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Detection of Some Active Compounds in Aqueous and Ethanolic Extracts of Iraqi Propolis and Examine Their Antibacterial Effects

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Abstract: The crude Iraqi propolis has been collected from an area surrounding by eucalyptus trees at the west of Baghdad city. The aqueous and ethanolic total soluble solids of crude Iraqi propolis were found to be 13.9 and 34.5% respectively. The quantitatively detection of propolis extract refer to the presence of phenolic compounds (such as flavonoids) as the mainly active components. Spectrophotometric analysis was used to determine flavonoids in the local propolis extracts. Flavones and flavonols were determined using aluminum chloride and expressed as quercetin equivalent and were found 2.1 and 6.71% in aqueous and ethanolic extracts respectively. The flavanones were determined using 2, 4-dinitrophenyl hydrazine and expressed as naringenin and found to represent 4.27 and 13.65% of aqueous and ethanolic extracts. Total phenolic, free phenolic and tannin compounds were determined in crude propolis using Lowenthal-Proctor method and were found 9.98, 8.32 and 1.66% by using aqueous extraction and 24.80, 22.72 and 2.08% by ethanol extraction respectively. The inhibitory action of aqueous and ethanolic extract with concentrations 120, 240…to 1200 and 2000, 2500…to 4000 ppm were examined against gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus* and *Bacillus stearothermophilus*) and gram-negative bacteria (*Escherichia coli*, *Salmonella enteritidis* and *Pseudomonas fluorescens*). The ethanolic extract of local propolis showed highly inhibitory percentages (up to 83.80% against gram-negative and 87.80% against gram-positive) as compared with aqueous extract (70.58% against gram-negative and 59.59% against gram-positive).

Key words: Iraqi propolis, active compounds, antibacterial agents

INTRODUCTION

Propolis is a natural resinous substance (bee glue) collected by honey bees (*Apis mellifera*) from buds and leaves of trees and plants mixing with pollen, bee wax and 13-glycosidase bee salivary enzymes, which causing the hydrolysis of the glycosyl flavonoids, originating flavonoids aglycones (Crane, 1998; Pereira *et al*., 2002). Propolis was applied the combs and walls of the hive, thereby insulating and reinforcing the hives as well as making the environment aseptic (Ildenize *et al*., 2004). The crude propolis is composed basically of 55% vegetable resins and balsam, 30% bee wax, 10% essential oil and 5% pollen (Ildenize *et al*., 2004). The chemical analysis revealed that propolis contains more than 300 constituents among them the phenolic compounds, including flavonoids as major components (Siegfried *et al*., 2003). Flavonoids, one of the main groups of phenolic compounds in propolis, are the key compounds for estimation of propolis quality. Flavonoids in propolis are aglycons (without the sugar component). These lipophilic flavonoids are chemically divided into subgroups of flavones, flavonols flavonones, dihydroflavonols and chalcones (Marcucci *et al*., 2001; Tapiero *et al*., 1998). The concentration of flavonoids in propolis depends on the geographic origin and plant sources (Nieva *et al*., 1999). It is known that the flavonoid concentration will affect the biological activity of propolis (Kujumgiev *et al*., 1999).

Propolis have been used as a folk medicine since antiquity, mainly as an antibiotic (Hegazi *et al*., 2000), anti-cancer agent (Banskota *et al*., 2001; Lyudmila *et al*., 2005) and wound healing promoter (Bretz *et al*., 1998; Vennat *et al*., 1998). Recently, propolis is used in cosmetic products such as face creams and lotions and has gained popularity as food supplement in numerous countries (mainly Asia) claimed to improve health and prevent diseases (Marcucci and Bankova, 1999; Monks *et al*., 1991; Pereira *et al*., 2002).

Although many active compounds have been identified in propolis and its antimicrobial activity over the entire world, no sufficient studies have been known dealing with Iraqi propolis, especially of the western region of Baghdad. The present work identifies the mainly active compounds in the local propolis and shows their inhibitory effects against some bacterial isolates.

