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Full Length Research Paper

Antibacterial effect of Jordanian propolis and isolated flavonoids against human pathogenic bacteria

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Propolis is a natural product widely consumed in folk medicine. The present study was carried out to investigate the antibacterial activity of Jordanian propolis, collected from two locations with two different dominant floras (Type1; Pine trees and Type II; Oak trees). Zones of inhibition and minimum inhibitory concentrations (MICs) were determined on methicillin resistant *Staphylococcus aureus* (MRSA), multidrug resistant *Escherichia coli* and standard strains of both bacteria. Propolis Type I and Type II showed antibacterial activity against MRSA (MIC 4.69 and 18.75 µg ml^{-1}, respectively). Crude propolis from Type I showed higher antibacterial activity than Type II against the tested bacteria. Three pure phenolic compounds (three flavonoids) namely, pinobanksin-3-O-acetate, pinocemberin and chrysin, were isolated from fractions I-2 and I-4, and screened *in vitro* for antibacterial activity. Pinobanksin-3-O-acetate and pinocembrin exhibited antibacterial activity especially against MRSA, while chrysin was only active against standard *S. aureus*. This is the first report that shows *in vitro* antibacterial activity of isolated flavonoids from Jordanian propolis against standard and resistant strains of *E. coli* and MRSA. Overall, results of this study highlight the important role of propolis botanical source on the antibacterial activity of such natural material which might affect its medical applications.

Key words: Antibacterial activity, human pathogens, flavonoids, propolis, methicillin resistant *Staphylococcus aureus*, *Escherichia coli.*

INTRODUCTION

Propolis is a natural product widely consumed in folk medicine. Its medicinal and antimicrobial properties have been widely investigated (Kujumgiev et al., 1999; Hegazi and Abd El Hady, 2002; Prytzyk et al., 2003; Onlen et al., 2007; Darwish et al., 2009). Different researchers (Sforcin et al., 2000; Trusheva et al., 2006; Katircio and Nazime 2006; Yaghoubi. et al., 2007) have reported that propolis antibacterial activity is attributed to a number of phenolic compounds, mainly flavonoids, phenolic acids and their esters. Some prenylated ρ-coumaric acids were isolated from propolis in several countries (Kosalec et al., 2004). The antibacterial activity of volatile compounds and diterpenes from Brazilian propolis was identified by Bankova et al. (2000). Propolis and some of its cinnamic acid derivatives and flavonoids were responsible for un-coupling the energy transducing cytoplasmic membrane inhibiting bacterial motility, which might contribute to the antibacterial action (Bankova et al., 2000).

It is reported that pathogenic isolates have a relatively large potential for developing antibiotic resistance (Fluit et al., 2000; Sahm et al., 2001; Schwaber et al., 2006). The increase in antibiotic resistant bacteria is largely due to the widespread use of antibiotics in medicine, in animal care and in agriculture. The problem is compounded by the lack of new antibiotics to attack bacteria in different

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Abbreviations: MICs, Minimum inhibitory concentrations; **MRSA,** methicillin resistant *Staphylococcus aureus*; **TLC,** thin layer chromatography; **MDR,** multi drug resistance; **CC,** column chromatography.

ways to circumvent the resistant genes. Therefore, finding antimicrobial agents which are effective or might enhance the antibiotic efficacy against resistant bacteria would be an advantage. *Staphylococcus aureus* and *Escherichia coli* are of the major causes of hospital-acquired infections (Karlowsky et al., 2004; Fluit et al., 2000; Huang et al., 2006). These organisms occur naturally in and on human body. However, certain strains can lead to infections and are becoming resistant to antibiotics.

Numerous researchers have reported the biological activities of propolis collected in different parts of the world. However, no work has been conducted on the anti-bacterial activity of Jordanian propolis or its components. Thus, the objectives of this research were to investigate the antibacterial activity of the crude propolis, separated propolis fractions and promising constituents from two different floras (pine and oak trees) against resistant and standard strains of two medically important bacteria *S. aureus* and *E. coli.*

MATERIALS AND METHIODS

Extraction of propolis

Two Jordanian propolis samples were collected by honey bees *(Apis mellifera*) from two locations with two different dominant floras: University of Jordan main campus, located in Amman in which common pine trees with the majority of *Pinus halepenses* were the source of Type I propolis and Al-Hashmeah, located about 12 km west of Amman and is characterized by intensive oak trees (*Quercus coccifera*) which were the source of Type II propolis. Collected propolis was stored at -20°C. A specimen was retained as a reference at the Chemistry Department, Faculty of Science, University of Jordan.

Type I and Type II propolis samples were cut into small pieces and extracted once with chloroform for one day and twice with methanol for two days each. The extracts were evaporated under reduced pressure. The residual material was partitioned between hexane and 10% aqueous methanol. The crude aqueous methanol extract of Type I and Type II propolis were chromatographed on a silica gel column grade, (230 g) packed in chloroform. Seven fractions (1 - 7) were obtained. All chemical analysis was carried out at the Chemistry Department, University of Jordan.

