Antimicrobial and Antioxidant Activities of Propolis Water and Ethanolic Extracts

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ABSTRACT

For consumers, natural, healthy food should be used as a natural additive, replacing chemical additives. Propolis is one of the natural substances that can fulfill that role. Propolis contains bioactive compounds with antimicrobial and antioxidant capacity. The antimicrobial and antioxidant action of two different of propolis extracts: (i)Water extract of propolis (WEP) and (ii) Ethanolic extract of propolis (EEP). It was found that WEP has no antimicrobial effect on the tested undesirable examined microorganisms, while EEP varied in antimicrobial effect on them. The inhibition zone indicated that the high effect was found against *Pseudomonas aeruginosa* (21.36 mm) followed by *Streptococcus pyogenes* (16.24 mm), the lowest effect detected against *Listeria monocytogenes ATCC19116* (10.50 mm) and had a moderate effect on *Staphylococcus aureus NCTC 10788* (12.90 mm). The results of quantitative analysis of phenols and flavonoids in propolis extracts. WEP showed high content of gallic acid, p-hydroxy benzoic acid, catechin, syringic acid, benzoic acid, cinnamic acid and quercitin. On the other hand EEP was higher than WEP in p-coumaric acid, o-coumaric acid. It was clear that WEP had more antioxidant activity than EEP.

Key words: Propolis, Antimicrobial, Antioxidant.

INTRODUCTION

Recently, the vast majority of consumers have increased their wellness about the side effects of chemical components that are used in food preservatives which led the scientists to shift towards finding natural alternatives that can be used in food preservatives. There are diverse natural components that can be used in a state of chemical components such as essential oils as well as natural biopolymers. Natural biopolymers can serve as transporters for a variety of active ingredients, such as essential oils, to ensure their prolonged release to the food items throughout storage. They may also show potential for safeguarding food goods from oxidation and microbiological spoilage (Hromiš *et al.*, 2017).

Bees produce propolis to fill the cracks in hives and prevent honeycomb from microbial infections which helps the hives maintain homeostasis inside the hives. Additionally, propolis has been utilized by humans as a traditional medicine from 300 BBC till now (Yousef *et al.*, 2019), Propolis has so far been found to include more than 400 chemical components, including amino acids, aromatic acids, polyphenolic acids, essential oils, and waxes (Anjum *et al.*, 2019; Rojczyk *et al.*, 2020 and Santos *et al.*, 2020).

Burdock (1998), these components have been proven to support the antioxidants, antitumor, antimicrobial, anti-inflammatory, prebiotic, antiviral, and immunomodulatory, and also help the protection of the liver neurons, and heart. Propolis also promotes wound healing. Propolis type and geographic origin are directly related to its functional characteristics. Propolis has been widely used in the food and pharmaceutical industries because of its natural origins, low cost, and strong bioactivities (Veiga *et al.*, 2018 and Pobiega *et al.*, 2019). Propolis is the general word for the resinous substance that honeybees gather from diverse plants, while it is also occasionally referred to as "bee glue." This resin is chewed, salivary enzymes are added, and this substance is partially digested, then combined with beeswax and used in the hive. Bees make use of propolis, which they make, as a highly sticky resinous substance to smooth out the interior walls of their hives, seal any holes, and fortify the entrance against intruders. Although *Populus balsamifera* L. (and other Populus species) is frequently the source of the resin, the particular composition of raw propolis differs depending on the source. It is made up of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% different ingredients, including organic debris. Water washing and solubilization in 95% ethanol are used to remove wax and organic debris from raw propolis, resulting in propolis tincture, 'propolis balsam', or ethanol extract of propolis.

Over the centuries humans have bred bees so they use their products. The many beneficial characteristics of both raw and processed propolis lend to its use in a variety of human endeavors. Propolis has been used since at least 300 BC and is still used today in topical home medicines and personal care products, as well as an ingredient in toothpaste and dental floss (1± 5% of the total product), and as a health-food/dietary

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supplement (recommended dosage, 200 mg/day) (Braakhuis, 2019; El-Seedi *et al.,* 2020 and Santos *et al.,* 2020). Nowadays propolis sales in the United States are expected to be 40,000 lb/yr. Propolis is also noticed as a secondary ingredient in beeswax and extracted honey. Although ethanolic extract of propolis (EEP) is the most commonly used, extracts with various solvents have been created for ingredient identification. The majority of the components recovered from propolis tincture are flavonoid pigments, which are found across the plants, and the flavonoids isolated from propolis correlate relatively well with those found in the plants from which honeybees collect propolis (Anjum *et al.,* 2019; Rojczyk *et al.,* 2020; and Santos *et al.,* 2020).

This work was carried out to get more information about the antimicrobial and antioxidant properties of propolis water and ethanol extracts aimed to using it as food preservative.

MATERIALS AND METHODS

Materials:

- Two samples of propolis were used:

- Propolis (1) (Date of collection September – October 2018 Kafr El-Sheikh and the trees planted there were willow, camphor, poplar and blackberry) from which at a water extract was prepared.
- Propolis (2) (Date of collection September – October 2020 from Cairo – Alexandria Desert Road farms, and the trees planted there were citrus, olives, and casuarina windbreaks) from which an ethanolic extract was prepared.

