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A Comparison between the Anti-microbial Activity of Native Propolis and the Anti-microbial Activity of Imported Ones against Different Health Microbes

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Abstract

Propolis is a gum-like product gathered by bees from various plants. It is known that the propolis has anti-bacterial, anti-carcinogenic, and immune-stimulating biological activities. In the present study, we have investigated the anti-microbial activity of propolis against certain important human microbes. The paper disc diffusion method was used to investigate the propolis activity and the inhibition zones were measured. Results revealed that the ethanolic and water extracts of propolis have a strong inhibitory potential against *Aspergillus brasiliensis* Varga and *Escherichia coli* Migula strain (ATCC 0157:H7) regardless of the time of propolis harvesting. The propolis ethanolic and water extracts were ineffective against *Escherichia coli* strain (ATCC 29522) and *Proteus mirabilis* Hauser. Jordan propolis was the most effective in inhibiting the *Enterobacter aerogenes* 35029 Hormaeche and Edwards than the Chinese, Turkish and Tablet propolis samples. Furthermore, the Jordan propolis and the Chinese crude 2 propolis were the most effective against the *Candida albicans* mold. The Chinese propolis was the most effective against *A. brasiliensis* mold.

Key words: Anti-bacterial activity, Anti-fungal activity, Human microbes, Propolis extract

1. Introduction

Propolis is a sticky, rubbery, brown, thermoplastic resin collected by bees from buds of trees. Honey bees use propolis in their hives as a repairing crevice, and as a surface cover, hardener and preservative. Also, it is probably used as a repellent since it is applied inside the beehive and around its entrance (Burdock, 1998).

There are a number of studies documenting the bio-cidal functions of propolis, its extracts and constituents (Marcucci *et al*., 2008; Mello *et al*., 2010). Several biological activities have been described for propolis, including anti-bacterial, anti-fungal, anti-protozoal, anti-viral, anti-tumor, immune-modulation and anti-inflammatory activities, beside other activities (Gomes, 2007). Fernandes *et al.* (1995) demonstrated the antimicrobial activity of propolis against bacterial and yeast pathogens isolated from human infection. Park *et al.* (1998) reported that the growth of the *Streptococcus*, an oral pathogen, was inhibited by the ethanol extract of propolis from various regions in Brazil. Moreover, it was reported that propolis is active against Gram-positive bacteria, yet it showed a limited activity against Gram-negative bacteria (Li-Chang *et al*., 2005). The anti-microbial activity of propolis is reflected in its constituents that may differ from area to area and from season to season depending on its chemical composition (Hegazi *et al*., 2000; Lu *et al*., 2003). Propolis has bactericidal and fungicidal properties, and it is used as an alternative treatment for infections. The wide range of action of propolis on various microorganisms is the result of the combined activities of flavonoids and aromatic compounds (Hemändez and Bemal, 1990; Sforcin *et al.*, 2000; Ivanĉajiĉ *et al*., 2010). On the other hand, Li-Chang (2005) reported that the mechanism of anti-microbial activity is complicated and could be attributed to synergism between flavonoids hydroxyl acids and sesquiterpenes. Krol *et al.* (1993) also observed this effect.

In Jordan, although a widely distributed propolis flora is present, that are of common use in the folk medicine,

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and high number of honeybee colonies, there are no further studies on the type of the propolis and/or the chemical composition of each, and their anti-microbial activities. Based on these observations, the aim of this study is to investigate the anti-microbial activity of the ethanolic and the water extracts of the propolis from Jordan in comparison to the other sources of propolis produces widely against different species and strains of bacteria and fungi including: *Aspergillus brasiliensis*, *Candida albicans* strain (ATCC 18814), *Enterobacter aerogenes* 35029, *Escherichia coli* strain (ATCC 25922), *Escherichia coli* strain (ATCC 0157:H7), *Klebsiella oxytoca* 18182, *Klebsiella pneumonia* 13883, *Methicillin* resistant *Staphylococcus aureus* 29974, *Proteus mirabilis*, *Proteus vulgaris* 13315, *Pseudomonas aeruginosa* 27253, *Salmonella typhimurium* 19430, and *Staphylococcus aureus* 25923.

