Comparison of Free Radicals Scavenging Activity, Phenolics Levels and Antibacterial Activities by Iraqi Propolis from Different Sources

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Date of acceptance 7/9/2005

Abstract

Propolis (bee glue) a resinous material derived by bees from many plant sources was known for its therapeutic properties. In this study, Iraqi propolis from different sources was extracted by 70 % ethanol using ultrasonic bath. Iraqi propolis extract for free radical scavenging by [1aR-(1aαb, 8, ß, 8α, 8bα)]-6-amino-8-{[(amino carbonyl)oxy]- methyl}-1,1a,2,8,8a,8b-hexahydro-8a-methoxy-5-methyl-azirone [2',3':3,4] pyrrolo[1,2-α]indole-4,7-dione (mitomycin C) was evaluated .The phenolic compounds concentrations were quantified by the Folin-Ciocalteu spectrophotometric method at 765 nm. The antibacterial effect of propolis extract was evaluated by an in vitro study testing the growth of two strains of bacteria (*Escherichia coli* and *Staphylococcus aureus*) in nutrient broth containing varying concentrations of propolis. The results indicate that Iraqi propolis extract exhibit strong potential free radical scavenging activity and contained a higher level of phenolic compounds. Additionally, this extract showed the most consistent results, with the minimum bactericidal concentration of the extracts ranging between 500 and 1400 µg/mL, for the species of two strains of bacteria .The present results support the use of ethanolic extract of propolis as a strong disinfectant in modern medicine and as antibiotic pharmaceutical.

Introduction:

Propolis (bee glue) is a sticky dark-colored material that honey bees collect from living plants, mix with wax and use in construction and adaptation of their nests. The term "Propolis" was used by authors in Ancient Greece: ***Pro*** (for, in front of, e.g., at the entrance to) and ***Polis*** (city or community); a substance that is for or in defense of the city or hive. It can be yellow, green or brown depending on its source and collected season (1).Propolis is a traditional medicine used as early as 300 BC and has been reported to exert aboard spectrum of biological functions, including anticancer (2) , antioxidant (3) , anti-inflammatory (4),antibacterial (5) , antifungal (6)and antiviral (7) activities have been reported in propolis and its constituents . For this reasons propolis is widely used as a popular remedy in folk medicine in apitherapy as a constituent of "biocosmetics","health food" and for numerous further purposes (8). The most important pharmacologically active constituents in propolis are flavanoids (flavones, flavonols, and flavonones), phenolics, and aromatics. Flavanoids are thought to account for much of the biologic activity in propolis (9). The active components of propolis showing an antimicrobial effect include pinocembrin, galangin, caffeic acid, and ferrulic acid (10). Because of the appearance of bacterial resistance to

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antimicrobial agents, more effort is being made to find alternative antimicrobial components. It had been suggested that natural products are preferable to synthetic ones. The study of natural compounds has been considered as a fruitful approach in the search of new drugs. Thus, propolis becomes a subject of research by biologist and chemists. In this study, propolis samples were collected from four different localities of Iraq (Tarmyia, Dhuluayia, Taji and Dujel) to compare free radical scavenging activity, phenolics levels and to evaluated antibacterial activities.

Materiales and methods

Propolis

Four Iraqi propolis **(Ip)** samples were collected from (Tarmyia **(Ip1)**, Dhuluayia **(Ip2)**, Taji **(Ip3)** and Dujel **(Ip4)**) in different seasons and stored at 4 ^{0}C.

Extraction and sample preparation

One gram of each propolis sample was cut into small pieces and extracted at room temperature with 50 ml of 70% ethanol using ultrasonic bath, Decon FS 300, England .The alcoholic extract was evaporated at 50 ^{0}C until dryness.

Stable free radical scavenging activity

The scavenging effect of propolis as well of vitamin C corresponding to the quenching intensity of [1aR-(1aαb, 8, ß, 8α, 8bα)]-6-amino-8 -{[(amino carbonyl)oxy]-methyl}-1,1a,2,8,8a,8b-hexahydro-8a-methoxy-5-methyl-azirone [2',3':3,4] pyrrolo[1,2-α]indole-4,7-dione (mitomycin C) . The sample solution of each tested (500 µl) material was mixed with the same volume of 60 µM mitomycin C solution and allowed to stand for 30 min at room temperature. The absorbance was then measured at 650 nm. The samples and mitomycin C were dissolved in ethanol .The percent scavenging effect was determined by comparing the absorbance of solution containing the test sample to that of content solution without the test sample taking the corresponding blanks. The result is the mean of 5 measurements for each sample. Vitamin C was used as positive control sample. The percent of mitomycin C decoloration of the sample was calculated according to the formula: (1- Abs sample / Abs control) x 100.

