Original Article

Zahedan Journal of Research in Medical Sciences

Journal homepage: www.zjrms.ir

Antibacterial Activity of Propolis Ethanol Extract against Antibiotic Resistance Bacteria Isolated from Burn Wound Infections

Farzaneh Zeighampour,[1] Maryam Mohammadi-Sichani,*[2] Elaheh Shams,[1] Nafiseh Sadat Naghavi[2]

1. Young Researchers and Elite Club, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
2. Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

Article information

Article history:
Received: 17 Mar 2013
Accepted: 10 Apr 2013
Available online: 26 May 2013
ZJRMS 2014; 16(3): 25-30

Keywords:
Antibacterial agent
Propolis
Metallo-Beta-lactamase
Staphylococcus aureus
Pseudomonas aeruginosa

*Corresponding author at:
Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
E-mail:
mohamadi_m@iaufala.ac.ir

Abstract

***Background*:** Burn wound is a suitable site for incidence of resistant infections; thus, the research for finding effective drugs against this infection is necessary. The purpose of this study was to determine antibacterial activity of Isfahan bee propolis extracts against beta-lactamase producing bacteria isolated from burn wound infections.

***Materials and Methods*:** Ethanol extract of Isfahan bee propolis was prepared by 28 g of propolis in 100 ml of 70% ethanol. Antibacterial activity of ethanol extracts were evaluated against beta-lactamase producing bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) isolated of burn wound infection by well diffusion method. Broth serial dilution method was used to determine MBC of extract. Beta-lactamase production of isolates was detected by iodometric test, imipenem-EDTA combined disk test and imipenem-EDTA double-disk synergy test.

***Results*:** Ethanol extract of propolis was found to be the most effective against *S. aureus* strains (inhibition zone=17.66±0.47 mm) than *P. aeruginosa* strains (inhibition zone=7 mm). The MIC and MBC values of the extracts against *S. aureus* strains were 0.0143 and 0.0286 mg/ml and these values against *P. aeruginosa* strains were 0.75 and 1.5 mg/ml, respectively. Among the *S. aureus* clinical isolates, all of strains produced beta-lactamase. Imipenem-EDTA double-disk synergy test showed that only one clinical isolate of *P. aeruginosa* was metallo-beta-lactamase positive.

***Conclusion*:** This study demonstrated that ethanol extract of Isfahan bee propolis is mainly active against *S. aureus* and it is effective on *P. aeruginosa* at higher concentration. Ethanol extract of propolis did not inhibit production of beta-lactamase enzyme in tested bacteria.

Introduction

The widespread use of antibiotics both inside and outside of medicine is playing a significant role in the emergence of resistant bacteria. The common pathogens isolated from burn patients include *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Antibacterial resistance in some of the most frequent bacterial species isolated from burn patients such *S. aureus* and *P. aeruginosa*, and other gram negative bacilli has reached to a worrying level [1, 2]. Beta-lactams are the most widely used antibiotics. The most common mechanism of beta-lactam resistance among bacteria involves the production of beta-lactamases. Beta-lactamases classified on the basis of their primary structure into four molecular classes (A through D). Class A and C are the most common and have a serine residue at the active site, as do class D beta-lactamases [3]. Class B comprises the metallo beta-lactamases (MBLs) [4, 5]. The prevalence of MBLs has been increasing worldwide, notably among *P. aeruginosa* and lately, among other gram negative bacteria as well. Therapeutic control of beta-lactamases producing bacteria has been a major clinical problem for more than 50 years [6]. Development of drug combinations containing the beta-lactamases inhibitors has given clinicians a novel approach to controlling resistant organisms [3]. So there is a requirement for new beta-lactamases inhibitors to fight against the resistant bacteria.