2. Material and methods

2.1. Propolis Extracts

Propolis was collected from the bee hives monthly for one year. The collection time of the local propolis was done in the periods from (February to April), (May to July), and (August to October) of 2012 to make sure that all propolis types were obtained during the whole year. The main plant sources of propolis during these periods were oak (in Badr, Naour and Salt areas), pine (in Badr, Naour and Salt areas) and almond trees (in Naour, North Ghour and Wadi Shuaib areas) where bee hives were located. The Jordan propolis was compared with other four non-Jordanian types. The imported Chinese propolis includes: the crude 1 propolis which is found in the local market in a liquid phase, and the crude 2 propolis which is found in a powder form. A tablet propolis form and a Turkish propolis (crude form) were also used in order to confirm the effect of area and environmental conditions on the propolis physiological activities.

Water extract of propolis (from different sources) was obtained as described by Suzuki (1990) with a slight modification according to Osman and Taha (2008), where 20.0g of propolis was suspended and extracted with 5 volumes of distilled water with shaking using shaker (GFL3005, Gesellschaft fur, D-30983, Germany) at 300 rpm. Then the mixture was boiled at 70°C for 7 hours and left at room temperature to cool down. The extract was centrifuged at 3000g for 15 minutes (Beckman Allegra 21R Refrigerated Bench Top Centrifuge, UK LABS Direct Ltd.), and the supernatant was taken. The obtained supernatant was then concentrated using a concentrator (Rotational vacuum concentrator for laboratory RVC 2-18 CD, CHARIST) at 45°C until the concentration reached 10 mg/100 microliter. Later, the extracts were collected and stored in tightly closed dark bottles at 4°C until being used in the assay. The ethanolic extract of propolis (from the different sources) was obtained as described by Valdes Gonzales *et al.* (1985), where 300g of finally grounded and dried propolis were placed in 700 ml of concentrated ethanol in a container to obtain a 30% of extract. The mixture was shaken for two weeks at room temperature in a dark place using a shaker at 450 rpm. The extract was filtered through a coffee filter and then filtered again with a filter paper (Whatman No. 1). The filtrate was concentrated by putting on glass Petri dishes and left at room temperature until all the ethanol is evaporated. The extract is then re-dissolved with ethanol and stored in tightly closed, dark bottles at 4°C until being used in the assay.

2.2. Test Organisms

Standardized pure cultures of fungi and bacterial species and strains, procured (Microbiologics Inc., Minnesota-USA) by the Department of Biotechnology, Al-Balqa Applied University, were used in the present study. The microbes were chosen according to the frequency in which they were used in previous studies and also according to the frequency of infections in human beings. Pure cultures of tested microorganisms were cultured in nutrient broth (Acumedia Manufacturers, Inc., Maryland) and stored at 4°C until being used. Each test microorganism was re-cultured in this manner three days in succession before the experiment.

2.3. Determination of Anti-microbial Activity

Stock cultures of bacteria were grown in nutrient broth at 26–27°C for 24 hours with shaking and enumerated using a serial dilution method. One ml of each of the microbes' cultures was separately poured in 9 ml of sterile distilled water, and hereafter 8-fold serial dilutions were made (Kango, 2010). Final cell concentrations were 10^7–10^8 cfu/ml. Disk diffusion assay was performed according to the protocol recommended by NCCLS (National Committee for Clinical Laboratory Standards, 2000) to detect the anti-microbial activity of the propolis extracts. Sterilized filter paper discs (5 mm in diameter) soaked with 15 ul of propolis extracts in absolute ethanol (Merck–Darmstadt, Germany) were put in the middle of Mueller-Hinton Agar plates inoculated with suspensions of cells adjusted to McFarland turbidity standards equals to 0.5 using Mueller-Hinton Broth. The absolute ethanol was used as a negative control and the penicillin (10 µg) as a positive control. The plates were incubated at 37°C and observed after 24 hours. A digital caliper was used to measure the diameters of the zones of inhibition.

2.4. Statistical Analysis

The mean values and the standard deviation were calculated from the data obtained from triplicate trials. Means were then compared using the least significant difference test (SAS, 2001). A probability level of 5% was considered statistically significant.