MATERIALS AND METHODS

The local propolis has been collected during spring season of 2010 from the bee hives at Al-Radhwaniah area-Abu Ghraib (to the west of Baghdad city) from an area surrounding by eucalyptus trees. The samples were stored under freezing conditions. 10 grams of

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slicing propolis have been refluxed with 100 mL of distilled water or 95% ethanol at the boiling point for 1 h. Primary filtration through a piece of cloth was done before using whattman filter paper No.1. The soluble extracts were placed into large petteridishes in an air oven (Thelco, model 15) at 50°C till complete dryness. The scraped matter was kept in clean screw test tubes. The propolis extracts (aqueous and ethanolic) were subjected to total phenolic, free phenolic, tannins and flavonoids compounds determination. A spectrophotometeric method (UV/VIS Spectroscan 80 D Spectrophotometer) were used (Ivan *et al*., 2004) to determined flavonoids. A standard curve of quercitine with 12.5, 25, 50, 80 and 100 µg/mL concentrations has been prepared to determine flavones and flavanols at 415 nm. The flavanones were determined by using naringenin standard curve with concentrations of 0.25, 0.50, 1.00, 1.50 and 2.00 mg/mL and the absorbance were records at 495 nm. The total phenolic, free phenol and tannins were determined according to Lowenthal-Proctor method (Harold *et al*., 1981).

The bacterial isolates (*Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas fluorescence*, *Bacillus cereus*, *Staphylococcus aureus* and *Bacillus stearothermophilus*) were obtained from Food Sciences and Biotechnology Department Laboratories, College of Agriculture, University of Baghdad. The cultures were maintained on nutrient agar slants for 18 hr at 37°C and 24 hr at 55°C (for *Bacillus stearothermophilus*). Different concentrations (120, 240,.....to 1200 and 2000, 2500,.....to 4000 ppm) of aqueous and ethanolic extracts were examined for their inhibitory effects against the bacterial isolates by using Total Plate Count (to account the inhibition percentages) (Alken-Murray Corporation, 2006) and Disc Diffusion Method (Rula *et al*., 2010). 0.1 mL of activated bacterial suspension was spread on nutrient agar dishes by using L-shape glass rode, 10 µL of each extract concentration was added to triplicate 4 mm discs. The plates were incubated and the inhibition activity for each concentration was determined by measuring the inhibition zone diameter. Control plates with and without alcohol have been done.

RESULTS AND DISCUSSION

The crude local propolis, which was collected from bee hives, has a dark greenish brown color and hardly sliced by using a kitchen knife. The dark color may due to the presence of the natural colored matters in buds and leaves of eucalyptus trees. The total soluble solids (TSS %) of aqueous and ethanolic of the local propolis were 13.97 and 34.50 respectively. These percentages are less as compared with Brazillian propolis extracts which gave 4-14% and 41-60% TSS respectively (Ildenize *et al*., 2004). The high percentage of TSS in alcoholic extract due to some extractable components of local propolis (like resins and bee wax) by 95% ethanol, in addition to the phenolic compounds, especially flavonoids, which are lipophilic compounds and more soluble in alcohol as compared with water, which is more polar. The quantitatively analysis of Iraqi propolis showed, that the inhibitory action due to the presence of phenolic compounds (such as flavonoids) rather than glycosides and alkaloids (Table 1). Many studies were reported, that the antibacterial activities of propolis extracts due to the presence of phenolic compounds (Rula *et al*., 2010; Yaghoubi *et al*., 2007; Christian *et al*., 2010; Melina *et al*., 2007; AnaCarla *et al*., 2006).

Table 1: The main active compounds in aqueous and ethanolic extracts of Iraqi propolis

The active compound (%)	Aqueous extract	Ethanolic extract
Total phenolic compounds	9.98	24.80
Free phenolic compounds	8.32	22.72
Tannins compounds	1.66	2.08
Flavones and flavonols	2.10	6.71
Flavanones	4.27	13.65

Table 2 showed the effect of aqueous extract concentration against gram-negative bacteria. There was no inhibitory action within the concentration below 960 ppm. The inhibitory effect of aqueous extract of local propolis against *Salmonella enteritidis* and *Pseudomonas fluorescens* appear at 960 ppm, while *Escherichia coli* resists the aqueous extract till concentration of 2000 ppm. No gradually inhibition has been associated with the increasing in aqueous extract concentrations.