Methods:

1. Water extract of propolis (WEP):

Water extract of propolis was obtained as described by Suzuki (1990) with slight modifications by Nagai *et al.* (2003) as follows: 20.0 g of propolis were suspended and extracted with 5 volumes of distilled water with shaking using shaker (Wise Shake ® Feedback Control Digital Program Function) at laboratory temperature (25 ºC) for 24 h. The extracts were centrifuged at 3000 g for 20 min., and the supernatants were taken. The residue was re-extracted under the same conditions. The obtained supernatants were combined and dialyzed against distilled water, and then dialysate was lyophilized by vacuum freeze dryer (model: FDF 0350; Korea).

2. Ethanolic extract of propolis (EEP):

The correct weight of propolis is measured and the correct volume of alcohol is measured. Becoming a dieter of alcohol. The specific gravity of pure ethanol is 0.794 compared to 1.00. Alcohols and other occasions therefore, weighing both propolis and solvent is the preferred method Krell (1996). As alcohol and propolis were poured into a bowl, closed the top and shaken it by using a stirrer at 160 revolutions per minute at room temperature for 24 h. Then, it was shaken once or twice a day during the period of leaving the mixture in the dark, but otherwise, the mixture was left in dark place for best results, propolis should be extracted for a week or two. Soaking for more than a week.

Then the alcohol is evaporated by placing the container in a warm water bath at a temperature of 40 ˚C to evaporate the alcohol. Some producers boil a mixture of alcohol and propolis for eight hours in order to dissolve all the resins. If the propolis contains wax, most of this will be dissolved by heating or must be removed prior to extraction. For a high-quality product. Then the liquid is filtered through a filter paper. For several hours or a day until filtering, better results are obtained. The filter must also be cooled before using it. The remains of the first filter can be washed or soaked in alcohol again. A clear, particle-free liquid filter of dark brown or slightly reddish color was obtained and kept in clean, dark, airtight containers of dark colour. The bottles should be kept in a cool dark place.

Ingredient for 10 % extract:

One part of propolis with nine parts of alcohol (1:9 w/w) or any multiple thereof.

3. Antimicrobial activity of propolis extracts:

Agar gel diffusion test: This method was adopted according to Grove & Randall (1955) and Kavanagh (1972), for assessing the antibacterial activity of the preparation extract. The microbiological examinations performed in aseptic conditions. During the microbiological study we determined the antimicrobial activity of the studied preparation, using solid growth media and the well technique. Resistance to preparation from natural material was examined in nutrient agar with standard cultures of *Klebsiella pneumoniac ATCC12296, Streptococcus pyogenes, Listeria innocuaa ATCC33090, Candida albicans ATCCMYA 2876, Yersinia enterocolitica ATCC23715, Staphylococcus aureus NCTC 10788, Pseudomonas aeruginasa, Escherichia coli BA12296 and Listeria monocytogenes ATCC19116.* Agar was poured into sterile petridishes 80 mm in dimeter (20 ml in eash dish) with 0.1 µl of 0.1 % of standardizaed bacterial stock conentration. The density of the bacterial suspension in the nutrient agar was 10^3 CFU/ml. Agar wells of 6 mm diameter were cut into solidified agar midia with the help of sterilized stainless steel borer . (0.1 µl of EEP10%) and (0.1 µl of WEP10%) was poured in the respective well and the plates were incubated at 37 ºC for for 24 h. The activity of extracts was determined by measuring the diameter of inhibition zone around each well by millimeter against the tested organism. The experiment was performed in triplicate under strict aseptic conditions.

4. Determination of total phenolic content in propolis extracts:

Measure the total phenolic content **(TPC)** in propolis extracts by using the Folin-Ciocalteu reagent (Singleton *et al.,* 1999 and Dewanto *et al.,* 2002). One mg of extract was dissolved in one ml of deionized water, and 500 µl of dissolved sample was mixed with a volume of 0.5 ml of distilled water and 0.125 ml of Folin-Ciocalteu reagent. The mixture was mixed and allowed to rest for 6 min before adding 1.25 ml of 7% Na_2CO_3. Distilled water was added to adjust the final volume to 3 ml and carefully mixed before incubating in the dark for 30 min and monitoring the absorbance at 650 nm versus the prepared blank. A standard curve was produced using various amounts of gallic acid (standard, 0-100 g/ml). It was calculated as gallic acid equivalent (GAE)/mg/g sample (TPC). All measurements were made in triplicate.

5. Determination of total flavonoid contents in propolis extracts:

A modified colorimetric approach reported by Sakanaka *et al.* (2005) was used to quantify the total flavonoid contents (TFC) of propolis extracts with concentrations that ranged from 20 to 200 µg/ ml using catechol as a reference. Distilled water (1.25 mL) was mixed with 250 µl of extracts or standard solution and 75 µl of 5% sodium nitrite ($NaNO_2$). 150 µl of 10% aluminum chloride ($AlCl_3$) solution was added after 5 min. After 6 min, 0.5 ml of 1 M sodium hydroxide (NaOH) and 0.6 ml of distilled water were added to thin. The mixture was then blended, and the absorbance at 510 nm was measured. The total flavonoid content was reported as catechol equivalent (CE) in the results. All measurements were made in triplicate.