Propolis is a resinous substance collected by worker bees (*Apis mellifera*) from the bark of trees and leaves of plants. This salivary and enzymatic secretions-enriched material is used by bees to cover hive walls to ensure a hospital-clean environment. Bees use propolis as a "chemical weapon" against pathogenic microorganisms. Propolis shows a complex chemical composition [7]. Biological properties and chemical compositions of propolis may vary according to different plant sources that bees could visit, collecting time and geographic locations [1, 2-8]. Many investigations have been carried out to identify the antibacterial activity of propolis. Researchers have reported that propolis antibacterial activity is attributed to phenolic compounds, flavonoids, phenolic acids and their esters [7-11]. Further studies revealed that propolis contain more than 200 constitutes. Among them are phenolic compounds predominant

including flavonoids as a major compounds, cinnamic acid derivatives, aminoacid, fatty acids and vitamins like B_1, B_2, B_6, C and E, minerals like (Mg, Ca, I, Na, Cu, Zn, Mn, Fe) and enzymes like (adenosine triphosphatase, succinic dehydrogenase, glucose 6-phosphatase). Some mechanisms of the activity of propolis on bacterial growth and inhibition of enzyme have been reported: 1) Inhibition of cell division, 2) Bacterial cytoplasm and 4) Protein synthesis inhibition. Calagin and caffeic acid from propolis ethanol extract are enzymatic inhibitors agents against bacteria [12]. The purpose of this study was to determine antibacterial activity ethanol extract of propolis of Isfahan against beta-lactamase producing bacteria isolated from burn wound infection and to compare with effects of some selected antibiotics.

Materials and Methods

Crude samples of *Apis mellifera* propolis were obtained from different region of Isfahan, Iran. Propolis samples were cut into small pieces and stored in 4ºC. Twenty eight grams of propolis were extracted by 100 ml of 70% (v/v) ethanol by orbital shaking at 150 rpm at room temperature for 5 days. Then ethanol extract of propolis was filtered. Various concentrations of 1500, 750, 375, 187, 93.8, 46.9, 23.4 and 11.7 µg/ml of ethanol extract of propolis were made. Samples were stored in the dark at 4ºC and used within 2 months of preparation [13].

The bacterial strains were isolated from clinical specimens in the Imam Musa-Kazem hospital (hospital of burn and hazards) Isfahan, Iran. Clinical isolates were included *S. aureus* and *P. aeruginosa*. *P. aeruginosa* (ATCC: 1074) and *S. aureus* (ATCC: 25923) were obtained from Iranian Research Organization for Science and Technology, Tehran, Iran. These strains were grown and maintained on Brain Heart Infusion agar (BHI, Merck) supplemented with blood. Bacterial cultures were diluted in sterile saline and the optical density was adjusted to that of tube 0.5 in McFarland's scale, to standardize the inoculums (1.5×10^8 cfu/ml).

Antibacterial susceptibility for all isolates was determined by Kirby-Bauer disk diffusion susceptibility method as per standard Clinical and Laboratory Standard Institute (CLSLs) guidelines. All *P. aeruginosa* strains were screened for MBL production by imipenem-EDTA combined disk and imipenem-EDTA double disk synergy test by the method published by Christensen. Simple iodometric test used for detecting beta-lactamase activity in *S. aureus* strains [14].

The well diffusion method was used to determine the antibacterial activity of propolis ethanol extracts. The Muller Hinton agar plates were inoculated with different selected strains of bacteria (about 10^8 cfu/ml) separately. Wells were made on the agar prepared surface with 6 mm cork borer. The lower portion was sealed with little molten agar medium. Forty µl of different concentrations of propolis ethanol extracts (1500, 750, 375, 187, 93.8, 46.9, 23.4 and 11.7 µg/ml) were poured into each well of inoculated plates aseptically. All plates were incubated at 37ºC for 24 h. They were inspected for the zone of inhibition of growth, which were measured in millimeters and the values obtained were recorded. A control experiment was carried out using ethanol 8.75% (v/v) for each of the test bacteria. The entire tests were performed in triplicate.