3. Results

Results revealed that both the ethanolic and the water extracts of the local propolis, harvested during different periods of the year, resulted in variable inhibitory effects on the tested microbes. The propolis ethanolic extracts were anti- *E. coli* strain (ATCC 0157:H7) and anti- *A. brasiliensis* regardless of the time of the propolis harvesting time ($F_{11,24}$= 33.8, P<0.0001). By the passage of time, however, the ethanolic extract activity extended to include *K. pneumonia* 13883 ($F_{11,24}$= 246.7, P<0.0001) (Table 1). The propolis water extracts were anti- *E. aerogenes*, anti- *E. coli* strain (ATCC 0157:H7), anti-

Methicillin resistant *S. aureus* 29974 and anti- *A. brasiliensis* ($F_{11,24}$= 192.3, P<0.0001). However, for the water extracts, their inhibition activities extended to include *P. vulgare* and *S. typhimurium* with the passage of time ($F_{11,24}$= 210.1, P<0.0001) (Table 1). *P. mirabilis, E. coli* strain (ATCC 29522) and *P. vulgare* were the most resistant microbes to the ethanolic extracts regardless of the time of the propolis harvesting time. *P. mirabilis, E. coli* strain (ATCC 29522) and *E. aerogenes* were the most resistant microbes to the water extracts (Table 1). Regarding the effect of propolis against microbes in respect to the time of propolis collection, the August-October propolis water extract was the most effective one against most of the microbes tested ($F_{11,24}$= 115.6, P<0.0001) followed by the August-October propolis ethanolic extract ($F_{11,24}$= 208.6, P<0.0001). The May-July propolis water extract was ranked the third in inhibiting the microbes ($F_{11,24}$= 115.6, P<0.0001) (Table 1). The standard antibiotic, penicillin, was superior over all the propolis extracts against 6 out of 12 microbes, these are *E. aerogenes, E. coli* strain (ATCC 29522), *E. coli* strain (ATCC 0157:H7), *Methicillin* resistant *S. aureus* 29974, *P. mirabilis, P. vulgare* and *S. aureus* 25923 (Table 1).

The impact of the Jordan propolis and the imported propolis on inhibiting different bacterial microbes is shown in (Table 2). The Jordan and Turkish propolis were effective against all microbes. The propolis crude 1 from China and the tablet propolis were effective against all tested microbes except against *P. mirabilis* (Table 2). Furthermore, the Chinese crude 2 propolis was effective only against two of the tested microbes; these are *E. coli* strain (ATCC 29522) and *E. coli* strain (ATCC 0157:H7) (Table 2). A comparison of the Jordan propolis with the others was done against each microbe.

Table 1. Effect of different local propolis ethanolic and water extracts against different microbes

Inhibition Zone (mm) ± Standard Error	Propolis extract[2]	Local Feb -April ethanolic ext	Local May-July ethanolic ext	Local Aug – Oct. ethanolic ext	Local Feb. - April water ext	Local May - July water ext	Local Aug. – Oct. water ext	Penicillin
	Aspergillus brasiliensis	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	-
	Enterobacter aerogenes 35029	14.3 ± 0.07 e A	11.0 ± 0.04 j B	13.0 ± 0.04 j A	10.8 ± 0.03 j B	12.5 ± 0.03 j A	13.5 ± 0.05 e A	5.0 ± 0.05 C
	Escherichia coli (ATCC 29522)	9.7 ± 0.03 e A	0.00 ± 0.00 k C	12.3 ± 0.03 j A	0.00 ± 0.00 j C	11.0 ± 0.07 j A	12.0 ± 0.07 e A	7.0 ± 0.04 B
	Escherichia coli (ATCC 0157:H7)	30.0 ± 0.00 a A	27.0 ± 0.07 b B	30.0 ± 0.00 a A	27.3 ± 0.06 b B	30.0 ± 0.00 a A	30.0 ± 0.00 a A	5.0 ± 0.04 C
	Klebsiella oxytoca 18182	17.0 ± 0.40 c A	20.0 ± 0.00 e A	24.3 ± 0.05 d A	15.5 ± 0.03 e A	25.0 ± 0.00 c A	12.3 ± 0.07 e A	0.0 B
	Klebsiella pneumonia 13883	15.0 ± 0.00 d C	27.0 ± 0.04 b A	26.8 ± 0.03 b A	17.8 ± 0.05 d B	25.5 ± 0.03 c A	25.3 ± 0.03 c A	0.0 D
	Methicillin resistant *Staphylococcus aureus* 29974	16.0 ± 0.00 c B	23.3 ± 0.12 d A	25.0 ± 0.00 c A	21.5 ± 0.10 c A	25.5 ± 0.03 b A	25.3 ± 0.03 b A	5.0 ± 0.03 C
	Proteus mirabilis	0.00 ± 0.00 f D	0.00 ± 0.00 k D	20.0 ± 0.0 e A	0.00 ± 0.0 j D	0.00 ±0.00 k D	13.3 ±0.03 e B	5.0 ± 0.04 C
	Proteus vulgaris 13315	12.0±0.00 e D	10.8 ±0.03 j D	13.3 ±0.05 f C	11.8 ± 0.03 f D	14.8 ± 0.03 e B	18.8 ± 0.0 d A	3.0 ± 0.03 E
	Pseudomonas aeruginosa 27253	25.0 ± 0.00 b A	20.5 ± 0.03 e B	21.0 ± 0.04 e B	20.5 ± 0.03 d B	23.8 ± 0.05 c A	23.5 ± 0.03 c A	0.0 C
	Salmonella typhimurium 19430	20.0 ± 0.00 c C	23.7 ± 0.06 c B	24.3 ± 0.05 d B	20.0 ± 0.00 d C	29.5 ± 0.05 a A	30.0 ± 0.00 a A	0.0 D
	Staphylococcus aureus 25923	19.3 ± 0.03 c A	14.8 ± 0.03 f A	19.0 ± 0.07 e A	18.8 ± 0.08 d A	20.5 ± 0.09 d A	17.0 ± 0.12 d A	10.0 ± 0.07 B