6. Reversed phase high performance liquid chromatography (HPLC) of Ethanolic extract of propolis (EEP) and Water extract of propolis (WEP):

A quantitative analysis of the phenols and flavonoids was performed by reversed phase HPLC with a chromatograph equipped with ymc pack odss –a column. The mobile phase as acetic acid: methanol: water (5:75:60, v/v); with a flow rate of 1 ml / min, and detection was with a diode array detector. Chromatograms were recorded at 254 nm. The quantities of flavonoids in the EEP and WEP were calculated by using authentic standards of flavonoids purchased from Extrasynthese A.A.Co ., France (Park *et al.,* 1998).

7. Determination of antioxidant activity in propolis extract:

DPPH (1,1-diphenyl – 2- picrylhydrazyl radical) assay:

Antioxidant (H-A) react with DPPH; which is a stable free radical and is reduced to the DPPH –H and as consequence the absorbance's decreased from the DPPH radical to the DPPH –H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The protocol of DPPH radical assay (Chen and Ho, 1995) and modified by Xu & Chang (2007) was followed. Chemicals used were 0.1 Mm solution of DPPH in methanol was prepared and 3.8 ml of this solution was added to 0.2 ml of propolis extract. Thirty minutes later, the absorbance was measured at 517 nm with UMCO UV-2100 spectrophotometer. A blank was prepared without adding propolis extract. Ascorbic acid at various concentrations (6 to 40 µg / ml) was used as standard. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation.

DPPH radical scavenging activity (%) =

$$\frac{\mathbf{A}\text{control} - \mathbf{A}\text{test}}{\mathbf{A}\text{control}} \times 100$$

Acontrol = Absorbance of the control reaction and

Atest = Absorbance in the presence of the sample of the extracts.

The antioxidant activity of propolis extracts is expressed comparing with standard ascorbic acid.

IC_{50}: The concentration of sample that scavenges 50 % of DPPH.

Statistical analysis:

The data were analyzed using IBM SPSS software package version 20.0**. (**Armonk, NY: IBM Corp**)**. Quantitative data were described using mean and standard deviation. Significance of the obtained results was judged at the 5% level (El-Nassag and Refaat, 2017).

The used tests were: 1 - One way ANOVA test

For normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (LSD) for pairwise comparisons.

RESULTS AND DISCUSSION

Antimicrobial activity of propolis extracts:

Antimicrobial activity for propolis extracts: The results presented in Table (1) and Fig. (1), (2) show a significant difference in the diameter of the inhibitory zone (DIZ) between the tested cultures obtaining propolis ethanolic and water extracts. There were significant changes in antibacterial activity among concentrations (0.001 g). Findings of the antibacterial activity evaluation of propolis samples obtained by the

technique of diffusion on agar wells showed strong antimicrobial activity against pathogens. It was found that WEP has no antimicrobial effect on the tested undesirable examined microorganisms, while EEP varied in antimicrobial effect on them. The inhibition zone indicated that the high effect was found against *Pseudomonas aeruginosa* (21.36 mm) followed by *Streptococcus pyogenes* (16.24 mm), the lowest effect detected against *Listeria monocytogenes ATCC19116*(10.50 mm) and has a moderate effect on *Staphylococcus aureus NCTC 10788* (12.90 mm). The same results were found by Al-Salmani and Hassan (2011) they showed that ethanol extract of Iraqi propolis at a concentration of 5 mg/ml was more active in inhibiting the growth of *Staphylococcus aureus, Escherichia coli and Klebsiella pneumonia* by agar diffusion technique. Wherein, inhibition zone diameters attained 22.30, 20.0, and 19.30 mm, respectively.

Chaillou and Nazareno (2009) it was stated that the antimicrobial effect of EEP on *Staphylococcus aureus* with inhibition zones of more than 9 mm diameter was regarded as substantial. Also, Rahman *et al.* (2010) revealed that propolis had higher antibacterial activity against *Staphylococcus aureus* when compared with honey and more susceptible than *Escherichia coli*.

Table 1. Antimicrobial activity of the ethanolic and water extracts of propolis against some undesirable microorganisms (Diameter (mm) of the Inhibition Zone (DIZ).

Undesirable bacteria	EEP	WEP
Streptococcus pyogenes	16.24 ± 0.28	0.0 ± 0.0
Yersinia enterocolitica ATCC23715	11.50 ± 0.51	0.0 ± 0.0
Escherichia coli BA12296	14.0 ± 0.80	0.0 ± 0.0
Bacillus subtilis D B 100 host	13.82 ± 0.05	0.0 ± 0.0
Candida albicans ATCCMYA 2876	15.16 ± 0.73	0.0 ± 0.0
Listeria innocua ATCC33090	11.13 ± 0.38	0.0 ± 0.0
Staphylococcus aureus NCTC 10788	12.90 ± 0.20	0.0 ± 0.0
Listeria monocytogenes ATCC19116	10.50 ± 0.47	0.0 ± 0.0
Pseudomonas aeruginosa	21.36 ± 0.40	0.0 ± 0.0
Klebsiella pneumonia ATCC12296	15.71 ± 0.53	0.0 ± 0.0