Fraction I-2 was chromatographed on a silica gel column, using ethyl acetate-benzene mixtures of increasing polarity for elution. The collected fraction eluted with ethyl acetate–benzene (5:95 v/v) was further purified by preparative thin layer chromatography (TLC), using methanol–chloroform (2:98) as the mobile phase. Two flavanes were isolated: pinocembrin 1 and pinobanksin-3-O-acetate 2.

Fraction I-4 was also chromatographed on a silica gel column as described for fraction I-2. The fraction eluted with ethyl acetate–benzene (20:80) afforded upon treatment with methanol a yellow solid, which was further purified by recrystallization from methanol to give the flavane chrysin 3.

The aqueous methanol extract of Type II propolis was fractionated in a similar manner to give seven main fractions.

Identification of the isolated compounds

Mass spectra for the flavones were obtained using a Varian MAT 112 spectrometer. ^{1}H-NMR spectra were recorded on a Bruker DPX-300 MH_z spectrometer with TMS as internal standard. ^{13}C-NMR spectra were recorded at 7S.5 MH_z. Silica gel DF (Fluka) was used for CC and silica gel glass plates G- UV_{254} (Macherey-Nagel) were used for TLC. All chemical procedures were carried out at the Chemistry Department, University of Jordan. Identified structures of the isolated compounds are presented in Figure 4.

Antimicrobial assay

Two resistant bacterial strains, methicillin resistant *S. aureus* (MRSA) and resistant *E. coli* were isolated from hospitalized patients at the Jordan University Hospital and approved by biochemical tests. Two standard bacterial strains *S. aureus* ATCC 6538P and *E. coli* ATCC 8739 were also used in the study. The susceptibilities of the isolates to the crude propolis extracts and the fractions were determined by the agar diffusion method on solid media with Mueller-Hinton agar plates (Merck, Germany) according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2000a). The isolates were grown overnight on a nonselective medium at 35°C. Bacterial inocula were prepared by suspending the freshly grown bacteria in sterile normal saline and adjusted to a 0.5 McFarland standard. The suspension was inoculated onto 9 cm plates of Miller-Hinton agar (Merck). Cups of 9 mm were filled with saturated solutions of the crude propolis or the fractions. The plates were incubated for 24 h at 37°C. Control cups containing the solvent alone were also included. The absence of growth was interpreted as antibacterial activity. The antibiotics clindamycin (2 units/disc), penicillin G (10 units/disc), cephalexin (30 µg/disc) and nalidixic acid (30 µg /disc) were used as standards to compare the activity of propolis in inhibiting the growth of bacteria. The antibacterial activity was determined by comparing zones of inhibition (mm) and complete inhibition (including the diameter of the disc) produced by the propolis or its fractions with those in the controls and standards. Each experiment was carried out three times and was correlated against the controls.

Determination of the minimum inhibitory concentration (MIC)

The MICs of the propolis and the fractions were conventionally determined in triplicate for each strain by the macrodilution broth method as described by the NCCLS (National Committee for Clinical Laboratory Standards, 2000b). Serial dilutions of each propolis and fractions were prepared in macrodilution tubes. Bacterial suspend-sions were adjusted to the 0.5 Mc Farland standards (approximately 1 to 2 × 10^8 cfu /ml). Final inocula were adjusted to the 10^4 cfu/ml. A constant amount of bacteria were added to all tubes and they were incubated at 37°C for 18 – 24 h. Each tube was examined for growth, comparing each tube to the control. The MIC was defined as the lowest concentration of propolis at which there was no visible growth of the organism. MICs of the antibiotics were determined in the same way. A positive growth control was included where bacterial suspension was added to a tube filled with nutrient broth without crude propolis. An uninoculated tube of nutrient broth was also added to serve as negative growth control.

Broth microdilution method for determining MIC of isolated flavanoids

The susceptibility of each bacterial isolate to the three compounds isolated from fraction I-2 and I-4 was tested by the NCCLS broth microdilution reference method (NCCLS, 2000b) with some modifi-cations. MIC test was performed in 96 flat bottom microtiter plates (TPP, Switzerland). Each test well was filled with a volume of 100 µl nutrient broth. Extracts and fractions under investigation (100 µl) were added to the first column of test-well and mixed. A series of

Table 1. Means zone of inhibition by four antibacterial agents on standard and resistant (MRSA) *S. aureus* and *E. coli.*

Treatment	*Zone of inhibition (mm) ±SD			
	Standard		Resistant	
	S. aureus	*E. coli*	MRSA	*E.coli*
Penicillin G	23 ± 2.5	23 ± 1.92	N	N
Cephalexin	23 ± 1.00	22 ± 1.51	N	N
Clindamycin	24 ± 0.67	24 ± 1.75	N	N
Nalidixic acid	18 ± 1.33	22 ± 2.00	N	N
Methanol	N	N	N	N

*Average of three rplicates; N: no zone of inhibition.

dilutions of each tested sample was then carried out across the plate using micropipette, changing the tips at each dilution step. Then, 10 µl of pre-adjusted overnight microbial culture was used to inoculate each well in the microtiter plate to achieve a final inoculum size of 5 x 10^6 cfu/ml.