* Values with different small letters in the same column are significantly different (P < 0.05)

** Values with different capital letters in the same row are significantly different (P < 0.05) (effect of time of propolis collection)

Table 2. Effect of different local Jordan and imported (Chinese and Turkish) propolis ethanolic extracts against different microbes

	Propolis extract	Chinese Propolis (crude1)	Chinese Propolis (crude2)	Jordan Propolis	Tablet Propolis	Turkish Propolis	Penicillin
Inhibition Zone (mm) ± Standard Error	*Enterobacter aerogenes* 35029	20.0 ± 0.12 a A	21.7 ± 0.03 c B	26.3 ± 0.07 a A	15.7 ± 0.03 b C	10.3 ± 0.03 b D	5.0 ± 0.06 E
	Escherichia coli (ATCC 29522)	20.3 ± 0.09 a B	35.7 ± 0.19 a A	16.0 ± 0.06 b C	21.3 ± 0.09 a B	21.3 ± 0.09 a B	7.0 ± 0.05 D
	Escherichia coli (ATCC 0157:H7)	23.0 ± 0.12 a B	25.7 ± 0.09 b A	11.3 ± 0.09 b D	20.3 ± 0.03 a C	9.7 ± 0.03 b D	5.0 ± 0.04 E
	Klebsiella pneumonia 13883	14.3 ± 0.12 b D	20.3 ± 0.03 c A	13.7 ± 0.18 b B	14.7 ± 0.03 b B	9.0 ± 0.06 b C	0.0 E
	Methicillin resistant *Staphylococcus aureus* 29974	22.0 ± 0.06 a A	23.0 ± 0.06 c A	6.0 ± 0.06 b B	20.7 ± 0.03 a A	7.0 ± 0.06 b B	5.0 ± 0.05 C
	Proteus mirabilis	9.3 ± 0.00 c B	19.0 ± 0.00 c A	9.7 ± 0.07 b B	11.3 ± 0.07 c B	9.3 ± 0.03 b B	5.0 ± 0.04 C
	Proteus vulgaris 13315	19.3 ± 0.12 a A	20.3 ± 0.03 c A	10.3 ± 0.03 b B	18.7 ± 0.03 a A	6.3 ± 0.03 b C	3.0 ± 0.02 D
	Pseudomonas aeruginosa 27253	20.0 ± 0.07 a A	17.3 ± 0.07 c B	10.3 ± 0.00 b C	20.0 ± 0.07 a A	7.0 ± 0.07 b D	0.0 E
	Salmonella typhimurium 19430	21.3 ± 0.06 a A	17.7 ± 0.07 c B	7.7 ± 0.03 b C	17.3 ± 0.00 a B	8.7 ± 0.03 b C	0.0 D
	Staphylococcus aureus 25923	13.0 ± 0.12 b C	13.0 ± 0.06 d C	17.0 ± 0.03 b B	19.7 ± 0.07 a A	8.0 ± 0.05 b E	10.0±0.06 D