Four replicate for each group; Data was expressed using Mean ± SD; SD: Standard deviation

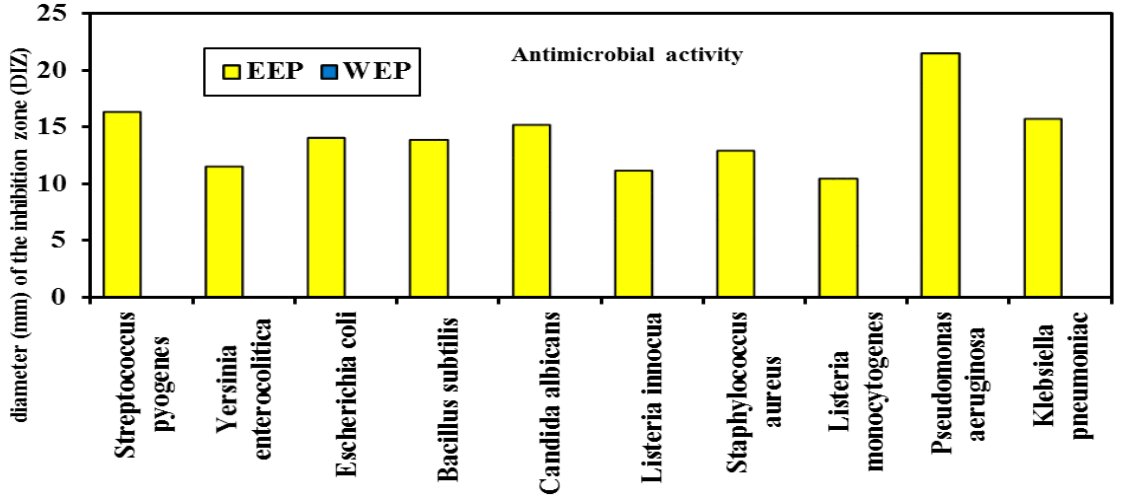
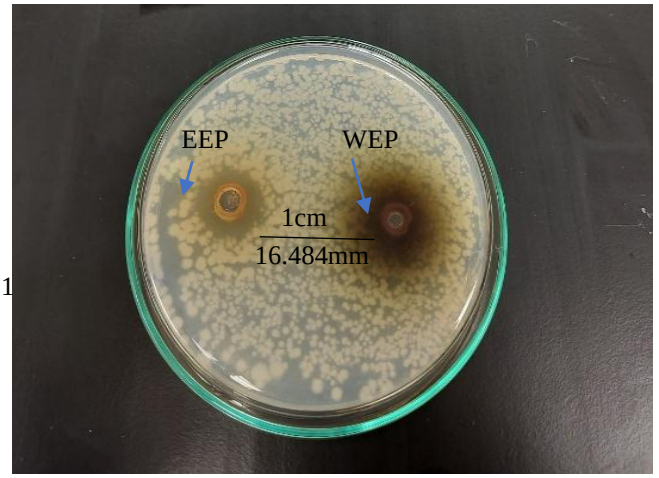
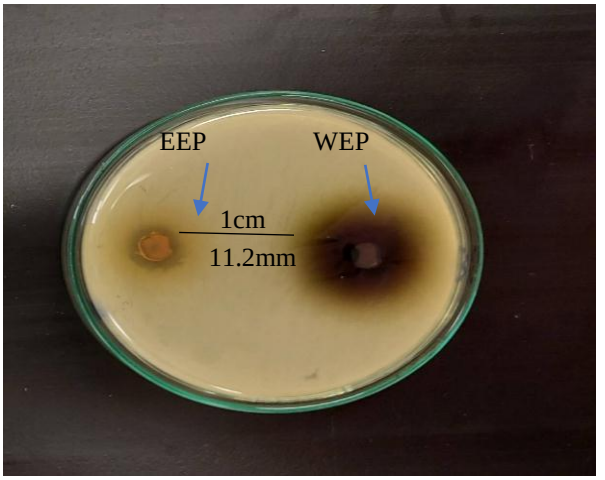


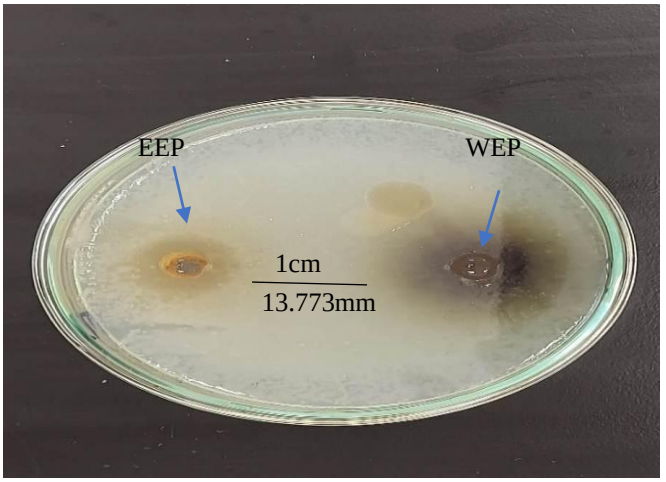
Fig.1. Antimicrobial activity of the ethanolic and water extracts of propolis against some undesirable microorganisms (Diameter (mm) of the Inhibition Zone (DIZ)