In all assays, positive growth controls (well with overnight culture, nutrient broth and bacterial inoculum but without any testing agents) and negative controls (well with broth but without inoculum) were included.

MICs were expressed as the average of two successive concentrations of the antimicrobial agent showing no growth and growth, respectively. The microorganism's growth was detected as turbidity, visualized by naked eyes, relative to the negative and positive controls. The following equation expresses the calculated MIC:

$$MIC = C\,n + C\,(n + 1) / 2$$

Where, C *n:* Concentration at well number n, where no turbidity occurs; C (n + 1): concentration at well number (n + 1), where turbidity occur.

MIC determination was carried out in triplicate (in same 96-well plate) and repeated twice for each bacteria and each tested agent.

Statistical analysis

Treatments of gram positive and negative bacteria were carried out under laboratory conditions using a split plot design with complete randomized design (CRD) arrangement and analyzed according to LSD test SAS (1990). Three replicates of Petri dishes were used for each treatment, mean zone of inhibition and standard deviations were calculated and presented in Table 1.

RESULTS AND DISCUSSION

The antibacterial activity of crude propolis and different fractions from Type I and II against standard *S. aureus* and MRSA was tested as presented in Figures 1 and 2 and Plates 1a, 1b, 2a and b. Type I crude propolis and fraction I-4 produced the highest antibacterial activity with zones of inhibition of 24.67 and 25.33 mm, respectively on standard *S. aureus* (Figure 1 and Plate 1a) and 17.00 and 16.67 mm, respectively on MRSA (Figure 2 and Plate 1b). The activity of fractions I-4 indicates that the active components are concentrated in this fraction. This is in agreement with reports of several researchers which indicate that each propolis sample contain 80 - 100 chemical compounds with different concentrations (Bankova et al., 2000; Kosalec et al., 2004; Trushera et al; 2004; Park et al., 2005; Yaghoubi et al., 2007).

Crude propolis Type II against standard *S. aureus* did not differ significantly from fraction II-4 and was less effective compared to Type I crude propolis. Type II and fraction II-4 exerted zone of inhibition of 18.67 and 18.00 mm, respectively (Figure 1 and Plate 2a). Their effect on MRSA was less, with inhibition zones of 14.67 and 14.33 mm, respectively (Figure 2 and Plate 2b).

The antibacterial activity of antibiotics used (penicillin G 2 units/disc, cephalexin 10 units/disc, clindamycin 30 µg/disc and nalidixic acid 30 µg /disc) was comparable with that of crude propolis Type I with zones of inhibition of 23, 23, 24 and 18 mm, respectively against standard *S. aureus* (Table 1). Fractions I-2 and I-4 of Type I gave inhibition zones of 22 and 25.33 mm against standard *S. aureus* which are comparable with that obtained by the antibiotics used (Figure 1 and Table 1) indicating that the inhibitory effect of the propolis is mainly attributed to these fractions. It is worth mentioning that the antibiotics used have no effect on MRSA (Table 1), while crude propolis Type I, Type II and their fractions inhibited the growth of this strain. Crude propolis Type I, Type II, fractions I-2 and I-4 and fractions II-2 and II-4 gave inhibition zones of 17.00, 14.67, 13.67, 18, 11.67 and 14.33 mm, respectively, against MRSA (Figure 2, Plates 1b and 2b).

The present results on standard *S. aureus* are in agreement with those obtained by several authors like Kujumgiev et al. (1999) who found that the inhibition zones obtained by propolis from Mongolia, Albania, Egypt, Brazil and the antibiotic streptomycin were 24, 21.8, 24.3, 21.8 and 28 mm, respectively. These results are also comparable with results obtained by Prytzyk et al. (2003) who measured the inhibition zone for Bulgarian propolis as 20 mm. They are also comparable with results obtained by Stepanović et al. (2003) who found out that the inhibition zone of propolis form different geographical areas of Serbia ranged from 18 - 23 mm.

