	+
1/256	0.085	+	+	+	+	+	+	+	+
1/512	0.043	+	+	+	+	+	+	+	+

(-) represents inhibition; (+) represents growth

Table (3) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of propolis against Gram- negatives

Dilution	Conc. (mg/ml) Propolis	MIC				MBC			
		E.coli	*S. enteritidis*	*K. pneumoniae*	*P. vulgaris*	*E.coli*	*S. enteritidis*	*K. pneumoniae*	*P. vulgaris*
1/2	10.96	-	-	-	-	-	-	-	-
1/4	5.48	-	-	-	-	-	-	-	-
1/8	2.74	-	+	+	+	-	-	+	+
1/16	1.37	+	+	+	+	+	+	+	+
1/32	0.68	+	+	+	+	+	+	+	+
1/64	0.34	+	+	+	+	+	+	+	+
1/128	0.17	+	+	+	+	+	+	+	+
1/256	0.085	+	+	+	+	+	+	+	+
1/512	0.043	+	+	+	+	+	+	+	+

(-) represents inhibition; (+) represents growth

Table (4) The mean of the diameters1 (mm) of microbial growth inhibited by different concentrations of different antibiotics

Microorganism	Nafcillin 10 µg	Doxycycline 30 µg	novobiocin 30 µg	Carbenicillin 100 µg	Methicillin 5 µg	Oxacillin 1 µg
S. aureus	8	10	8	8	8	8
S. epidermidis	10	8	10	14	9	8
B. subtilis	12	12	8	8	8	8
B. cereus	8	8	10	14	8	8
S. enteritidis	8	10	8	12	8	8
E. coli	12	12	14	10	8	8
K. pneumoniae	8	8	10	10	8	8
p. vulgaris	8	10	12	8	8	8

Values expressed are averages of three replicates.

The TLC screening of phytochemical the ethanol extract of propolis (EEP) showed contained bactericidal flavonol galangin, kaempferol, naringenin, apigenin and caffeic acid (Table 5,6). These were indicated by spots of different colors at different distances on the TLC plate upon spray by chemical reagents as shown in (Fig. 3).

Table (5) TLC analysis of Ethanolic extracts of propolis

RF	Color of fluorescence (365 nm)	galangin	kaempferol	naringenin	apigenin	caffeic acid	not identified components
0.88	light green	-	-	-	-	-	+
0.83	dark brown	-	-	-	-	-	+
0.72	greenish blue	+	-	-	-	-	-
0.67	dark brown	-	-	-	-	-	+
0.59	Yellow	-	+	-	-	-	-
0.55	yellowish brown	-	-	+	-	-	-
0.50	yellowish green	-	-	-	+	-	-
0.44	blue	-	-	-	-	+	-

Notable fluorescence (+), no fluorescence (-)

Rf **= distance of spot from the base**
Distance the solvent moved from the base

Table (6) *Rf* values of standard solutions used in TLC analysis of propolis ethanolic extracts

Symbol	Compound	*Rf* value	Colour
1	Caffeic acid	0.43	blue
2	galangin	0.75	Greenish -blue
3	Apigenin	0.51	Green
4	naringenin	0.59	yellow-brown
5	Kaempferol	0.44	yellowish

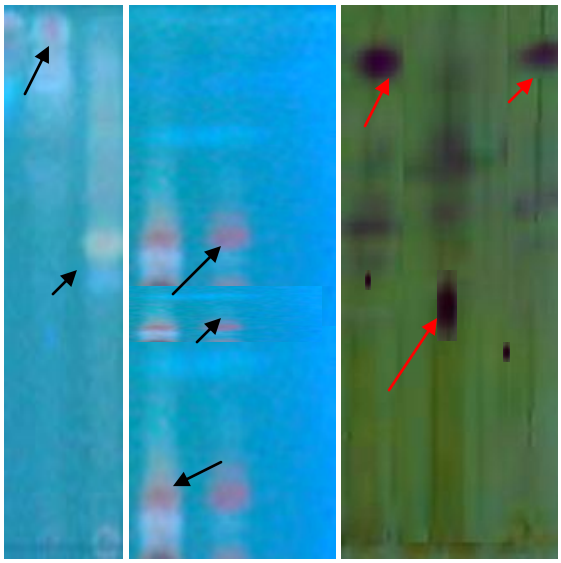

Fig. (3) TLC Pattern of EP

Bioautography of the TLC plate (Fig. 4) shows a large area containing substances that inhibited the growth of *S. aureus* and *E.coli* over the region containing the components with high and medium polarity.