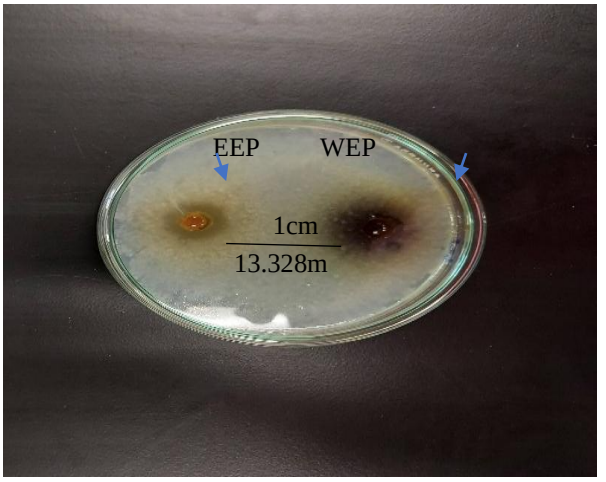
Streptococcus pyogenes



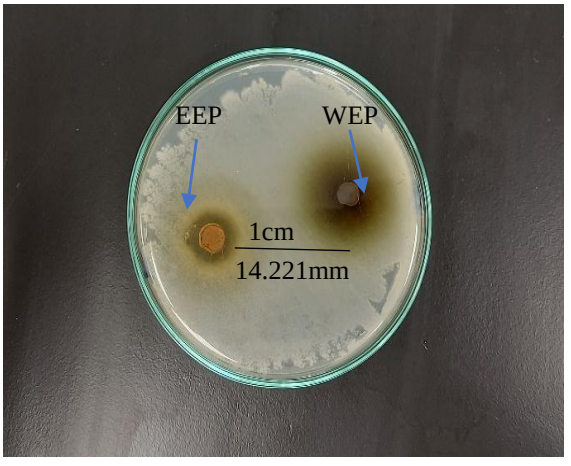
Yersinia enterocolitica ATCC23715



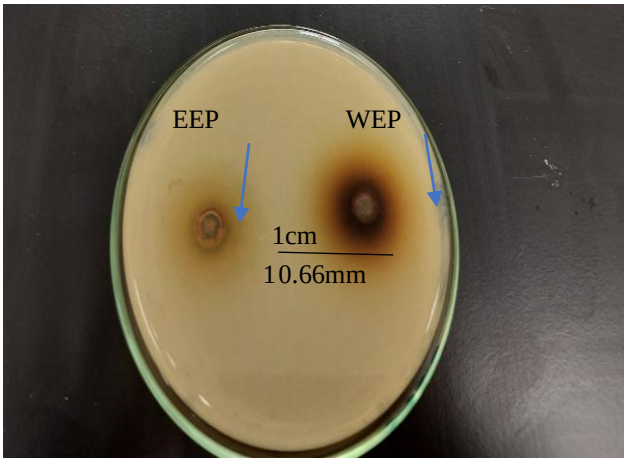
Bacillus subtilis D B 100 host



Escherichia coli BA12296

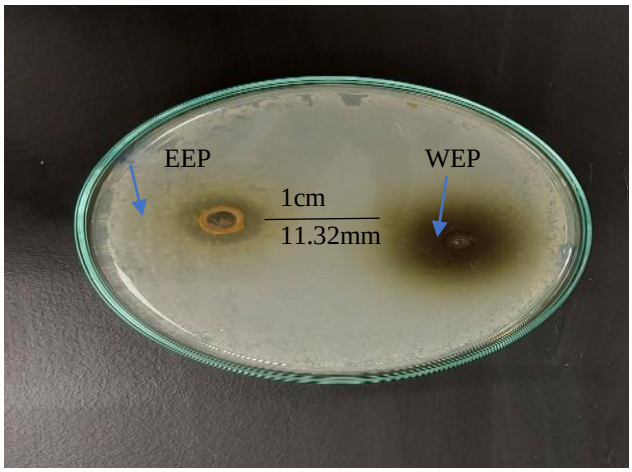


Candida albicans ATCCMYA 2876

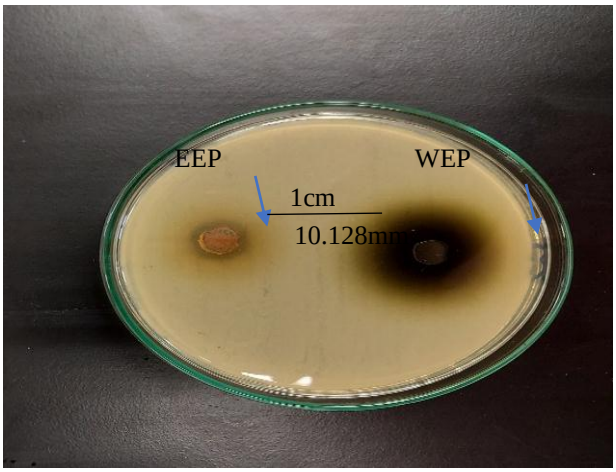


Listeria innocua ATCC33090

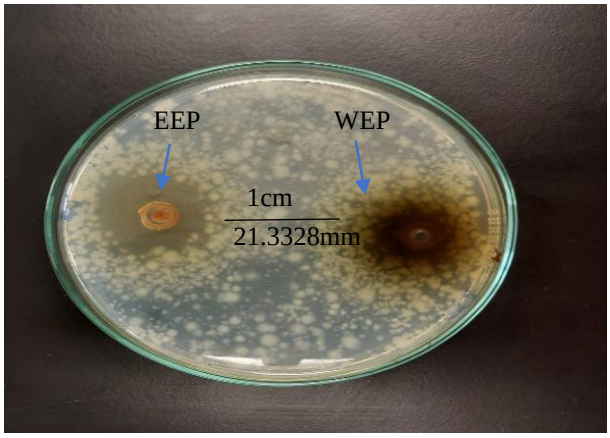
Fig. 2. Effect of the ethanolic extract of propolis (EEP) and water extract of propolis (WEP) against *Streptococcus pyogenes, Yersinia enterocolitica (ATCC23715), Bacillus subtilis D B 100 host, Escherichia coli (BA12296), Candida albicans ATCCMYA 2876, Listeria innocua ATCC33090.*



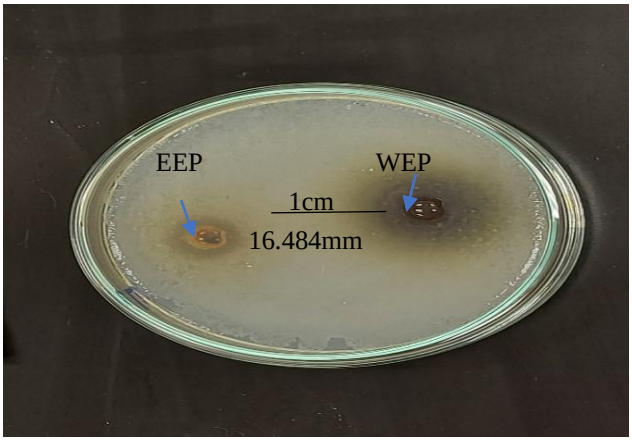
Staphylococcus aureus NCTC 10788



Listeria monocytogenes ATCC19116



Pseudomonas aeruginosa



Klebsiella pneumonia ATCC12296

Fig 3. Continued, Effect of the ethanolic extract of propolis (EEP) and water extract of propolis (WEP) against *Staphylococcus aureus NCTC 10788, Listeria monocytogenes ATCC19116, Pseudomonas aeruginosa Klebsiella pneumonia ATCC12296.*

Total phenolics content and total flavonoids:

The total phenolic amount (TPC) of propolis ethanol and water extracts is shown in Table (2). Water propolis extract had the largest quantity of TPC (367.29 mg/100g) gallic acid, while ethanolic propolis extract had the lowest quantity of TPC (262.20 mg/100g) gallic acid. And show the total flavonoids content of the ethanol and water extracts of propolis. It can be noted that water propolis extract had the highest amount of total flavonoids content being117.59 mg/100g, while the total flavonoids content of ethanol propolis extract was 108.81 mg/100 g, respectively. Further analysis for total phenols and flavonoids of propolis extracts compounds by HPLC. The results of quantitative analysis of phenols