The broth microdilution method was used to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ethanol extract of propolis as recommended by the National Committee for Clinical Laboratory Standard (NCCLS) [15]. The ratio of ethanol propolis extract in test medium furnished the required concentration ranging from 1500, 750, 375, 187, 93.8, 46.9, 23.4 and 11.7 µg/ml for *S. aureus* and *P. aeruginosa*. MIC of 70% ethanolic solution was tested against studied bacterial strains in order to determine the effects of ethanolic solvent of propolis. The test well of a 96-well microtiter plate were filled with 100 µl of exponentially growing culture (about 10^8 cfu/ml) and added with 100 µl of different concentration of propolis ethanol extract (1500, 750, 375, 187, 93.8, 46.9, 23.4 and 11.7 µg/ml) separately. Eight sets of test wells were prepared containing broth medium and decreased ethanol concentration (8.75, 4.37, 2.18, 1.09, 0.54, 0.27, 0.13, 0.068 v/v), allowed to grow at 37ºC for 24 h as control of the solvent effect. The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 630 nm. The plate was incubated at 37ºC for 24 h, agitated and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. The MIC was defined as the lowest concentration (highest dilution) of propolis that inhibited the visible growth (no turbidity) compared to the control [16].

To determine the MBC, 10 µl of each well was transferred to Mueller Hinton agar plates and incubated at 37ºC for 24 h. The MBC was considered as the lowest concentration of propolis associated with no visible growth of bacteria on the agar plates [15]. All samples were tested in triplicate.

In vitro study inhibition activity of ethanol extract of propolis on beta-lactamase enzyme was determined by serial dilution of propolis ethanol extract by micro dilution method. The ratio of ethanol extract of propolis in test medium were furnished concentration ranging from 1500, 750, 375, 187, 93.8, 46.9, 23.4 and 11.7 µg/ml. Bacterial suspensions were adjusted to the 0.5 McFarland standards (approximately 1 to 1.5×10^8 cfu/ml). Final inoculate were adjusted to the 10^4 cfu/ml. A constant amount of bacteria were added to all wells and they were incubated at 37ºC for 18-24 h. After incubation, 0.1 ml of each concentration of propolis ethanol extract for *P. aeruginosa* strains and *S. aureus* strains were spread on Muller-Hinton agar. After incubation at 37ºC for 24 h, enzyme inhibition by iodometric, imipenem-EDTA combined disk and imipenem-EDTA double disk synergy test, was recorded for each of bacteria [17]. The experimental results were expressed as mean±standard deviation (SD) of triplicates. The data were subjected to one-way analysis of variance (ANOVA), using the SPSS-17 software. The $p<0.05$ were regarded as significant.

Results

In this study, eight strains of *P. aeruginosa* and three strains of *S. aureus* were isolated from burn infections. Standard antibiograms showed that all of the clinical isolates of *P. aeruginosa* were resistant to cefotaxime and ceftriaxone. Six isolates of *P. aeruginosa* were resistant to imipenem and one of the strains was resistant to gentamicin (Fig. 2). Clinical isolates of *S. aureus* were resistant to cefotaxime, ceftriaxone and two of the strains were resistant to imipenem. All of the *S. aureus* strains were susceptible to vancomycin (Fig. 3).

Result acquired from iodometric, imipenem-EDTA combined-disk and imipenem-EDTA double-disk synergy test showed in figure 1. Beta-lactamase production of *S. aureus* isolates was detected by iodometric test. Results showed that all of clinical isolates of *S. aureus* produced beta-lactamase.

Imipenem-EDTA double-disk synergy test showed that only one clinical isolate of *P. aeruginosa* produce metallo-beta-lactamase (Fig. 1).

Variation in diameters of inhibition zone among bacterial species and propolis samples were showed in table 1.

Minimum inhibitory concentration and minimum bactericidal concentration of propolis ethanol extract against of *P. aeruginosa* and *S. aureus* strains were showed in table 2.