Results reveal that there is antibacterial activity of

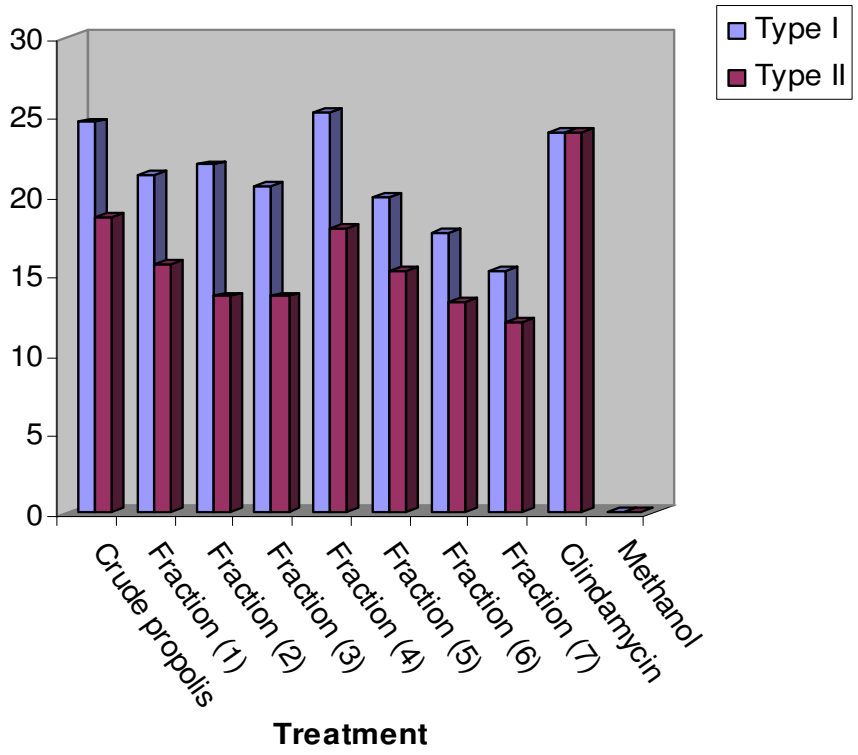


Figure 1. Mean zone of inhibition by various propolis treatments against standard *S. aureus* ATCC 6538P.