Determination of total phenolics

The concentration of total phenolic compounds in the fractions was determined spectrophotometrically using Folin-Ciocalteu reagent (11). The **Ip** extract (0.1 ml) was diluted with deionized water (7.9 ml) .Folin-Ciocalteu reagent (0.5ml) was added, and the contents were mixed thoroughly .After 1 min, 0.2 ml 0f 7.5% (w/v) sodium carbonate solution was added, and the mixture was mixed thoroughly .The absorbance of the blue color produced was measured with a spectrophotometer at 765 nm .The level of Phenolic content was expressed in percent of dry weight and determined by comparing the absorbency with data established using known phenol standards in the same assay system (Fig.1).

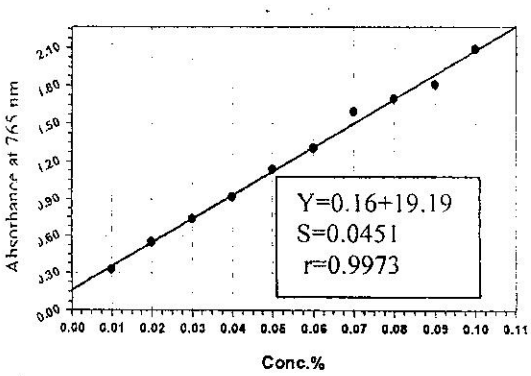

Figure 1: The standard curve for the determination of total phenolic content of Iraqi propolis.The absorbance was read at 765 nm.

Antibacterial assay

Two bacterial strains were used: ***Escherichia coli*** and ***Staphylococcus aureus*** .These bacteria were kindly

supplied by the Biotechnology department; college of science, university of Baghdad, Baghdad, Iraq .The bacterial suspension was prepared and adjusted by comparison against 0.5 Mc-Farland turbidity standard (5x 10^7 cell /ml) tubes. It was further diluted to obtain a final of 5 x 10^6 cell/ml. ***Escherichia coli*** was enriched on nutrient agar. While ***Staphylococcus aureus*** was enriched on MacConkey agar. Both bacteria were sub- culture on nutrient broth (12). The broth was inoculated by the 0.2 ml/10ml broth either with ***Escherichia coli*** and ***Staphylococcus aureus***, then added 1 ml of (2 and 4 mg) propolis. The tubes were incubated at 37 C^0 for 24 h. The growth of control bacterial growth due to propolis was measured by turbidity at 492 nm wavelength. The mean values of inhibition were calculated from triple reading in each test. The minimum bactericidal concentration (MBC) of propolis was determined by the ten-fold dilution method against bacterial strains in ***in vitro***.

***Statistical data analysis*:** Data were statistically analyzed using SPSS statistical software (version 11.5) by computing independent –samples T test. The values are given as mean ± standard error.

Results

Free radical scavenging activity

The results of the free radical scavenging effect of the four propolis samples, and positive control (**Vitamin C**) in mitomycin C –free radical system were determined (table 1). The values of the free radical scavenging effect of **Ip** showed a concentration – dependent activity, the free radical scavenging ranged from 19.10±1.10 to 38.15±2.25 % at a concentration of 1 **µg** while the free radical scavenging of Vitamin C was 14.30±2.30 % but the activity at a concentration of 100 **µg** was ranged from 40.60±4.00 to 68.30±2.30 % while the free radical scavenging of Vitamin C was 32.50±2.00 % .The results indicate that **Ip** extract exhibit strong potential free radical scavenging activity.

Table 1: The mitomycin C free radical scavenging effect of Iraqi propolis .The free radical scavenging effect was measured by the absorbance of mitomycin C radical at 650 nm.

