الفعالية المضادة للبكتريا في الزجاج ومحتوى الفينولات الكلية في المستخلص الكحولي للعكبر

بسام العقلة* رضوان بدر الدين* ميادة حاج علي*

الملخص

هدفت الدراسة الحالية إلى تحري الفعالية المضادة للبكتريا في 25 عينة عكبر مجموعة من 9 مناطق جغرافية مختلفة في سورية و3 عينات من عمان، الأردن ومقارنتها بـ3 عينات تجارية، تجاه 8 أنواع من البكتريا الممرضة المنقولة بالغذاء (4 أنواع إيجابية غرام: *Bacillus cereus*، *B. subtilis*، *Staphylococcus haemolyticus*، *S. lugdunensis*، و4 أنواع سلبية غرام: *Citrobacter braakii*، *Enterobacter cloacae*، *Proteus mirabilis*، *Salmonella enteritidis*). كان محتوى الفينولات الكلي في المستخلصات الكحولية للعكبر (EEP) بين 281 و 3046 ملغ/100 غ. تم اختبار الفعالية المضادة للبكتريا لـ EEP في مجال التمديدات بين 1: 20 و 1: 1280 في مرق مولر هنتون (MHB). كانت الأنواع الأكثر مقاومة لمستخلصات العكبر هي *Salmonella enteritidis* و*Bacillus cereus*، بينما كان النوعان *Enterobacter cloacae* و *Staphylococcus haemolyticus* هما الأكثر حساسية من بين جميع البكتريا السلبية والإيجابية غرام.

الكلمات المفتاحية: العكبر، محتوى الفينولات، الفعالية المضادة للبكتريا، البكتريا الإيجابية غرام، البكتريا سلبية غرام.

* الهيئة العامة للتقانات الحيوية دمشق – سوريا.

In Vitro Antibacterial activity and total phenolic content of propolis alcoholic extracts

Bassam Al-oklah* **Rudwan Badr Al-Deen***
Mayadah Hajali*

Abstract

The aim of the present study was to investigate the antibacterial activities of 25 propolis samples collected from 9 various geographical provinces of Syria and 3 samples from Amman, Jordan, compared with 3 commercial samples, against 8 food-borne bacterial pathogens (4 gram-positive: *Bacillus cereus*, *B. subtilis*, *Staphylococcus haemolyticus*, *S. lugdunensis* and 4 gram-negative: *Citrobacter braakii*, *Enterobacter cloacae*, *Proteus mirabilis*, *Salmonella enteritidis*). The total phenolic content of ethylnolic extracts of propolis (EEP) ranged between 281 and 3046 mg/100 g. Antibacterial activities of EEP samples were tested in dilutions of 1:20 to 1:1280 dilutions of Meuller Hinton Broth (MHB). The most resistant species to propolis extracts were *Salmonella enteritidis* and *Bacillus cereus*, whereas *Enterobacter cloacae* and *Staphylococcus haemolyticus* were the most sensitive among all Gram negative and positive tested bacteria**.**

Keywords: Propolis, Phenolic Content, Antibacterial Activity, Gram-Positive Bacteria, Gram-negative Bacteria.

*National Commission for Biotecnology (NCBT), Damascus-Syria

Introduction:

Propolis, from the Greek 'pro'='in front' or 'in defense' and 'polis'='the city', meaning 'defense of the hive', is a strongly adhesive natural mixture manufactured by

honey bees (Apis mellifera L.) (El-Guendouz *et al*., 2019). Propolis is a non-toxic natural substance (Borrelli *et al*., 2002). It is a mixture of buds (Bertrams *et al*., 2013), shoots, and wounds of various plant species and mixed with mandible secretions (Barrientos *et al*., 2013). Bees use propolis mainly to protect their hive from adverse weather conditions or invaders by sealing internal walls, holes, and cracks of the beehive or to embalm dead insects in order to prevent hive infections. Mankind has been using propolis for ages, and especially in traditional medicine the application of propolis has a long history with first reports dating back to 300 BC (Bertrams *et al*., 2013).