* Values with different capital letters in the same column are significantly different ($P < 0.05$)

** Values with different small letters in the same row are significantly different ($P < 0.05$)

Jordan propolis ranked the first as anti- *E. aerogenes* ($F_{4,10}$= 141.86, P<0.0001) and the second against *K. pneumonia* 13883 ($F_{4,10}$= 18.28, P<0.0001), Methicillin resistant *S. aureus* 29974 ($F_{4,10}$= 129.34, P<0.0001), *P. mirabilis* ($F_{4,10}$= 40.71, P<0.0001), *P. vulgare* ($F_{4,10}$= 129.34, P<0.0001) and *S. aureus* 25923 ($F_{4,10}$= 51.24, P<0.0001) (Table 2). The Turkish propolis was effective against only three of the microbes; it ranked the second against *E. coli* strain (ATCC 29522) ($F_{4,10}$= 45.43, P<0.0001), Methicillin resistant *S. aureus* 29974 and *P. mirabilis.* The Chinese crude 1 propolis was super anti-microbe against all microbes except for *K. pneumonia* 13883 and *S. aureus* 25923. The Chinese crude 2 propolis ranked the first or second against all microbes tested except for *S. aureus* 25923. The tablet propolis ranked the first as anti-Methicillin resistant *S. aureus* 29974, *P. vulgare, P. aeruginosa* 27253 ($F_{4,10}$= 69.07, P<0.0001) and *S. aureus* 25923. Penicillin was superior over the Jordan propolis and also superior over the other four types of propolis against all microbes except *K. pneumonia* 13883, *P. aeruginosa* 27253 and *S. typhimurium* (Table 2).

A comparison of the impact of the Jordan propolis and the imported propolis on inhibiting two different fungi species is shown in (Figure 1) based on the ethanolic extracts. The Chinese crude 1 propolis was the most effective against the *A. brasiliensis* and the Turkish propolis was the least effective (Figure 1). The Chinese crude 2 propolis was the most effective against the *C. albicans*, while the Turkish propolis was also the least effective. Moreover, the Jordan propolis ranked the second in its effectiveness against *C. albicans* among the tested propolis of different sources (Figure 1).

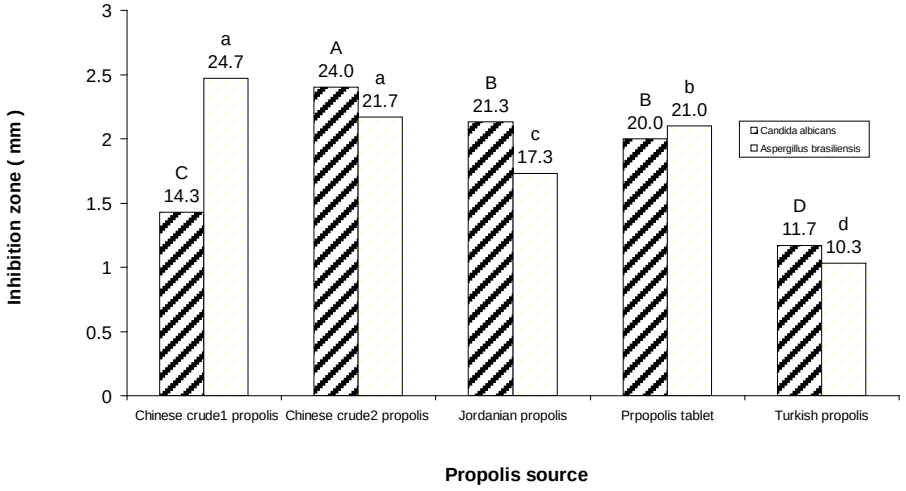

Figure 1. Effect of local Jordan ethanolic extracts and imported Chinese, Turkish and Tablet propolis against *Candida albicans* and *Aspergillus brasiliensis* molds. Means in columns for each fungus with same letter are not significantly different using LSD at 0.05.