and flavonoids in propolis extracts are shown in Table (3) and Figs. (3,4). There are some components are not found in EEP such as pyrogallol, quinol, ferulic acid, rosemarinic while corresponding amounts in WEP were 47.40858, 299.05875, 1518.22288 and 722.68156 mg/kg respectively. Also, it was clear that some components are not found in WEP such as ellagic acid, myricetin, kaempferol rosemaries, while corresponding amount in EEP was 258.52961, 317.88251, 25.69605 mg/kg respectively. WEP showed high content of gallic acid, p-hydroxy benzoic acid, catechin, syringic acid, benzoic acid, cinnamic acid and quercetin. On the other hand, EEP is higher than WEP in p-coumaric acid, o-coumaric acid. From the above result is clear that WEP has more antioxidant activity than EEP. Propolis was found to have antioxidant properties due to its components galangin and pinocembrin (El-Guendouz *et al.*, 2017). The aqueous extract of propolis was more effective than the ethanolic extracts because of the

increased polyphenol content. The biological basis of propolis's anti-oxidant property is related to phenolic chemicals, which donate hydrogen ions to free radicals to protect cells from oxidation reactions as well as food storage from oxidation and poisoning. Free radicals, which are the principal cause of lipid, nucleic acid, and protein oxidation, could be removed by propolis (Chandna *et al.*, 2014).

Hegazi and El-Hady (2002) found that caffeic acid and vitamin C at concentrations of 1, 10 and 100 µg showed the highest activity as free radical scavenger compared to the same concentration of propolis samples collected from a reclaimed land in Egypt. Ahn *et al.* (2007) observed that propolis samples collected in various area of China showed free radical scavenging activity and there was positive correlation between the activities and total polyphenol contents.