It has over 300 compounds, among which polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, sugars and amino acids have been detected in raw propolis, but its composition is qualitatively and quantitatively variable, depending on the vegetation at the site from which it was collected and the collection season (Koo *et al*., 1999; Bankova, 2005; Tosi *et al*., 2007; Valencia *et al*., 2012). Flavonoids and phenolic acids represent the most active constituents of propolis (Lahouel *et al*., 2010).

Propolis has been used as a popular remedy for several centuries, mainly due to its antimicrobial properties, present in propolis from different origins , but it is also taken orally and applied externally for a series of diseases, ranging from tumors to parasites (Sawaya *et al*., 2011). It is also considered as an alternative in the treatment and prevention of many infectious diseases, since it displays a wide range of antimicrobial activity against a variety of bacteria, fungi, parasites and virus (Kujumgiev *et al*., 1999; Sforcin *et al*., 2000). Propolis has been used as a popular remedy for several centuries for a wide array of ailments. Its antimicrobial properties, present in propolis from different origins, have been extensively studied. But, more recently, antiparasitic, anti-viral/immune stimulating, healing, anti-tumor, anti-

inflammatory (Marcucci, 1995). However, a sensitizing or even allergenic potential has also been reported (Hausen *et al*., 1992; Giusti *et al*., 2004), which is partly caused by certain caffeic acid derivatives like caffeic acid benzyl ester or caffeic acid phenethyl ester, i.e. compounds which, at the same time, are associated with the aforementioned health-beneficial effects (Banskota *et al*., 2001b).

The current study aimed to determine the antibacterial activity and total phenolic contents of propolis ethanolic extracts.

Materials and methods:

Bacterial strains, subculture and maintenance:

Bacterial strains (*Bacillus cereus*, *B. subtilis*, *Staphylococcus haemolyticus*, *S. lugdunensis*, *Citrobacter braakii*, *Enterobacter cloacae*, *Proteus mirabilis*, *Salmonella enteritidis*) were isolated from food samples in Syria; and identified by morphological, physiological and biochemical tests. The strains were preserved in broth medium (Luria–Birtani (LB) broth + 20% (v/v) glycerol), and stored at -80°C. Ten µL of stored culture, were subcultured by streaking on LB agar plates and incubation at 37°C for 24 h. Good isolated colonies were chosen to prepare bacterial suspension for antibacterial activity assay.

Propolis samples:

Twenty five samples of propolis were collected from 9 province: Qunaitera, Swaidaa, Damascus countryside, Hamah, Edlib, Tartous, Latakia, Aleppo and Al-Hasakah, and three samples form Amman in Jordan (Naour, Jarash, Al-Jubaiha), and three commercial samples (Hungarian, Chinese and Saqqa-Ameeni).

Determination of total phenolic contents:

Total phenolic contents of propolis ethanolic extracts (PEE) were determined by photometric assay according to Folin-Ciocalteu method (Savikas *et al*., 2005) with some modifications. 30 ml of absolute ethanol was added to one gram of propolis sample, the mixture was kept in dark for half an hour, 4 ml of sodium carbonate solution (7%

m/v), 3 ml of distilled water and 0.3 ml of Folin-Ciocalteu reagent were added to 2 ml of PEE, then the volume was made up to 10 ml with distilled water and the mixture was kept in dark for 2 hours. The optical density measured at 750 nm using spectrophotometer (Optizen 3000 plus, Mecasys Inc., Korea). The results were expressed as Gallic Acid Equivalent (GAE).