Table 3 shows the unexpected resistance of gram-positive bacteria (*Bacillus stearothermophilus* and *Bacillus cereus*) as compared with gram-negative (*Salmonella enteritidis* and *Pseudomonas fluorescence*, Table 2) against the inhibitory action of aqueous extract of the local propolis. In general, the cell walls of gram-negative bacteria have more complicated structure than do those of gram-positive bacteria (Anthony, 1971). Outside the cytoplasmic membrane is the periplasm, which contains the thin layer of peptidoglycan. The peptidoglycan in gram-negative cell contains less cross-linking than in gram-positive cells with no peptide linker. Covalently bound to the peptidoglycan is Braun's lipoprotein, which has a hydrophobic anchor in the outer membrane that helps to strongly bind the peptidoglycan to the outer membrane. In gram-positive cell walls there was a teichoic acid, which it is a phosphodiester polymer of glycerol or ribitol joined by phosphate groups. Amino acids such as D-alanine are attached. Teichoic acid is covalently linked to muramic acid and stitches various layers of the peptidoglycan mesh together. Teichoic acid stabilizes the cell wall and makes it stronger (Pastian, 2010). According to the previous explanation, we can declare, there was a cross-linking take place between the amino acids fragment of teichoic acid and the phenolic compounds in propolis extract.

Table 2: The antibacterial effect of aqueous extract of Iraqi propolis against gram-negative bacteria

Extract	The diameter of inhibition zone (mm) and the inhibition percentages					
	Escherichia coli		*Salmonella enteritidis*		*Pseudomonas fluorescence*	
Conc. ppm	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition
120	-	00.00	-	00.00	-	00.00
240	-	00.00	-	00.00	-	00.00
360	-	00.00	-	00.00	-	00.00
480	-	00.00	-	00.00	-	00.00
600	-	00.00	-	00.00	-	00.00
720	-	00.00	-	00.00	-	00.00
840	-	00.00	-	00.00	-	00.00
960	-	00.00	4.5	10.00	4.8	16.67
1080	-	00.00	5.6	28.57	5.4	25.93
1200	-	00.00	7.5	46.66	6.6	39.39
2000	05.00	20.00	8.2	51.21	7.6	47.36
2500	06.20	35.40	9.0	55.55	9.1	56.04
3000	07.60	47.37	10.5	61.90	10.8	62.96
3500	09.80	59.18	12.4	67.74	12.0	66.66
4000	10.20	60.78	13.1	69.46	13.6	70.58

Table 3: The antibacterial effect of aqueous extract of Iraqi propolis against gram-positive bacteria

Extract	The diameter of inhibition zone (mm) and the inhibition percentages					
	Staphylococcus aureus		*Bacillus cereus*		*Bacillus stearothermophilus*	
Conc. ppm	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition
120	-	00.00	-	00.00	-	00.00
240	-	00.00	-	00.00	-	00.00
360	-	00.00	-	00.00	-	00.00
480	-	00.00	-	00.00	-	00.00
600	-	00.00	-	00.00	-	00.00
720	-	00.00	-	00.00	-	00.00
840	-	00.00	-	00.00	-	00.00
960	04.70	14.90	-	00.00	-	00.00
1080	05.00	20.00	-	00.00	-	00.00
1200	05.60	28.57	-	00.00	-	00.00
2000	06.10	34.42	-	00.00	-	00.00
2500	06.80	41.17	-	00.00	-	00.00
3000	07.60	47.36	-	00.00	-	00.00
3500	08.80	54.54	04.60	13.00	-	00.00
4000	09.90	59.59	05.50	27.27	-	00.00

Table 4: The antibacterial effect of ethanolic extract of Iraqi propolis against gram-negative bacteria

Extract	The diameter of inhibition zone (mm) and the inhibition percentages					
	Escherichia coli		*Salmonella enteritidis*		*Pseudomonas fluorescence*	
Conc. ppm	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition
120	-	00.00	-	00.00	-	00.00
240	-	00.00	-	00.00	-	00.00
360	05.20	23.00	-	00.00	-	00.00
480	06.40	37.50	04.90	18.37	-	00.00
600	08.20	51.21	05.70	29.82	06.00	33.33
720	08.80	54.54	07.20	44.44	09.00	55.56
840	09.70	58.76	10.60	62.26	12.50	68.00
960	10.20	60.78	11.40	64.91	13.50	70.37
1080	11.90	66.38	13.60	70.58	14.20	71.83
1200	13.10	69.46	14.40	72.22	16.60	75.90
2000	13.70	70.80	15.30	73.85	18.30	78.14
2500	14.60	72.60	16.20	75.31	19.20	79.16
3000	15.60	74.35	18.10	77.91	20.70	45.67
3500	16.10	75.15	19.10	79.05	22.80	82.45
4000	17.50	77.14	20.50	80.48	24.70	83.80

Table 5: The antibacterial effect of ethanolic extract of Iraqi propolis against gram-positive bacteria