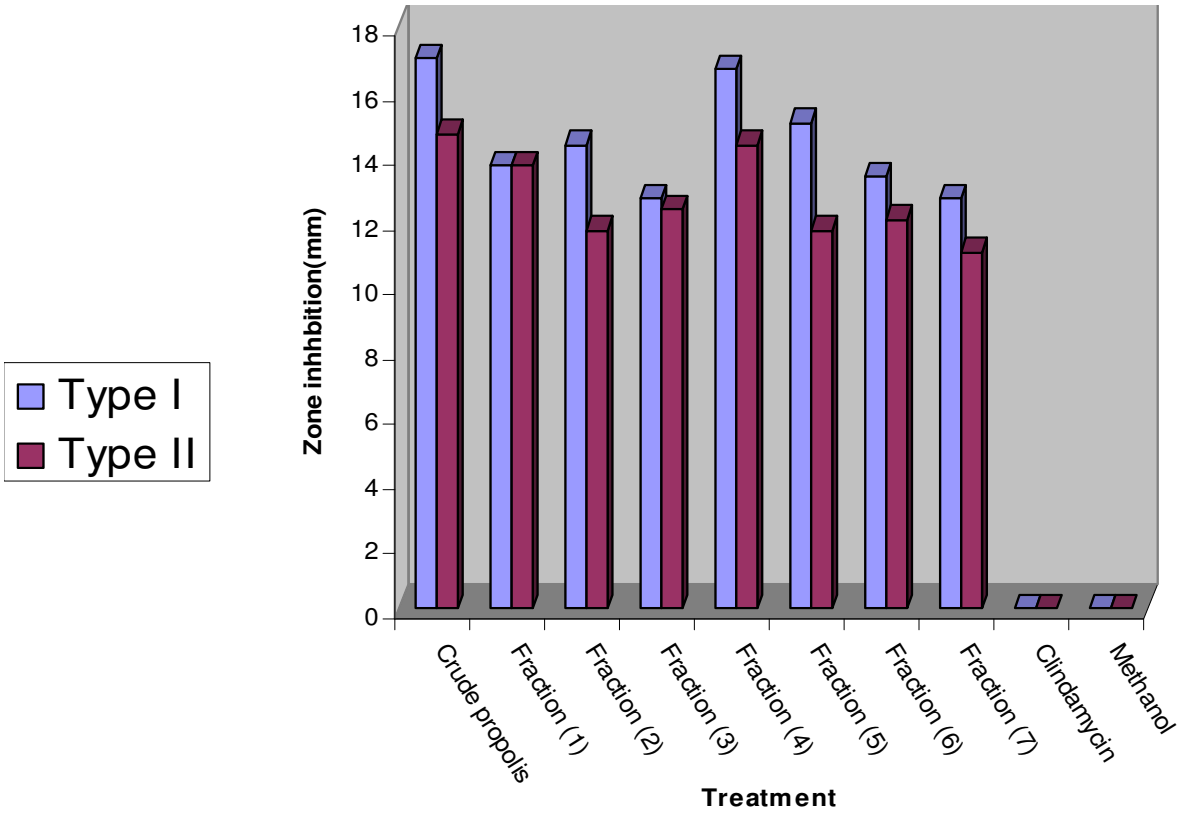
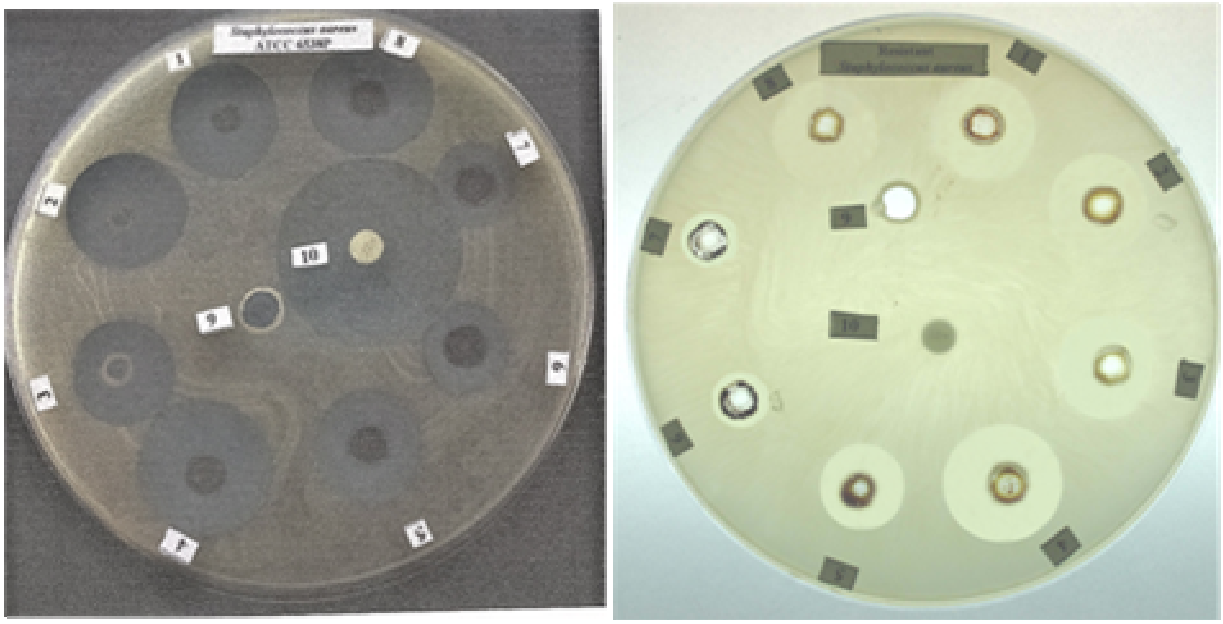
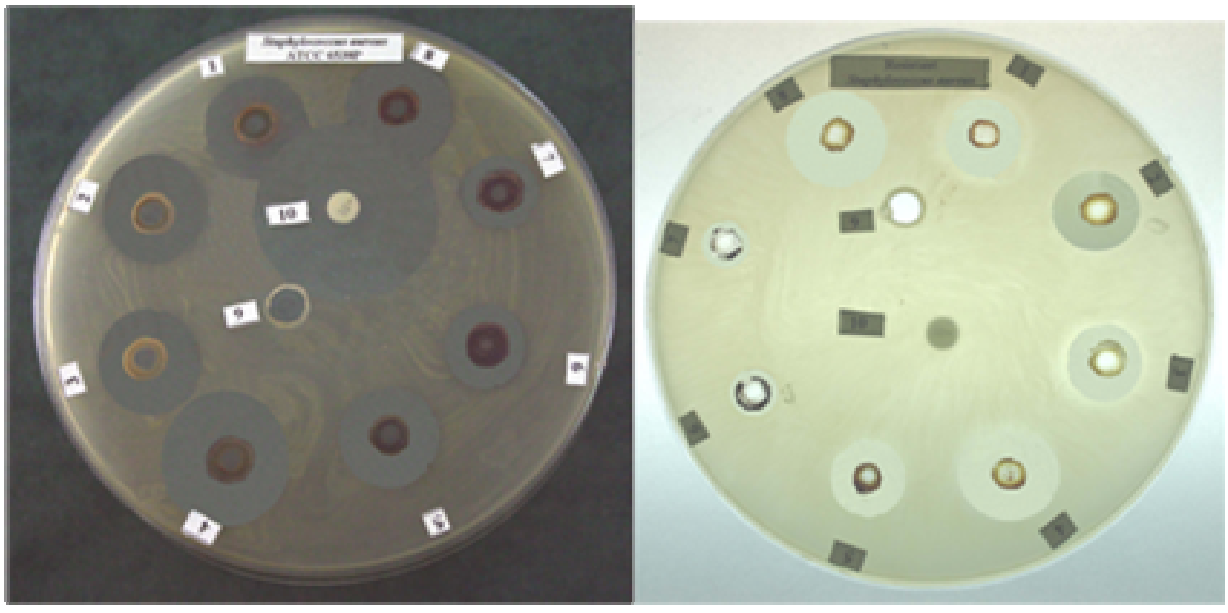


Figure 2. Mean zone of inhibition by various propolis treatments against resistant *S. aureus* MRSA.