Preparation of Propolis Ethanolic Extracts (PEEs)

Propolis ethanolic extracts were prepared according to the method described by Wozniak *et al*. (2019), with some modifications (the extraction time was reduced from 5 days to 2 days). Ethanol (70%) was used in the current study to prepare propolis extracts, because it is superior to other solvents (Kubiliene *et al*., 2018; kubilien *et al*., 2015). One hundred ml of ethanol 70% was added to 10 g of propolis in amber vessels. The mixtures were shaken at rotary shaker for 48 hours at room temperature. The mixture was filtrated using filter paper (Wattmann No. 1). The filtrate was evaporated to dryness using rotary evaporator (Heidolph, Germany) at 40°C .The yield of extraction was calculated by dividing the mass of residue resulting from rotary evaporator by the mass of propolis sample. Results were expressed in %. The percentage yields were calculated according to following equation (Pobiega *et al*., 2019a):

$$\textbf{Yield} = \frac{\textbf{The weight of dried extract}}{\textbf{The weight of crude propolis}} \times \textbf{100}$$

The residue was resolved in 10 ml distilled water to obtain the concentration 1: 10. Then 1: 20, 1: 40, 1: 80, 1: 160, 1: 320, 1: 640 and 1: 1280 dilutions were prepared and used to estimate the antibacterial activity.

Determination of *in vitro* antibacterial activity:

One ml of Mueller Hinton broth (MHB) (Criterion, Hardy, USA) was added to 1 ml of each double-strength dilutions to obtain the final single-strength concentrations.

Bacterial suspensions of 0.5 McFarland were prepared for each species (Ristivojevic *et al*., 2016): 4 Gram positive bacteria (*Bacillus subtilis, B. cereus, Staphylococcus haemilyticus, S. lugdunensis*), and 4 Gram negative bacteria (*Enterobacter cloacae, Citrobacter braakii, Proteus mirabilis, Salmonella enteritidis*) from the colonies grown on Luria Bertani agar (LBA) plates. MBC values were determined according to (Nascimento *et al*., 2013), with some modification. Briefly, 10 µl of each bacterial suspension was added to a tube containing the mixture of MHB and propolis to estimate the minimum bactericidal concentration (MBC). The mixture was incubated at 37°C for 24 hours. To determine the bactericidal effect, 10 µl of the incubated solution was cultured on LBA plates. The plates were then incubated at 37°C for 24 hours and growth was observed. The least concentration that did not show any growth of tested organisms was considered as the MBC value of the tested propolis extract against the tested bacterial species.

Results and discussion:

1- Extraction yield:

Extraction of crude propolis samples was performed with ethanol 70% (v/v). Table 1 revealed that the extraction yield was between 4.46 and 81.16% in Jubaiha and Al-jurd, respectively, and in average 35.72. The high differences in extraction yield are due to the high content of waxes in low extract percent samples. The extraction yield in this study was higher than that recorded by Pobiega *et al*. (2019a), who found that the extraction yield was between 5.76 and 15.92% in 5 samples of Polish propolis.

Table (1): the extraction yield (%) and total phenols (mg GAE/ 100 g propolis) in propolis samples

Samples	Extraction yield (%)	Total phenols (mg GAE/ 100g propolis)
Chinese	**25.12**	**3046.18**
Saqqa-Ameeni	**46.97**	**2977.55**
Damascus countryside Al-Ghota	**30.09**	**2781.47**
Damascus countryside Al-Qalamon	**32.17**	**2781.47**
Damascus countryside Al-Nabek	**53.56**	**2624.61**
Damascus countryside Al-Jurd	**81.16**	**2193.24**
Hungarian	**27.04**	**2006.96**
Jarash (Jordan)	**32.84**	**1905.00**
Aleppo1	**20.51**	**1663.82**
Tartous1	**50.12**	**1575.59**
Jubaiha (Jordan)	**40.46**	**1555.98**
Hamah	**41.90**	**1487.35**
Latakia2	**31.18**	**1457.94**
Aleppo2	**28.35**	**1379.51**
Swaidaa	**33.84**	**1006.96**
Edlib1	**40.56**	**967.75**
Al-Hasakah2	**35.91**	**967.75**
Tartous2	**68.12**	**820.69**
Qunaitera-Sendianah	**40.56**	**761.86**
Latakia1	**28.16**	**634.41**
Naour (Jordan)	**41.9**	**526.57**
Al-Hasakah1	**50.12**	**477.55**
Aleppo3	**68.12**	**350.10**
Qunaitera-Ain Aishah	**38.25**	**340.29**
Edlib2	**35.91**	**281.47**
Mean ± standard deviation	**35.72±16.48**	**1462.88±883.94**