Extract	The diameter of inhibition zone (mm) and the inhibition percentages					
	Staphylococcus aureus		*Bacillus cereus*		*Bacillus stearothermophilus*	
Conc. ppm	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition	Diameter (mm)	% inhibition
120	-	00.00	-	00.00	-	00.00
240	-	00.00	-	00.00	-	00.00
360	-	00.00	-	00.00	05.00	20.00
480	-	00.00	08.40	52.38	08.20	51.20
600	-	00.00	14.60	72.60	12.30	67.47
720	-	00.00	18.60	78.50	15.90	74.84
840	-	00.00	22.00	81.81	18.60	78.49
960	04.60	13.00	23.40	82.91	21.20	81.13
1080	05.80	31.00	24.60	83.74	24.20	83.47
1200	08.70	54.02	26.30	84.79	26.20	84.73
2000	11.50	65.21	27.20	85.29	28.40	85.91
2500	13.00	69.23	27.90	85.66	29.40	86.39
3000	14.30	72.02	28.70	86.06	29.70	86.53
3500	15.70	74.52	29.10	86.25	30.90	87.05
4000	18.40	78.26	30.00	86.66	32.80	87.80

Such binding will scavenge some of the active phenolic compounds such as flavonoids. This phenomenon looks like the stringency effect of tannins on the saliva proteins.

Table 4 and 5 show the highly inhibitory effect of ethanolic extract of propolis as compared with the aqueous extract, due to the ability of 95% ethanol to extract the active components of local propolis, especially flavonoids, which are lipophilic compounds and more soluble in alcohol as compared with water, which is more polar. The flavonoids of propolis are easily soluble in organic solvents, such as ethanol and poorly soluble in water. The polarity of high concentration of ethanol is weak, while that of water is very strong (Zhang *et al*., 2005). Dizaji *et al*. (2008) mentioned, that the ethanolic extract of propolis exhibited an inhibition in the growth of all examined microorganisms including bacteria and fungi showing the highest antibacterial activity against gram-positive bacteria such as *staphylococcus*.

Conclusion: The active compounds in Iraqi propolis are a reflex to the trees from which it's collected by the honey bees. The mainly active components of the local propolis are the phenolic compounds, especially flavonoids (Flavones, Flavonols and Flavanones). The ethanolic extract of the local propolis is more effective as compared with aqueous extract at the same concentration. High extract concentration gave high inhibitory effect. In general, the gram-negative bacteria are more resistible than gram-positive. The aqueous and ethanolic extracts of propolis (especially, the ethanolic) can serve as natural potential preservative for processed foods. Furthermore, the literatures are talking about the antioxidant characteristics of the phenolic compounds (especially flavonoids) to scavenge the bad effects of free radicals, so the propolis extracts play an important role as anticancer. It is also known to exhibits some pharmacological activities, such as antiviral, antifungal, anti-inflammatory in addition to its anesthetic and cystostatic properties. We must thinking of the propolis as additive in our future diets.

REFERENCES

Alken-Murray Corporation, 2006. Plate Count Procedure. Qualitics Control Method-1. Count of Aerobic Microorganisms and Spore Count. Revised by Valerie Anne Edwards on Oct. 30: 2006, pp: 1-8.

AnaCarla, P.O., S.S. Cristiane, L. Renata and L.F. Selma, 2006. Antifungal activity of propolis extract against yeast isolated from onychomycosis lesions. Mem Inst Oswaldo Cruz, Rio de Janeiro, 101: 493-497.

Anthony, H.R., 1971. Chemical Microbiology: An Introduction to Microbial Physiology (3rd Edn.). Butterworth and Co. (Publishers) Ltd. London, pp: 30-60.

Banskota, A.H., Y. Tezuka and S. Kadota, 2001. Recent progress in pharmacological research of propolis. Phytother. Res., 15: 561-571.

Bretz, W.A., D.J. Chiego Jr., M.C. Marcucci, I. Cunha, A. Custodio and L.G. Schneider, 1998. Preliminary report on the effect of propolis on wound healing in the dental pulp. Z. Natuforsch, 53c: 1045-1048.

Christian, L.H., A. Marysol, B. Leticia, M. Gloria and A.S. Luis, 2010. The antifungal effect of six commercial extracts of Chilean propolis on *Candida* sp. Cien. Inv. Agr., 37: 75-84.

Crane, E., 1998. The past and present importance of bee products to man. In: Mizarahi, A. and Y. Lensky. Bee Products: Properties, application and apitherapy. Plenum Press, New York, pp: 1-14.

Dizaji, A.A., E. Valizadeh, H.M. Alishah, A. Shaddel and N. Maheri Sis, 2008. Chemical composition analysis and antimicrobial activity of Iranian propolis. Res. J. Biol. Sci., 3: 448-450.