A B

Plate 1. Growth inhibition of *S. aureus* ATCC 6538P by various treatment of pine trees propolis, (a): Standard (b): resistant. Numbers 1 - 7 indicate propolis fraction number, 8: crude propolis, 9: methanol (solvent), 10: antibacterial penicillin.



A B

Plate 2. Growth inhibition of *S. aureus* ATCC 6538P by various treatment of oak trees propolis, (a): Standard (b): resistant. Numbers 1 - 7 indicate propolis fraction number, 8: crude propolis, 9: methanol (solvent), 10: antibacterial penicillin.

propolis Types I and II against the different studied bacteria (including gram positive and negative bacteria) and that Type I propolis has better antibacterial activity than Type II. This indicates that propolis from the different

Table 2. Minimal inhibitory concentration (MIC)* of crude propolis against standard and resistant strain of *S. aureus and E. coli*.

Microorganism	Crude propolis (mg ml^{-1})		Antibacterial (µg ml^{-1})			
	Type I	Type II	Penicillin G	Cephalexin	Nalidixic acid	Clindamycin
Staphylococcus aureus ATCC 6538P	0.585	4.690	4	32	8	7.02
Escherichia coli ATCC 8739	4.69	ND	4	35	6	225
MRSA	4.69	18.75	ND	ND	ND	ND
Resistant *Escherichia coli*	18.75	ND	ND	ND	ND	ND

*Average of three replicates; ND: not detected.

regions in Jordan exhibits antibacterial effect. This again supports the commonly reported statement in literature, that sensitivity of microbes and differences in chemical composition of propolis are greatly affected by variations in geographical origins (Bankova et al., 2000; Abd El Hady and Hegazi, 2002; Kartal et al., 2003; Trusheva et al., 2006).

Results in Table 2 emphasize the effect of different flora on the antibacterial activity. MICs of Type I and II propolis against standard *S. aureus* were 0.585 and 4.69 mg ml $^{-1}$, respectively. The MIC of Type I propolis is similar to what was reported by Sforcin et al. (2000) on propolis collected from Brazil. Our MIC results from pine propolis (Type I) on standard *S. aureus* (0.585 mg ml $^{-1}$) are better than those reported by Hegazi and Abd El Hady (2002) in Egypt which were 2.2 and 2.6 mg ml^{-1}, respectively. However, Moreno et al. (1999) reported that propolis collected from Argentine had lower MIC value of 0.04 mg ml^{-1} against the same strain. Again, this difference was related to the different constituents of propolis collected from different geographical regions (Bankova et al., 2000; Abd El Hady and Hegazi, 2002). It is worth adding that Jordanian crude propolis Type I and II were also active against MRSA with 4.69 and 18.75 mg ml^{-1}, respectively (Table 2). This bioactivity on resistant bacterial strains opens the door for practical application.

Several researchers (Kujumgiev et al., 1999; Moreno et al., 1999; Sforcin et al., 2000; Stepanović et al., 2003; Gonzalez et al., 2005) reported that there was no effect of propolis from different geographical regions on standard *E. coli*. Our results however, show that there is some antibacterial effect of propolis on gram negative bacteria, but it is rather limited with a zone of inhibition of 17.33 and 15.67mm for crude propolis Type I and fraction I-4 treatments, respectively (Figure 3). Meanwhile, propolis Type II has no effect. This again might reflect the fact that chemical composition of propolis differs greatly from one region to another (Burdoc, 1998; Bankova et al., 2000; Abd El Hady and Hegazi, 2002; Prytzyk et al., 2003; Stepanović et al., 2003).

The MIC value against standard *E. coli* was 4.69 mg ml^{-1} for Type I propolis, while Type II propolis showed no activity (Table 2). This MIC value is better than that reported by Sforcin et al. (2000) of 8 mg ml^{-1} on the same strain. However, it was less than the MIC obtained by Hegazi and Abd El Hady (2002) who reported an MIC value of 1.6 mg ml^{-1} against the same bacteria. The variation might reflect the difference in the composition of the propolis, since the bacterial strain used is the same. The low sensitivity of *E. coli* shown in Table 2 and Figure 3 is in agreement with the findings by many researchers where this bacterium showed either very low sensitivity or total lack of sensitivity against propolis (Marcucci, 1995; Kujumgiev et al., 1999; Gonzalez et al., 2005). This emphasizes the fact that, gram negative bacteria are less sensitive than gram positive strains, which is in agreement with several previous reports (Burdoc, 1998; Moreno et al., 1999; Sforcin et al., 2000; Abd El Hady and Hegazi, 2002; Gonzalez et al., 2005). The most possible explanation for the low sensitivity of gram negative bacteria is that their outer membrane inhibits and/or retards the penetration of propolis (Tegos et al., 2002). Another possible reason is their possession of multi drug resistance (MDR) pumps, which extrude amphipathic toxins across the outer membrane (Tegos et al., 2002).