2-Total phenols

Table 1 revealed differences in total phenols according to the samples and geographic region. The highest content of total phenols was noticed in Chinese propolis sample (3046.18 mg/100 g), while the lowest content was found in Edlib2 (281.47 mg/100 g), the average was 1462.88 mg/100 g. These contents were lower than that recorded by Yaghoubi *et al*. (2007) in Iranian propolis (7300-36000 mg/100 g), and Savickas *et al*. (2005) in Lithuanian and Czech propolis (180-

16400 mg/100g), and Yaghoubi *et al*. (2007) in Turkish propolis (36000 mg/100 g), and Medić-Šarić *et al*. (2013) in Croatian propolis (28000 mg/100 g), and Alencar *et al*. (2007) in red Brazilian propolis (4300-23200 mg/100 g), and Salah *et al*. (2014) in 12 samples of Argentinean propolis (1725-23575 mg/100 g), and Yang *et al*. (2011) in Chinese propolis (17470-23560 mg/100 g). However, the content of total phenols in current study was higher than that recorded by Barrientos *et al*. (2013) in Chilean propolis (340-2140 mg/100 g), while the highest content found in current study was close to the average recorded by Ibrahim (2011) in Iraqi propolis (3125 mg/100 g). The differences in total phenolic content is due the differences in the geographical regions and the vegetation, from which the bees collects propolis.

3-*In vitro* Antibacterial activity:

The results of antibacterial activity of PEEs revealed that Hungarian and Saqqa-Ameeni (commercial samples), and Al-Qalamon and Al-Ghota had the highest antibacterial activity (the lowest values of MBC) (Table 2). Moreover, *Bacillus cereus* and *Salmonella enteritidis* were the most resistant species, while *Enterobacter cloacae* and *Staphylococcus haemolytics* were the most sensitive species among tested bacteria.

The most resistant Gram negative bacteria against PEEs was *Salmonella enteritidis* where the lowest MBC values were 50 mg/ml in 7 samples (Saqqa-Ameeni, Aleppo1, Aleppo2, Latakia2, Hamah, Tartous1 and Hasakah2) while no antibacterial activity (MBC values were higher than 50 mg/ml) in the other PEEs. These values were higher than that recorded by Paul *et al*. (2014) in Cameroonian brown propolis (0.1-0.2 mg/ml) and the value found by Hames-Kocabas *et al*. (2013) in popolis samples collected from North East Anatolia, and Times *et al*. (2011) (1.2 mg/ml) and Ibrahim (2011) (5.48 mg/ml), and Pobiega *et al*. (2019b) (16-32 mg/ml). The higher values of MBC against *Salmonella enteritidis* in the current study are due to the lower content of total phenols in the ethanolic extracts (281.47-3046.18 mg

GAE/100 g in our study versus 5265-10029 mg GAE/100 g in the last study); but our results were similar to that recorded by Ivančajić *et al*. (2010) who found that no antibacterial effect of PEEs on *Salmonella cholerae*, *S. galinarum* and *S. typhimurium* using disk diffusion method in neutral medium in their study on Serbian propolis, and the results recorded by Yaghoubi *et al*. (2007) who concluded that there is no antibacterial effect of Iranian propolis on *S. entriditis*.

Proteus mirabilis was resistant against PEEs. The lowest MBC for this species was 3.125 in Aleppo1, then Saqqa-Ameeni (25 mg/ml), followed by Aleppo2, Hamah, Tartous1 and Al-Hasakah (50 mg/ml), these values were higher than that recorded by Segueni *et al*. (2014) (0.1-1.2) in two Algerian propolis samples, and Pobiega *et al*., (2019b) (16 mg/ml), because the lower content of total phenols in the current study (281.47-3047.18 mg GAE/100 g) compared with the values recorded by Pobiega *et al*. (2019b) (5256-10029 mg GAE/100 g) in 5 Polish propolis samples.