4. Discussion

Over the last several years, a worldwide trend has been observed in the use of natural products like propolis due to its safe and its multidirectional biological properties (Topcuoglu *et al.*, 2012). It has been used commercially on the market as a component of toothpaste, mouth rinses, lozenges, and so forth. Both ethanolic and water extracts of the local propolis used in the present study demonstrated an anti-bacterial and anti-fungal activity against the tested human microbes. It inhibited the growth of ten out of the twelve microbes investigated; whereas *E. coli* strain (ATCC 29522) and *P. mirabilis* were not inhibited. Propolis composition is mentioned to be responsible for the anti-bacterial and anti-fungicidal biological properties (Marcucci, 1995). Chang *et al.* (2002) reported that the propolis samples collected in Taiwan contained various amounts of flavones, flavonols, flavanones, and isoflavones. Also, these constituents are generally regarded by Hemändez and Bemal (1990) and Sforcin *et al.* (2000) to be responsible for the anti-microbial activity of the propolis. Furthermore, Marcucci (1995) attributed the anti-bacterial effect of propolis mainly to the flavonoids.

The local propolis ethanolic extracts showed weak potential effects against the *S. aureus* 25923. This finding is in disagreement with the results of Ehsani *et al.* (2013) who found an anti-bacterial activity of the ethanolic extract against this microbe. On the other hand, our results confirmed the results of Ehsani *et al.* (2013) regarding the weak effect of the propolis aqueous extract against the *S. aureus* 25923. Moreover, Kashi *et al.* (2011) reported that the Iranian propolis ethanolic extract showed a bactericidal activity against *S. aureus* 25923. Lu *et al.* (2003) also observed that the ethanolic extracts of propolis samples from different regions in Taiwan also exerted various extents of anti-bacterial activities.

All the inhibited bacteria (*E. aerogenes*, *E. coli*, and *K. pneumonia* 13883) by the local Jordan propolis are gram negative bacteria. This type of propolis-bacteria relationship is demonstrated by Ozan *et al.* (2007). Furthermore, Ozan *et al.* (2007) found that these propolis solutions showed moderate effect on the fungus *Candida* strains. This finding is in full agreement with the results obtained in this study due to the Jordan propolis against the *C. albicans* in which the local propolis ranked the second when compared with the Chinese propolis, the Turkish propolis and the Tablet propolis. Same results were also obtained by Possamai *et al.* (2013) where the adsorbed Brazilian propolis to polyethylene glycol microspheres had a stimulatory effect on these cells to assist in combating the *C. albicans*. Moreover, Egyptian propolis ethanol extract in the concentration range 25 - 125 ng/µL were used to inhibit the adhesion of oral *Candida* and, therefore, preventing its colonization (Gomaa and Gaweesh, 2013). Moreover, the results of Khosravi *et al.* (2013) proved that the propolis inhibits the growth of pathogenic yeasts and confirmed the efficiency of propolis as an anti-*Candida* agent.

Jordan is mostly classified within the arid desert zone and the rainy season may extend from November to April at best (Al-Eisawi, 1996). The situation is largely different in China which is divided into tropical, subtropical and temperate zones. Most of China (www.warriortours.com/climate/) and Turkey (Weather Online, UK) lies in the warm temperate zone. Consequently, the vegetations and the biodiversity of China and Turkey flora are different from the Jordan flora. Hence, the chemical composition of the propolis from these countries is also different (Burdock, 1998). Therefore, their propolis efficacy is expected to be different. Results obtained here confirmed this assumption, while all types of propolis differed significantly in their inhibition ability to the investigated microbes (Table 2). The role of the geographic origin on the propolis anti-bacterial activity is supported by many researchers (Cheng and Wong, 1996; Kujumgiev *et al.*, 1999; Nieva Moreno *et al.*, 1999; Santos *et al.*, 1999). Identifying the active ingredient that could be responsible for the biological activities of the local propolis, revealed in the present study, is of vital importance.

In conclusion, the study showed a positive inhibitory influence of the local propolis ethanol and water extracts with respect to the *A. brasiliensis*, *E. coli* strain (ATCCO 157:H7), and *K. pneumonia* 13883. The Jordan propolis was superior to the Chinese propolis, the Turkish, and the Tablet propolis in its inhibitory effect against *E. aerogenes*. Moreover, Jordan propolis ranked second in inhibiting the *C. albicans* mold.

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