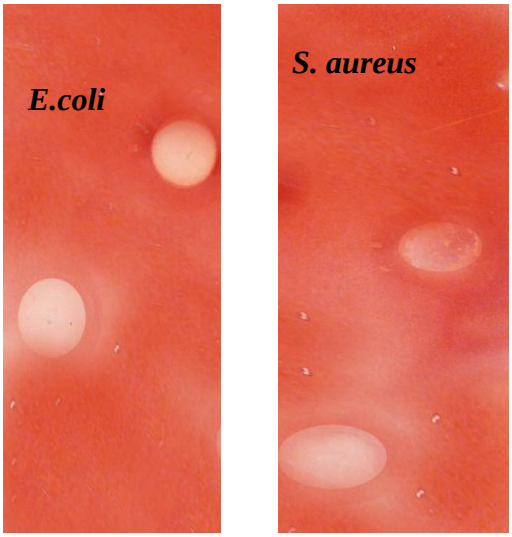

Fig. (4) Bioautography of TLC plate of EEP indicate zone inhibition growth of *S. aureus* and *E.coli after covered with* triphenyltetrazolium chloride

Flavonoid contents of raw propolis sample determined by aluminum chloride colorimetric method were generally lower than those determined by 2,4-dinitrophenylhydrazine colorimetric method. The formers 5.37 ± 0.05% while the letters 18.50 ± 0.17 % (Table 7).

Table (7) The flavonoid contents of raw propolis samples determined by aluminum chloride and 2,4-dinitrophenylhydrazine colorimetric methods

Sample	Flavonoid content (%)[a]		
	AlCl3[b]	2,4-D[c]	Total
propolis	5.37 ± 0.05	18.50 ± 0.17	23.87 ± 0.22

a: Results were presented as mean ± SD (n=3).

b: Flavonoid content (%) = quercetin equivalent (µg/mL) × total volume of ethanol extract (mL) ÷ sample weight (g) × dilution factor × 10-6 (g/µg) × 100.

c: Flavonoid content (%) = naringenin equivalent (µg/mL) × total volume of ethanol extract (mL) ÷ sample weight (g) × dilution factor ×10-6 (g/µg) × 100.

Propolis extracted yield the percentage of total phenolic content about 31.25 ± 1.52 of the extract,(Table 8). (Fig. 5) shows the standard graph that was used to determine total phenolics as described in analytical procedure. The figure also shows the regression equation, correlation index (r) and standard deviation (SD.) for the curve.

Table (8) Phenolic content of Propolis extracted

sample	yield (PE) %	Total phenolics a(mg/g)
Propolis	**34.12 ± 1.42**	**31.25 ± 1.08**

a: Results were presented as mean ± SD (n=3).

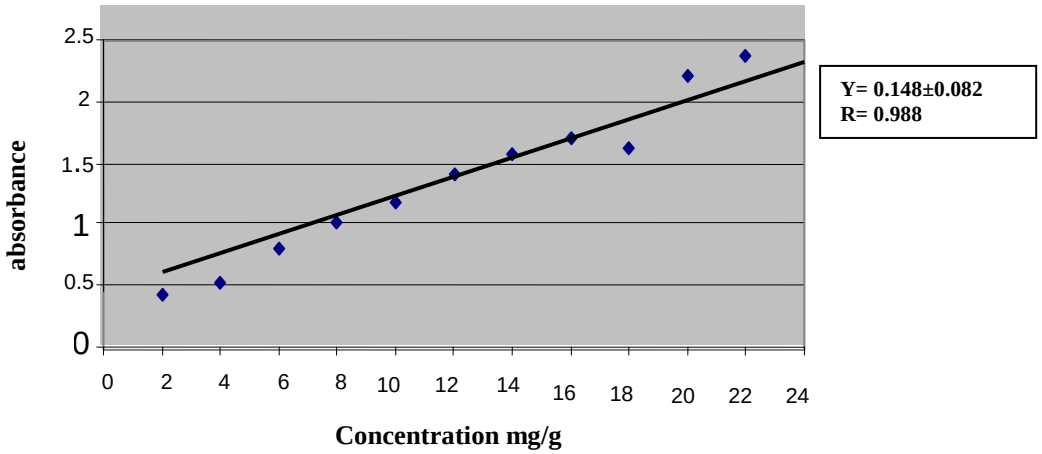

Fig (5) Standard curve for determination total phenolics by Folin-Ciocalteau method