Figure1. Detection of MBL production with imipenem-EDTA combined disk in down and imipenem-EDTA double disk synergy test in top

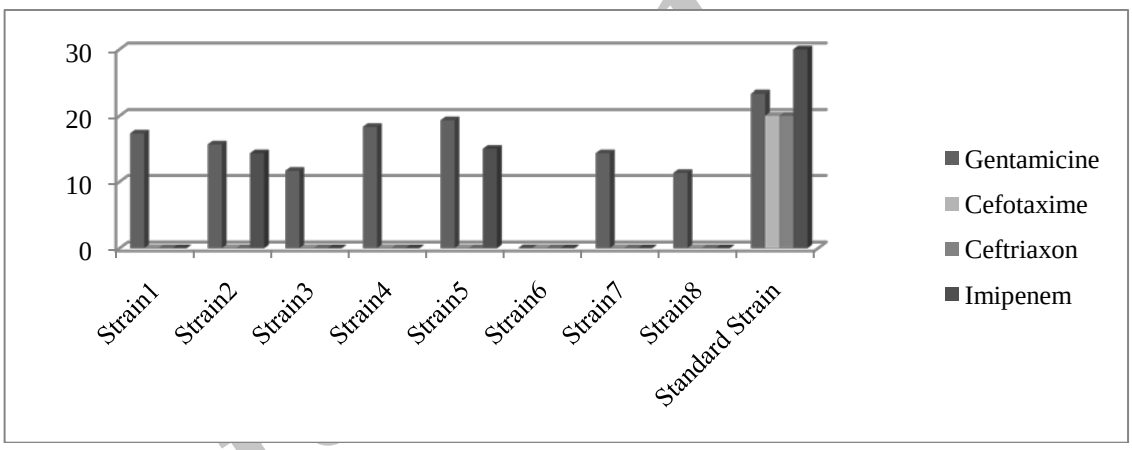

Figure 2. Antibiotic susceptibility pattern of P. aeruginosa isolates zone diametres (mm)

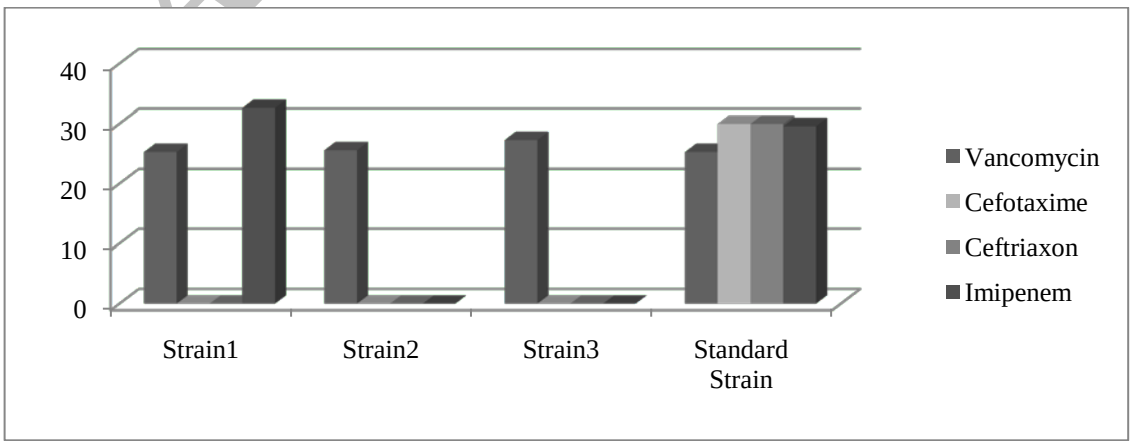

Figure 3. Antibiotic susceptibility pattern of S. aureus isolates zone diameters (mm)

Table 1. Antibacterial activity of ethanol extracts of propolis against multidrug resistant bacteria by well diffusion method