Table 2. Total phenolics and total flavonoids content in propolis extracts

Propolis extract	Total phenolics (mg/g)	Total flavonoids (mg/g)
Water extract	367.29±2.56	117.59±4.42
Ethanolic extract	262.20±3.54	108.81±2.59

Reported values are the mean ± SD of three replicates. Means in the same column followed by different lower-case letters are significantly different ($p \leq 0.05$). Total phenolics was expressed as Gallic acid equivalents (GAE) mg/g sample. Total flavonoids was expressed as mg catechol equivalents g sample.

Table 3. Identification of phenols and flavonoids of propolis extracts component by HPLC (mg/kg).

Name	EEP Amount [mg/kg]	WEP Amount [mg/kg]
Pyrogallol	0	47.40858
Quinol	0	299.05875
Gallic acid	15.17558	217.72368
3-Hydroxytyrosol	0	0
Catechol	0	0
p-Hydroxy benzoic acid	1.09389e4	1428.95102
Catechin	2.75277e4	4995.69425
Chlorogenic	0	0
Vanillic acid	0	0
Caffeic acid	0	0
Syringic acid	219.47458	8729.47454
p- Coumaric acid	698.85250	108.99796
Benzoic acid	2.19153e4	4.35537e5
Ferulic acid	0	1518.22288
Rutin	0	0
Ellagic	258.52961	0
o- Coumaric acid	746.37740	319.17806
Resvertol	0	0
Cinnamic acid	18.86185	921.64906
Quercitin	1082.00151	1251.55130
rosemarinic	0	722.68156
Neringein	0	0
Myricetin	317.88251	0
Kampherol	25.69605	0

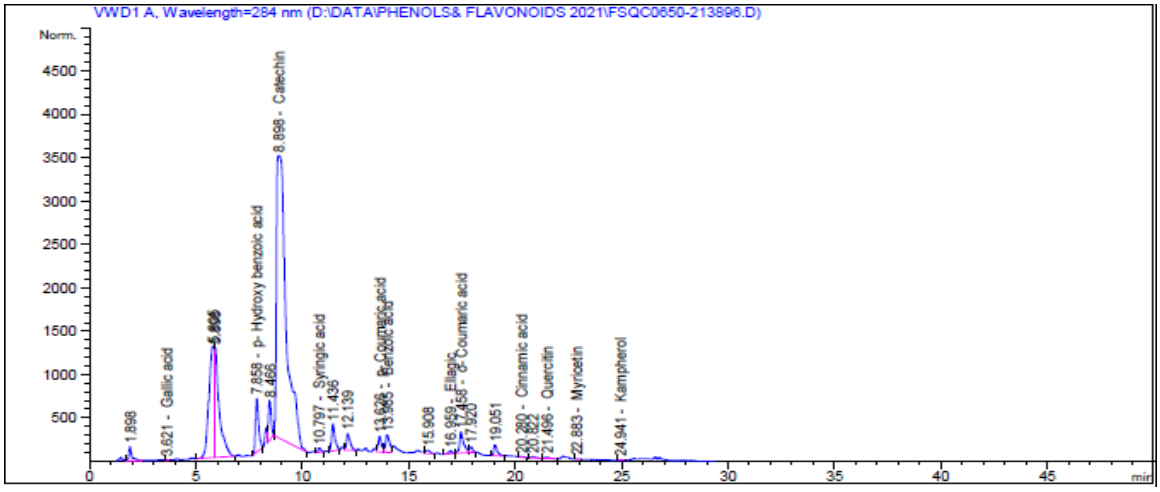


Fig.3. HPLC chromatogram of the ethanolic extracted propolis.

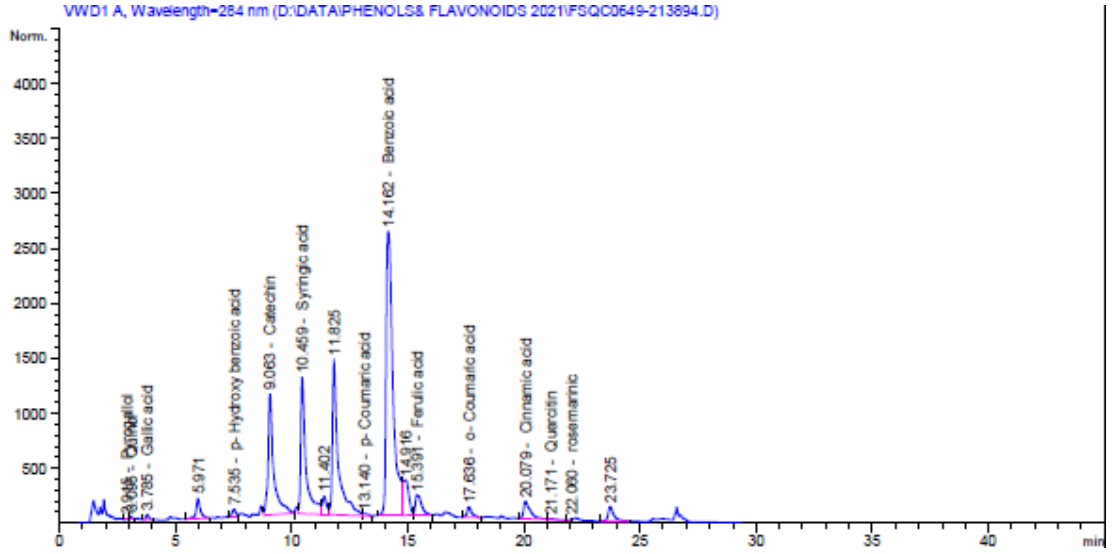


Fig.4. HPLC chromatogram of the water extracted propolis.