Discussion

The results of the antimicrobial activity of popolis showed that all the bacterial were sensitive to EP at different concentrations. Propolis at low concentration was effective to inhibit Gram-negatives bacteria and significant response was noticed in disk diffusion assay compared with Gram-positives bacteria similar results were reported by(23). Larger inhibition zones were verified for the Gram-positive bacteria *S. aureus* and *B. subtilis*, compared with the Gram-negative *E. coli* and *S. enteritidis*. Among the Gram-positive bacteria, *S. aureus* had larger inhibition zones than *B. subtilis* but such difference was not statistically significant. The antibacterial effect of EP on *S. aureus* , *B. subtilis* ,*S. epidermidis* and *B. cereus* agreed with those reported by other authors. (24,25,26) among others, have reported that *S. aureus* is susceptible to propolis effects. Varied inhibition zones by EP have also been reported: 10–12mm by (27), 13 mm by(28), suggesting there is variability in the biological activity of EP, depending on its botanical origin and thus on its chemical composition. The antimicrobial activity of EP was demonstrated by (26) against *B. subtilis* and (29) against *Salmonella* sp. In Gram-negative bacteria, the highest antibacterial activity was recorded for *E.coli* compared with *S. enteritidis, K. pneumoniae, and P.vulgaris*. However, the differences in their inhibition zones diameters were not statistically significant. There are conflicting data on the susceptibility of *E. coli* to EP. (26,29) reported that EEP were ineffective against *E. coli*. on the other hand, (30,31) reported complete or minimal susceptibility. Grange and Davey (32) showed *P.aeruginosa* inhibition by EEP. The present results allow the conclusion that Gram-positive bacteria (*S. aureus, B. subtilis, S. epidermidis and B. cereus*) are more susceptible than Gram-negative bacteria (*E. coli, S. enteritidis, K. pneumoniae, and P.vulgaris*) to propolis. These findings agree with earlier reports

by(33). However, susceptibility of Gram-negative bacteria to EP is still an important subject for further investigations. It can also be concluded that the extraction procedures determine the EP antibacterial activity. This result may be due to the non-abuse of propolis by patients. Propolis is costly to buy and therefore is not within reach by low income earners and its antimicrobial activities have not been fully exploited and abused by both patients and healthy individual in the study area. The major selective force favouring the emergency of antibiotics resistance is their extensive use ether due to their low cost or personal rescriptive. The MIC results from propolis of the present study observed that (1.37 , 2.74 mg/ml) is effective against *S. aureus* and *E.coli* respectively. Similar MIC results from propolis (2.01 to 3·65 mg/ml) was obtained by (23,33) investigated propolis against *S. aureus* where MIC values ranged from 0.080 to 0.100 mg/ml. These MIC results are different than the MICs found to be active in this study because of different methodologies to determine antibacterial activity. Propolis against *S. aureus* was studied by agar dilution method and found average MIC was 22.5 mg/ml(34). Propolis is active against Gram-positive bacteria, showing limited activity against Gram-negative ones (35). From the above results it appears that *E. coli* is more resistant than *S. aureus* against propolis. *E. coli* is considered a particularly dangerous pathogen because of its resistance to many commonly used antibiotics. Therefore, to prevent contamination from *E. coli* higher concentrated propolis or more effective disinfectant/ antibiotic should be applied. *E. coli* is a gram negative bacteria and it is notorious for its resistance to many antibiotics due to the permeability barrier afforded by its(26). The effectiveness of antibiotic properties of propolis found that was equal to or slightly more effective than common antibiotics, Nafcillin, Doxycycline, novobiocin and Carbenicillin, in killing gram-positive and gram-negative bacteria and all test bacteria are resistance to Methicillin and Oxacillin. Similar results were reported by(27). Thin layer chromatography (TLC) analysis and bioautography was observed mainly because of the presence of most active compounds such as galangin, kaempferol, naringenin, apigenin and caffeic acid which a higher inhibitory activity against *S. aureus* (36). (23) suggested that effectiveness of propolis depends on differences in chemical composition, bee species and geographic region. Estimation of Flavonoid and phenolic contents showed the presence of a high content of polyphenols and flavonoids in alcoholic extracts of propolis 23.87 ± 0.22, 31.25 ± 1.08 respectively is associated with significant microbial activity (32, 28). These results indicate that the antimicrobial activity of EP against Gram--positive and negative bacterial strain does not depend upon the concentration of particular flavonoids but on the synergistic effect of all phenolic compounds. Future researches should be focused on establishing the possible synergistic antimicrobial activity of individual flavonoids and phenolic acids. Also, the relation between the chemical structures of flavonoids and phenolic acids and their antimicrobial activity on different bacterial species and yeasts should be investigated.

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