Bacteria	Extract concentrations (µg/ml)						
	1500	750	375	187	93.7	46.8	Control
P. aeruginosa (METp)	7	0	0	0	0	0	0
P. aeruginosa (ATCC: 1074)	7	0	0	0	0	0	0
S. aureus (strain 1)	17.66±0.47	15±0.81	13.66±0.47	11.66±0.47	10.00±0.47	0	0
S. aureus (strain 2)	17.66±0.47	15.66±0.47	13.00±0.81	10.66±0.47	0	0	0
S. aureus (strain 3)	17.00±0.47	14.66±0.47	12.66±0.47	10.66±0.47	0	0	0
S. aureus (ATCC: 25923)	15.66±0.47	11.66±0.47	10.66±0.47	0	0	0	0

METp: Metallo-beta-lactamase producing strain, Negative control: Ethanol 17.5% v/v

Table 2. The MIC and MBC (µg/ml) of propolis ethanol extract against selected P. aeruginosa and S. aureus strains

Bacterial strains	MBC (mg/ml)	MIC (mg/ml)
S.aureus (ATCC: 25923)	23.4	11.7
S. aureus(strain 1)	31.2	15.6
S. aureus(strain 2)	23.4	11.7
S. aureus(strain 3)	31.2	15.6
P. aeruginosa (ATCC: 1074)	1500	750
P. aeruginosa (METp strain)	1500	750

METp: Metallo-beta-lactamase producing strain

Discussion

The study focused on antibacterial activity of ethanol extracts of Isfahan bee propolis against beta-lactamase producing *S. aureus* and *P. aeruginosa* isolates. In other words, this study is about how ethanol extract of propolis effect on the growth of the bacteria and inhibition of beta-lactamase enzyme. The result of this study showed that the ethanol extract of propolis has antibacterial activity on beta-lactamase producing bacteria (*S. aureus* and *P. aeruginosa*) isolated of burn wound and its effect is better than selective antibiotics (Table 1, 2). Propolis is a natural product from the honey bee (bee glue). The ethanol extract of propolis is an extremely complicated mixture of natural substances and contains amino acids, phenolic acids, phenolic acids esters, flavanoids, cinnamic acid, terpenes and caffeic acid.

These substances have been shown to exert a variety of medical properties, such as antimicrobial activity [10, 11]: that is; the application of ethanol extract of propolis can prevents bacteria growth; furthermore, we used ethanol extracts of propolis from different regions of Isfahan because propolis composition in organic and inorganic basis is different depending to the region where bees collected the sample. Composition of propolis could be also changed dramatically in the same region with a few km distances because of the changing plant distribution. In the present study first, we isolated beta-lactamase producing bacteria (*S. aureus* and *P. aeruginosa*); then investigated the sensitivity of bacteria to ethanol extract of propolis.

In burn wounds the most commonly isolated organisms were *S. aureus* and *P. aeruginosa* [1-4]. Therefore, we selected two common bacteria strains in burn wounds including *S. aureus* and *P. aeruginosa*. Among the *S. aureus* clinical isolates, all of the strains produced beta-lactamase. All of the *S. aureus* clinical isolates produced beta-lactamase which makes them resistant to ceftriaxone and cefotaxime. Imipenem-EDTA double disk synergy test showed that only one clinical isolate of *P. aeruginosa* produce metallo-beta-lactamase and was resistance to all beta-lactam antibiotics and carbapenems such as imipenem, cefotaxime and ceftriaxone (Fig. 3). The increasing use of penicillins and cephalosporins in clinical settings places a highly selective pressure that favors pathogenic microorganisms through the development of various resistance mechanisms, the most prevalent of which is the generation of beta-lactamases bacterial enzymes [3].

In our study beta-lactamase production of *S. aureus* isolates was detected by iodometric test and metallo-beta-lactamase production of *P. aeruginosa* isolates were determined by imipenem-EDTA combined disk test and imipenem-EDTA double disk synergy test. One of resistance mechanisms in *P. aeruginosa* is production of metallo-beta-lactamase. Several non molecular techniques have been studied, all taking advantage of the enzyme's zinc dependence by using chelating agents, such as EDTA or 2 mercaptopropionic acid to inhibit its activity [5, 6].