IC_{50} (the efficient concentration of the ethanolic and water extracts of propolis in mg/ml required to decrease initial DPPH radical concentration by 50%) was obtained by interpolation from linear regression analysis. The higher DPPH radical scavenging activity is associated with a lower IC_{50} (good antioxidant activity). Show in Table (4) and Fig (5) the lowest value of IC_{50} was detected for water propolis extract (21.22 mg/ml), while ethanolic extract was (25.13 mg/ml).

Table.4: IC_{50} of DPPH radical scavenging activity of propolis extracts

Propolis extracts	DPPH (IC_{50}) µg/ml
Ascorbic acid	5.23± 0.153
Water extract	21.22± 0.453
Ethanolic extract	25.13± 0.290

Each reported value is the mean ± SD of three replicates. Means in the same column followed by different upper case letters are significantly different ($p \leq 0.05$). IC_{50} (µg/ml): inhibitory concentrations at which 50%of DPPH radicals are scavenged.

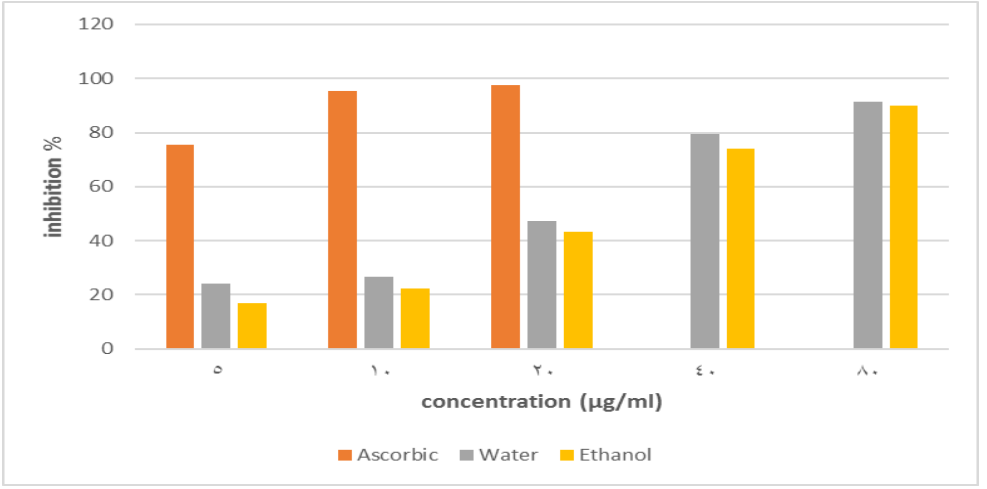


Fig. 5. DPPH radical scavenging activity (%) of propolis extracts.

CONCLUSION

From the previous results it can be concluded that the ethanolic extracts of propolis exhibit a varied antimicrobial activity against the examined microorganisms while water extracts of propolis didn't show any antimicrobial activity on them. The quantitate analysis of antioxidant and flavonoids are varied in both extracts and water extract showed more antioxidant activity than ethanol extract.

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الملخص العربي

النشاط المضاد للميكروبات والمضاد للأكسده لمستخلصي البروبوليس المائي والكحولي

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لاستهلاك أغذية صحية طبيعية يستوجب معها مضافات غذائية طبيعية لإستبدال المضافات الصناعية. البروبوليس يعتبر من المواد الطبيعية التي يمكنها القيام بهذا الدور. حيث وجد البروبوليس يحتوي علي مكونات لها نشاط حيوي مضاد للميكروبات ومضاد للأكسده. وقد تم تحضير مستخلص مائي ومستخلص كحولي من البروبوليس لتقدير قدراتهم علي تثبيط مجموعه من الميكروبات الغير مرغوبه في الصناعات الغذائية وكذالك تقدير قدراتهم كمضاد للأكسده لأطاله فتره حفظ الأغذية. أظهرت النتائج قدرة المستخلص الكحولي من البروبوليس علي تثبيط العديد من الميكروبات المختبره مثل*Streptococcus pyogenes*، *Pseudomonas aeruginosa* وأقل تأثير تثبيطي مع *Listeria monocytogenes* ATCC19116. ولم يظهرالمستخلص المائي أي تأثير تثبيطي علي الميكروبات المختبره. وعند تقدير النشاط المضاد للأكسده أظهر المستخلص المائي قدره أعلي كمضاد للأكسده لأحتواء علي نسبه أعلي من gallic acid, p-hydroxy benzoic acid, catechin, syringic acid, benzoic acid, cinnamic acid and quercitin.، ومن ناحية أخري أحتوي المستخلص الكحولي علي نسبة أعلي من المستخلص المائي في p-coumaric acid, o-coumaric acid.