Table (2): antibacterial activity of propolis samples (expressed as MBC) in mg/ml

Propolis sample	Bacterial species							
	E.cloacae	*B.cereus*	*B.subtitus*	*S.enteritidis*	*C.braakii*	*P.mirabilis*	*S.lugdunensis*	*S.haemolyticus*
Chinese[3]	12.5	25	25	50>	50>	50>	25	25
Hungarian[3]	6.25	25	12.5	50>	6.25	50>	12.5	12.5
Naour[2]	50>	50>	50>	50>	50>	50>	50>	50>
Al-Nabek[1]	50>	50>	50>	50>	50>	50>	50>	50>
Al-Jubaiha[2]	50>	50>	50>	50>	50>	50>	50>	50>
Al-Jurd[1]	12.5	25	3.125	50>	25	50>	25	12.5
Al-Qalamon[1]	1.5625	25	50>	50>	3.125	25	12.5	12.5
Al-Ghota[1]	12.5	25	12.5	50>	25	50>	25	12.5
Al-Swaidaa[1]	50>	50>	50>	50>	50>	50>	50>	50>
Ain-Aishah[1]	50>	50>	50>	50>	50>	50>	50>	50>
Jarash[2]	50>	50>	50>	50>	50>	50>	50>	50>
Sendianah[1]	50>	50>	50>	50>	50>	50>	50>	50>
Saqqa-Ameeni[3]	3.125	12.5	6.25	50	12.5	25	12.5	6.25
Aleppo1[1]	3.125	50	50	50	50	3.125	50	50
Aleppo2[1]	50	50>	50	50	50	50	50	50>
Aleppo3[1]	50>	50>	50>	50>	50>	50>	50>	50>
Latakia1[1]	50>	50>	50>	50>	50>	50>	50>	50>
Latakia2[1]	50	50	50>	50	50>	50>	50	50
Edlib1[1]	50>	50>	50>	50>	50>	50>	50>	50
Edlib2[1]	50>	50>	50>	50>	50>	50>	50>	50>
Hamah[1]	50	50	50	50	50	50	50	50
Tartous1[1]	50	50	50	50	50	50	50	50
Tartous2[1]	50>	50>	50>	50>	50>	50>	50>	50>
Al-Hasakah1[1]	50	50>	50>	50>	50>	50>	50>	50>
Al-Hasakah2[1]	50>	50	50	50	50	50	50>	50

[1] Samples collected from Syrian provinces
[2] Samples collected from Amman (Jordan)
[3] Commercial samples

It can be noticed that *C. braakii* was sensitive for PEEs in general. The most effective sample was Al-Qalamon (MBC=3.125 mg/ml), followed by Hungarian sample (MBC=6.25 mg/ml) then Saqqa-Ameeni (MBC=12.5 mg/ml), and Aleppo1, Aleppo2, Hamah, Tartous1 and Al-Hasakah2 (MBC>50 mg/ml). All these values were higher than that recorded by Kačániová *et al*. (2012) who found that this species can grow in the presence of 0.512 mg/ml.

We concluded that *E. cloacae* was the most sensitive species among both Gram positive and Gram negative bacteria. The lowest MBC value was 1.5625 mg/ml for Al-Qalamon sample, 3.125 mg/ml for Saqqa-Ameeni and Aleppo1, 6.25 mg/ml for Hungarian sample, 12.5 mg/ml for Chinese, Al-Jurd and Al-Ghota, 50 mg/ml for Aleppo2, Latakia2, Hamah, Tartous1 and Al-Hasakah. It can be noticed that all MBC values were higher than that recorded by Temiz

et al. (2011) who found that the minimal inhibitory concentration was 0.4 mg/ml.