In 1991, Japan reported the first plasmid-mediated metallo-beta-lactamase in *P. aeruginosa* [6]. So in our study, all *P. aeruginosa* strains were screened for metallo-beta-lactamase production by imipenem-EDTA combined disk and imipenem-EDTA double disk synergy test and all *S. aureus* strains were screened for beta-lactamase producing by iodometric test.

This study demonstrated that ethanol extract of Isfahan bee propolis have antibacterial activity on beta-lactamase producing *S. aureus* and *P. aeruginosa* isolates from burn wound infection and its effect is better than selective antibiotics. In present study propolis is mainly active against *S. aureus* and it is effective on *P. aeruginosa* at higher concentrations. That is sensitivity of *S. aureus* strains against antibacterial activity of ethanol extract of propolis with mean inhibitory diameter (17.66±0.47-10 mm) followed by *P. aeruginosa* strains (7 mm); moreover, the mean MIC and MBC values against *S. aureus* strains with 0.0143 and 0.0286 mg/ml and *P. aeruginosa* strains with 0.75 and 1.5 mg/ml were recorded, respectively (Table 1, 2). Similar results have been reported in other studies [18].

Najmadeen et al. reported that ethanol extract of propolis had antibacterial activity against *S. aureus* and *P. aeruginosa* [13]. Davey related that preparations of propolis ethanol extract (3 mg/ml) completely inhibited the growth of *P. aeruginosa* and *Escherichia coli*, but had no effect on *Klebsiella pneumonia* [19]. Different result were achieved by Nieva et al. that reported antibacterial activity against *S. aureus* but had not effect against *P. aeruginosa* and *E. coli* [20]. Data of Kilic et al. study

demonstrated that ethanol extract of propolis sampled from Turkey possess potent antimicrobial activity, providing an alternative therapy against infections caused by resistant strain such as vancomycin-resistant *Enterococcus faecalis* and methicillin-resistant *S. aureus* [8]. Variation in the antimicrobial activity of propolis has been attributed to the differences in its chemical components [10-13]; likewise, generally the higher activity ethanol extract of propolis against Gram-positive were greater than Gram-negative bacteria, it may be due to differences in the structure and composition of Gram-negative Gram-positive bacteria cell wall [18].

In the present study by means of our suggestive method, beta-lactamase production in bacteria was not inhibited by ethanol extract of propolis. Therapeutic control of beta-lactamases producing bacteria has been a major clinical problem for more than 50 years. Development of drug combinations containing the beta-lactamases inhibitors has given clinicians a novel approach to controlling resistant organisms. However the current marketed inhibitors (tazobactam, clavulanate and sulbactam) are not active against all beta-lactamases, so there is a requirement for new beta-lactamases inhibitors to fight against the resistant bacteria [6].

Alahi et al. showed that extract of *Rumex vesicarius* L. had beta-lactamase inhibiting effect [21]. This study demonstrated that ethanol extract of Isfahan bee propolis have antibacterial activity against most of bacteria from burn wound infection and its effect is better than selective antibiotics; however, we need more in vitro and in vivo investigation.

Acknowledgements

This study was conducted according to the Young Researchers and Elite club of Islamic Azad University of Falavarjan plans to the number 88994. Study co-authors are grateful to scientific support of Department of Microbiology in Islamic Azad University of Falavarjan.

Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

Funding/Support

Islamic Azad University, Isfahan.