Treatment	Concentration (µg) x 10^{-3}		
	1 µg	10 µg	100 µg
$n = 3$	Activity (%)	Activity (%)	Activity (%)
Control **	0.00	0.00	0.00
Ip1	19.10 ± 1.10	64.90 ± 4.00	68.30 ± 2.30
Ip2	38.15 ± 2.25	46.90 ± 2.00	51.40 ± 3.60
Ip3	23.90 ± 1.90	65.60 ± 4.00	64.00 ± 2.00
Ip4	23.90 ± 1.50	29.80 ± 1.00	40.60 ± 4.00
Vitamin C++	14.30 ± 2.30	16.30 ± 2.30	32.50 ± 2.00

** Mitomycin C; ++ Positive control; *n* Values are the mean of three replicates ± SE. ; Ip1 propolis collected from Tarmyia ; Ip2 propolis collected from Dhuluayia ; Ip3 propolis collected from Taji ; Ip4 propolis collected from Dujel.

Total phenolic content

The content of the extraction was 420, 190, 600 and 130 mg / dry weight respectively for **Ip** extracts (table 1).The total phenolic content of **Ip** extracts ranged from 30.84 ± 3.38 to 70.68 ± 3.35 %. In general, **Ip1** was found to have higher phenolics than other **Ip** extracts, the lowest amounts of phenolics were found in **Ip4**.

Table 2: Extract dry weight and total phenolic content of Iraqi propolis.

Propolis sample	Ip1	Ip2	Ip3	Ip4
Extract dry weight (mg)	420	190	600	130
Total phenolic content (%)	70.68 ± 3.35	54.52 ± 3.21	64.97 ± 3.18	30.84 ± 3.38

Ip1 propolis collected from Tarmyia; Ip2 propolis collected from Dhuluayia; Ip3 propolis collected from Taji; Ip4 propolis

Inhibition of bacterial growth by Iraqi propolis

The antibacterial activities of propolis collected from four localities of Iraq were evaluated in two strains of bacteria (***Escherichia coli*** and ***Staphylococcus aureus***). As shown in table 3, treatment with propolis samples showed a dose dependent inhibition in growth of the examined pathogens .The results indicated that **Ip1** collected from Tarmyia had the highest antibacterial activity against ***Escherichia coli*** was 0.21 ± 0.050 at 2 mg/ml of propolis extract , while was 0.09 ± 0.017 at 4 mg/ml of propolis extract. The **Ip1** and **Ip3** (collected from Taji) had the highest antibacterial activity against ***Staphylococcus aureus*** (0.16 ± 0.005, 0.15 ± 0.011; 0.29 ±0.023, 0.27±0.034 at 2 and 4 mg /ml, respectively).The MBC are illustrated in table 3 there were differences in their minimum inhibitory concentration. The MBC of **IP** ranged from 600 to 1400 µg/ml for ***Escherichia coli*** while it was 500 to 1000 µg/ml for ***Staphylococcus aureus.***

Table 3: Antibacterial activity of Iraqi propolis against two strains of bacteria (***Escherichia coli*** and ***Staphylococcus aureus***).

Treatment		*Escherichia coli*		*Staphylococcus aureus*	
		Growth*	MBC (µg/ml)**	Growth	MBC (µg/ml)
Control		0.7± 0.018	-	0.73± 0.002	-
Tetracycline (30 µg)		0.44± 0.017	-	0.13± 0.010	-
Ip1	2 mg	0.21 ± 0.050	600	0.16 ± 0.005	500
	4 mg	0.09 ± 0.017		0.15 ± 0.011	
Ip2	2 mg	0.45 ± 0.028	1100	0.34 ± 0.024	900
	4 mg	0.20 ± 0.013		0.33 ± 0.016	
Ip3	2 mg	0.37± 0.019	750	0.29 ± 0.023	800
	4 mg	0.16 ± 0.023		0.27 ± 0.034	
Ip4	2 mg	0.49 ± 0.012	1400	0.38 ± 0.011	1000
	4 mg	0.22 ± 0.034		0.35 ± 0.005	

* Growth = Inhibition of the growth measured by absorbance at 492 nm.
** MBC minimal bactericidal concentration.
Ip1 propolis collected from Tarmyia ; Ip2 propolis collected from Dhuluayia ; Ip3 propolis collected from Taji ; Ip4 propolis collected from Dujel.