Harold, E., S.K. Ronald and S. Ronald, 1981. Pearsons Chemical Analysis of Foods. Churchill Livingstone, Longman group limited, Great Britain, pp: 1-290.

Hegazi, A.G., F.K. Abdelhady and F.A. Abdallah, 2000. Chemical composition and antimicrobial activity of European propolis. Z. Naturforsch, 55c: 70-75.

Ildenize, B.S.C., C.H.F.S. Alexandra, M.C. Fabio, T.S. Mario, C.M. Mario, T.D. Flavia, S.P. Giovanna and C.O. Patricia de, 2004. Factors that influence the yield and composition of Brazillian propolis extracrs. J. Braz. Chem. Soc., 15: 964-970.

Ivan, K., B. Marina, P. Stjepan and V. Sand, 2004. Quantitative analysis of the flavonoids in raw propolis from north Croatia. Acta Pharm., 54: 65-72.

Kujumgiev, A., I. Tsvetkova, Yu Serkedjieva, V. Bankova, R. Christov and S. Popov, 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J. Ethnopharmacol., 64: 235-240.

Lyudmila, B., G. Galina, N. Rossen, D. Sirigan, L. Elena, K. Nikola, M. Ivan and K. Zacharii, 2005. Activity of Bulgarian propolis against 94 *Helicobacter pylori* in vitro by agar-well diffusion, agar dilution and disc diffusion methods. J. Medical Microbiol., 54: 481-483.

Marcucci, M.C. and V. Bankova, 1999. Chemical composition, plant origin and biological activity of Brazilian propolis. Curr. Topics Phytochemistry, 2: 115-123.

Marcucci, M.C., F. Ferreres, C. Garcia-viguera, V.S. Bankova, S.L. DeCastro, A.P. Dantos, P.H.M. Valente and N. Paulino, 2001. Phenolic compounds from Brazillian propolis with pharmacological activities. J. Ethnopharmacol., 74: 105-112.

Melina, P.P., S.B. Vassya, B. Stefan, T. Iva, N. Christo, L.M. Gian and Anna-Gloria, 2007. Chemical characteristics of poplar type propolis of different geographic origin. Apidologie, 38: 306-311.

Monks, A., D. Scuderio, P. Skehan, R. Shoemaker and K. Paull, 1991. Feasibility of high-flux anticancer drug screen using a diverse parel of cultured human tumor cell lines. J. Nat. Cancer Inst., 83: 737-766.

Nieva Moreno, M.I., M.I. Isla, N.G. Cudmani, M.A. Vattuoene and A.R. Sampietro, 1999. Screening of antibacterial activity of Amaicha del Valle (Tucuman, Argentina) propolis. J. Ethnopharmacol., 68: 97-102.

Pastian, T., 2010. The Microbial World: Thecell wall surrounds and hold in the microbe. Textbook Publishing Manifesto. Chapter 2, pp: 25.

Pereira, A.D.S., F.R.M.S. Seixas and F.R. Aquino Neto, 2002. Propolis: 100 years of research and future perspectives. Quimica Novo, 25: 321-326.

Rula, M.D., J.A.F. Raed, H.A.Z. Musa and K.N. Ibrahim, 2010. Antibacterial effect of Jordanian propolis and isolated flavonoids against human pathogenic bacteria. Afr. J. Biotechnol., 9: 5966-5974.

Siegfried, A., R. Dirk and L. Uwe, 2003. Propolis and some of its constituents down-regulate DNA synthesis and inflammatory cytokine production but induce TGF-β1 production of human immune cell. Z. Naturforsch, 58c: 580-589.

Tapiero, H., A. Arvouet-Grand and A. Pourrat, 1998. Polyphenols: Do they a role in the prevention of human pathologies? Biomed. Pharmacother., 56: 200-207.

Vennat, B., A. Arvouet-Grand and A. Pourrat, 1998. Skin healing preparations: Compared *in vitro* diffusion of the active ingredients. Drug. Dev. Ind. Pharm., 24: 253-260.

Yaghoubi, S.M.J., G.R. Ghorbani, S. Soleimanian Zad and R. Satari, 2007. Antimicrobial activity of Iranian propolis and its chemical composition. DARU, 15: 45-48.

Zhang, S., T. Xi and C. Wang, 2005. High hydrostatic pressure extraction of flavonoids from propolis. J. Chem. Technol. Biotechnol., 80: 50-54.