References

1. Sepehriseresht S, Najarpeerayeh S, Sattari M and Rezaee M. Production of plasmid mediated β-lactamases in Pseudomonas aeruginosa isolated from burn patients. Hakim Res J 2007; 10(1): 61-5.
2. Afrasiabian S, Heidari M. Infection burning and antimicrobial resistance pattern of aerobic isolates from burn patients in Sanandaj hospital. Iran J Clin Infect Dis 2008; 13(42): 61-5.
3. Drawz SM, Bonomo RA. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev 2010; 23(1): 160-201.
4. Saderi H, Karimi Z, Owlia P. Phenotypic detection of metallo-beta-lactamase producing Pseudomonas aeruginosa strains isolated from burned patient. Iran J Pathol 2008; 3(1): 20-4.
5. Jacoby G, Munoz-price L. The new β-lactamase. N Engl J Med 2005; 325(4): 380-91.
6. Yang Z, Yang X, Niu Y. A novel and rapid method to screen for β-lactamase inhibitors for extracts of herps. Int J Integr Biol 2009; 2(6): 62-4.
7. Mohammadzadeh S, Shariatpanahi M, Hamedi M, et al. Chemical composition, oral toxicity and antimicrobial activity of Iranian propolis. Food Chem 2007; 103(4): 1097-1103.
8. Kilic A, Baysallar M, Besirbellioglu B, et al. In vitro antimicrobial activity of propolis against methicillin-resistant Staphylococcus aureus and vancomycin-resistant Enterococcus faecium. Ann Microbiol 2005; 55(2): 113-7.
9. Barbaric M, Miskovic K, Bojic M, et al. Chemical composition of the ethanolic propolis extracts and its effect on HeLa cells. J Ethnopharmacol 2011; 135(3): 772-8.
10. Katircioglu H, Mercan N. Antibacterial activity and chemical composition of Turkish propolis from different region. Afr J Biotecnol 2006; 5(11): 1151-3.
11. Seidel V, Peyfoon E, Watson D and Fearnley J. Comparative study of the antibacterial activity of propolis from different geographical and climatic zones. Phytother Res 2008; 22(9): 1253-66.
12. Yaghoubi S, Ghorbani G, Soleimanian-Zad S and Satari R. Antimicrobial activity of Iranian propolis and its chemical composition. DARU 2007; 15(1): 45-8.
13. Najmadeen H, Kakamand A. Antimicrobial activity of propolis collected in different regions of Sulaimaniprovince-Kurdistan region/Iraq. J Duhok Univ 2009; 12(1): 233-9.
14. Yong D, Lee K, Yum J, et al. Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase-producing. J Clin Microbiol 2002; 40(10): 3798-801.
15. Liu JW, Jang TN, Cheng YJ, et al. Comparison of the E test and broth microdilution method for tigecycline susceptibility testing against clinical isolates of Acinetobacter baumannii from Taiwan. Int J Antimicrob Agents 2010; 35(2): 201-2.
16. Wiegand I, Hilpert K, Hancock E. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008; 3(2): 163-75.
17. Bokaeian M, Qureshi M. Study of beta lactamase production in coagulase positive Staphylococci by iodometric and acidimetric methods and their antibiotic resistance pattern. J Med Sci 2007; 7(1): 150-3.
18. Prajapati SB, Vegad MM, Mehta SG. An evaluation of two different phenotypic methods for detection of metallo-β-lactamase producing pseudomonas isolates. J Cell Tissue Res 2011; 11(1): 2601-4.
19. Grange J, Davey R. Antibacterial properties of propolis (bee glue). J R Soc Med 1990; 83(3): 159-60.
20. Nieva M, Isla M, Cudmani N, et al. Screening of antibacterial activity of Amaicha del Valle (Tucuman, Argentina) propolis. J Ethnopharmacol 1999; 68(1-3): 97-102.

21. Alahli AA, Abdulkhair WM. Inhibition of beta-lactamase enzyme of Pseudomonas aeruginosa by clavulanic acid of Rumex vesicarius L. Afr J Agric Res 2011; 6(12): 2908-15.

Please cite this article as: Zeighampour F, Mohammadi-Sichani M, Shams E, Sadat-Naghavi N. Antibacterial activity of propolis ethanol extract against antibiotic resistance bacteria isolated from burn wound infections. Zahedan J Res Med Sci (ZJRMS) 2014; 16(3): 25-30.