Discussion

Propolis, a complex mixture of plant metabolites, possesses a broad spectrum of biological activity including antibiotic, anticancer, antioxidant and anti inflammatory activities (1-4).The chemical composition of propolis is complicated and varied .Location, season and environmental condition are important factors influencing of propolis (13,14). Our results showed that all **Ip** extract exhibited an inhibitory action against two strains of bacteria (***Escherichia coli*** and ***Staphylococcus aureus)***, but **Ip1** activity was the strongest one. The variation in the antibacterial activity seems to be due to the differences in chemical composition of different propolis samples. These are in agreement with Sawaya et al. (15), Abd El Hady and Hegazi (7), Sforcin et al. (16) and Rhajaoui et al. (5) who found that the antimicrobial activity differs according to the differences in the chemical composition. The activity of **Ip** can probably be attributed to the presence of 2, 6-bis-(pentanyloxy)-4-pentanylphenethanol. This result seems to indicate that propolis activity is not due to the presence of one of particular substance but is a resultant of the complex action of various aromatic structures and of flavonoid compounds. Effectively we suggest that the antibacterial effect of our propolis is the result of their flavonoid contens, like myricitine [3,7,4',5' tetra methyl ether] , quercetine [3,7,3 trimethyl ether] (17), Galangin [3,5,7 trihydroxy flavone] (7,18), chrysin [5,7 dihydroxy flavone], Pinocembrin [5,7 dihydroxy flavone] (5,7), and to their various esters of caffeic acid (19).The variation of the antibacterial activity of propolis from area to area , which had a synergistic effect of various phenolic

compounds. The preliminary Folin-Ciocalteu investigation of the 70% alcoholic extract showed differences of total phenolic content between the four propolis samples. Regarding to the results of the free radical scavenging effect of **Ip** in a mitomycin C free radical system showed a concentration dependent activity with a slight variation and **Ip1** extract also showed relatively exerted high free radical scavenging activity as compared with **Ip2, Ip3** and **Ip4**. We suggest that **Ip1** contained higher levels of phenolic compounds and scavenged free radicals more efficiently. These findings suggest that plants and season are key factors influencing propolis composition and agreement with the finding of Bankova et al. who studied different fraction of European propolis and Brazilian propolis (20, 21). The present investigation support the use of ethanolic extract of propolis as a strong disinfectant in folk and modern medicine. These data demonstrate that **Ip** contains potent antioxidant compounds, powerful antibacterial effects so, we try to extend these study of inhibitory effect of ethanolic extract of propolis on growth of microorganisms such as parasites, yeasts, virus and molds.

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مقارنة لفعالية اخماد الجذور الحرة ومستويات المركبات الفينولية والفعاليات المضادة للبكتريا للعكبر العراقي من مصادر مختلفة

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الخلاصة

العكبر (صمغ النحل) مادة راتنجية يجمعها النحل من مصادر نباتية مختلفة ،معروف بخصائصه العلاجية . في الدراسة الحالية استخلصت المواد الفعالة من العكبر العراقي المجموع من مناطق مختلفة بواسطة كحول الايثانول تركيز ٧٠% وباستخدام جهاز الموجات فوق الصوتية. تم تقييم فعالية اخماد الجذور الحرة لمستخلص العكبر العراقي بدراستها على المركب - [1aR-(1aαb, 8, ß, 8α, 8bα)]-6-amino-8 {[(amino carbonyl)oxy]-methyl}-1,1a,2,8,8a,8b-hexahydro-8a-methoxy-5-methyl-azirone [2',3':3,4] pyrrolo[1,2-α]indole-4,7-dione (مايتومايسين C) . تم تحديد تراكيز المركبات الفينولية طيفيا عند ٧٦٥ نانوميتر باستعمال طريقة فولن – سيوكالتيو . تم تقييم الفعالية المضادة للبكتيريا لمستخلص العكبر في الزجاج على سلالتين من البكتيريا (*Escherichia coli* و *Staphylococcus aureus*) منماة على وسط المرق المغذي يحتوي تراكيز مختلفة من مستخلص العكبر . اظهرت النتائج ان مستخلص العكبر العراقي يمتلك فعالية تثبيطية عالية في اخماد الجذور الحرة وكذلك احتوائه على مستويات مرتفعة من المركبات الفينولية . بالاظافة الى ذلك فان مستخلص العكبر اظهر نتائج جيدة للتركيز الادنى للتثبيط البكتيري تراوحت بين ٥٠٠–١٤٠٠ مايكروغرام/مليليتر لكلا السلالتين المستخدمتين . اسندت النتائج الحالية استخدام مستخلص العكبر الايثانولي بكونه مطهر قوي في الطب الحديث وكمضاد حيوي دوائي.