Results in Table 2 show an MIC value of 18.75 mg ml^{-1} for Type I propolis against resistant *E. coli*, while Type II propolis showed no activity on this strain. The results showed that crude propolis of both Types I and II and their fractions had no (or slight) antibacterial activity on resistant *E. coli*. However, they had activity on resistant *S. aureus (*MRSA*)*. It is worth mentioning that the antibiotics used had no effect on the same strains (Table 2).

Overall results indicate that the antibacterial activity of the crude propolis, fraction I-2 and I-4 are superior to other fractions, indicating that the bioactive components are concentrated in these fractions. Our preliminary studies indicate that the compounds in these fractions are flavonoids. This is in agreement with the findings in the literature, which indicate that the active compounds present in propolis include mainly flavonoids, phenolic acids and esters (Marcucci, 1995; Burdoc, 1998). Major compounds from the active fractions (I-2 and I-4) were isolated by a combination of silica gel column chromatography (CC) and TLC. Fraction I-2 afforded the flavans 1 (Jung et al., 1990) and 2 (Fang et al., 1988) while fraction 4 afforded flavan 3 (Chen et al., 2003) as the major constituents (Figure 4). The three compounds were identified by spectroscopic methods including ^{1}H-NMR,

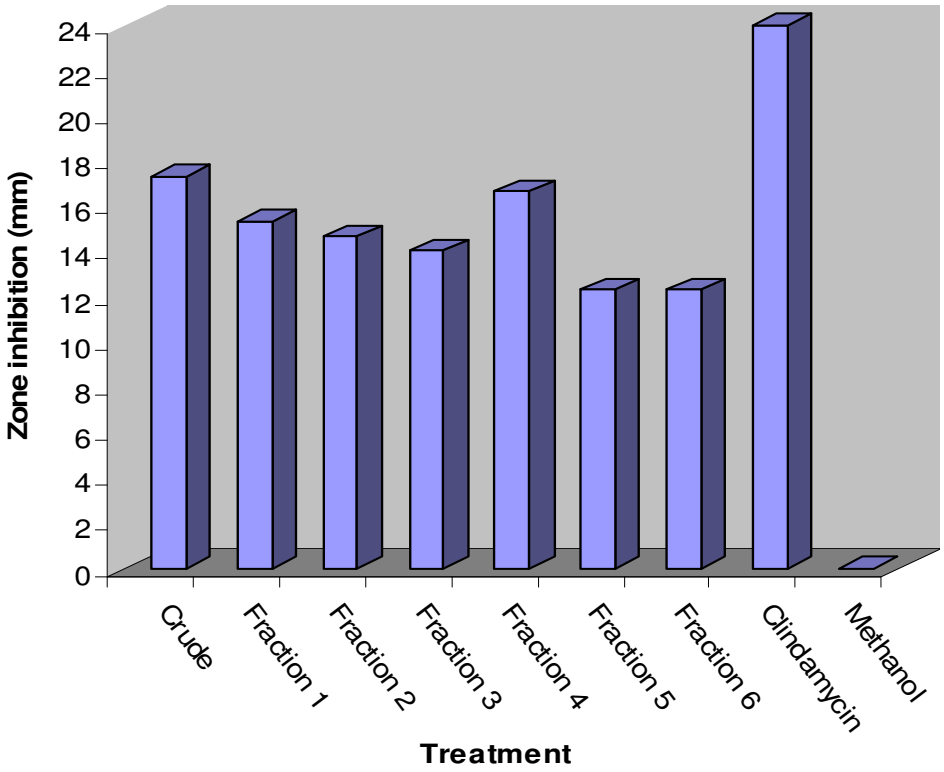


Figure 3. Mean zone of inhibition by crude and seven pine propolis fractions Type I against standard *E. coli*.