Tabe 2 revealed that MBC value for *S. haemolyticus* ranged between 6.25-50 mg/ml. The most effective sample was Saqqa-Ameeni (MBC=6.25 mg/ml), followed by Hungarian, Al-Jurd, Al-Qalamon and Al-Ghota (MBC=12.5 mg/ml). The MBC values for *S. lugdunensis* ranged between 12.5 and 50 mg/ml. The lowest value for MBC recorded in Hungarian, Al-Qalamon and Saqqa-Ameeni (12.5 mg/ml), followed by Chinese, Al-Jurd and Al-Ghota (MBC=25 mg/ml), then Aleppo1, Aleppo2, Latakia2, Hamah and Tartous1 (MBC=50 mg/ml). There are no reference studies about the latter two species for comparing.

MBC values for *B. subtilis* were between 3.125 and 50 mg/ml, the highest activity was recorded in Al-Jurd sample (MBC=3.125 mg/ml), followed by Saqqa-Ameeni and Al-Ghota (12.5 mg/ml), followed by Chinese (25 mg/ml). These values were higher than that recoded by Times *et al*. (2011) who found that the MIC value was 0.1 mg/ml, and Yaghoubi *et al*. (2007) who recorded that the concentration of 8.3 µg/ml resulted a inhibitory zone equal to 0.5 mm on the solid medium in the Iranian samples, and Ivančajič (2010) who found that the inhibitory diameter was 6 mm, and Ibrahim (2011) (MBC=1.37 mg/ml).

MBC value for *B. cereus* ranged from 12.5 and 50 mg/ml. the highest antibacterial activity was in Saqqa-Ameeni (12.5 mg/ml), followed by Chinese, Hungarian, Al-jurd, Al-Qalamon and Al-Ghota (MBC=25 mg/ml). These values were higher than that recorded by Times *et al*. (2011) (MIC=0.1 mg/ml), and Ibrahim (2011) (2.74 mg/ml) and Yaghoubi *et al*. (2007) (2.1 mm inhibitory zone at 4.1 µg/ml) and Ivančajič (2010) who found that the diameter of ethanolic extract of propolis was 4.8 in neutral medium.

In 1989 Abdulsalam *et al*. performed a study on the antibacterial activity of PEE and concluded that all Gram positive species (*B. cereus,B. subtilis, S. aureus, S. epidermidis, S. pyogenes*) stopped growth at a concentration of 100 ppm, while the Gram negative

bacteria stopped at higher concentrations (400 ppm for *E. cloacae* and *P. mirabilis*, 800 ppm for *P. aeruginosa* and *Serratia marcescens*, 1200 ppm for *E. coli, K. pneumoniae* and *Salmonella typhimurium*) (Hegazi, 2000). Cihangir *et al*. (2005) recorded that MIC values for Gram negative bacteria were 10-100 times higher than that for Gram positive bacteria in their study on PEEs from different regions of Turkey against three Gram positive bacteria (β -hemolytic Stre*ptococcu*s sp., *B. subilis*, *S. aureus*) and three Gram negative bacteria (*P. mirabilis, S. typhi, E. coli*), and Uzel *et al*. (2005) noticed that the Gram positive bacteria were more sensitive to low concentrations of propolis extracts, because Gram positive bacteria more sensitive to phenolic compounds than Gram negative bacteria (Raphaelli *et al*., 2019).

Conclusion:

Propolis is an important source of valuable active phenols. The current study aimed to estimate the total phenolic compounds in the ethanolic extracts of propolis samples collected from Syria and Jordan, and to evaluate antibacterial activity against some foodborne Gram-positive and Gram-negative bacteria. We found moderate contents of phenolic compounds, expressed as gallic acid equivalent (GAE), in propolis ethanolic extracts, ranged between 281.47 and 3046.18 mg GAE/100 g of crude propolis. Results revealed that Minimum Bactericidal Concentration (MBC) values against different Gram–positive and Gram–negative bacteria ranging between 3.125 and >50 μg/ml.

Recommendation:

We suggest investigating the potential of propolis ethanolic extracts to preserve food products, as they possess antibacterial properties, and Generally Recognized As Safe (GRAS) to be used in food industry**.**

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