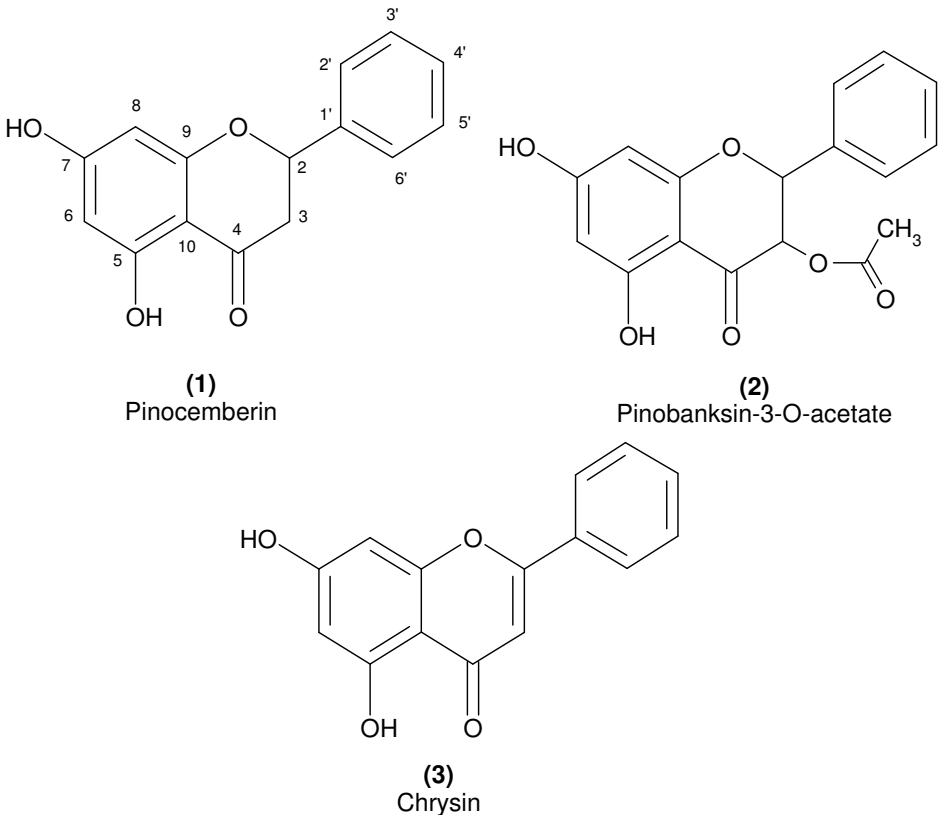


Figure 4. The chemical structure of the isolated compounds.

Table 3. Mean zone of inhibition by pure compounds isolated from fractions I-2 and I-4 against standard and resistant strains of *S. aureus* and *E. coli*.

Bacteria	*Zone of inhibition(mm)			
	Blank (solvent)	Pinobanksin-3-O-acetate	Pinocembrin	Chrysin
Standard *S. aureus*	N	13	13	10
MRSA	N	22	21	N
Standard *E. coli*	N	10	N	N
Resistant *E. coli*	N	12	N	N

Diameter of filter paper disc is 6 mm; *Average of three replicates; N; no zone of inhibition.

Table 4. Minimal inhibitory concentration (MIC) of pure compounds isolated from fractions I-2 and I-4 against standard and resistant strains of *Staphylococcus aureus* and *Escherichia coli.*

Bacteria	*MIC (µg ml^{-1})		
	Pinobanksin-3-O-acetate	Pinocembrin	Chrysin
Standard *S. aureus*	250	300	250
MRSA	250	250	ND
Standard *E. coli*	500	ND	ND
Resistant *E. coli*	>500	ND	ND

*Average of three replicates; ND; not detected.

^{13}C-NMR (with DEPT) and MS.

Screening of the antibacterial activity of the pure compounds isolated from fractions I-2 and I-4 shown in Table 3 reveal that, pinobanksin-3-O-acetate and pinocembrin which were isolated from fraction I-2 have good activity against both strains of *S. aureus*. Their effect was more promising against the resistant strain with inhibition zones of about 22 mm compared to 13 mm against standard strain (Table 3). Chrysin which was isolated from fraction I-4 inhibited the growth of standard *S. aureus* only (zone of inhibition was 10 mm). The inhibition of growth of standard and resistant *E. coli* was noticed only with pinobanksin-3-O-acetate with an inhibition zone of 10 and 12 mm, respectively (Table 3). The promising antibacterial effect of the isolated compounds may be due to the fact that ring B in all of the three flavonoids is not oxygenated; such a structural feature is uncommon in flavanoids. MICs of pinobanksin-3-O-acetate, pinocembrin and chrysin against standard *S. aureus* were similar (about 250 µg ml^{-1}) (Table 4). These MICs were high when compared with antibiotics MICs. Clindamycin, penicillin G, cephalexin and nalidixic acid MICs were 7.02, 4, 32 and 8 µg ml^{-1}, respectively (Table 2). This is in agreement with the results obtained by Koo et al. (2002) where the MICs for flavanoids isolated from propolis were greater than 500 µg ml^{-1}. Pinobanksin-3-O-acetate and pinocembrin were both effective against MRSA with MICs of 250 µg ml^{-1} (Table 4), while the antibiotics used were not effective against this strain. Pinobanksin-3-O-acetate was the only effective compound of the three tested against standard and resistant *E. coli.* However, the MIC value was 500 and >500 µg ml^{-1}, respectively (Table 4).

The antibacterial activity of the pure compounds was less than expected suggesting that the antibacterial activity of propolis could involve a synergistic interaction between its components. This is in agreement with the study by Hegazi and Abd El Hady (2002) and would explain the effect of crude propolis on bacteria (Kujumgiev et al., 1999).

In conclusion, propolis samples could be used for the standard and resistant strain-infection treatment such as an alternative therapy. The resistant strains are increasing rapidly all over the world; these urge further studies to be done with other microorganisms.

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