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Comparison of Antibacterial Activities of Walnut (*Juglans regia L.*) and Pine (*Pinus halepensis Mill.*) Leaves Alcoholic Extracts against Bacteria Isolated from Burn Wound Infections

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Summary

The threat of infections caused by drug resistant microorganisms is a global problem, so it is essential to carry out research on alternative antimicrobial drugs. Burn wound is an ideal environment for the development of drug resistant microorganisms. Walnut (*Juglans regia L.*) and pine (*Pinus halepensis Mill.*) leaves are ancient plants with phytochemical biological compounds. The aims of this study were to evaluate the antibacterial effects of walnut and pine leaves al-

coholic extracts against bacteria isolated from burn wounds infections, and compare them with selected antibiotics. Accordingly, the ethanolic extracts of walnut and pine leaves were prepared, analyzed using Agilent 7890B gas chromatography, and main phytochemicals compounds of them were identified. The antibacterial activities of alcoholic extracts against clinical isolates (n=6 isolates for each bacteria) and standard strains of *Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Acinetobacter baumannii, Escherichia coli, Staphylococcus epidermidis* and *Staphylococcus saprophyticus* were determined by agar diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods. The result of this study showed that the walnut and pine leaves extract had antimicrobial activity against all above clinical isolates. In conclusion, the findings of this study showed that the walnut leaves extract had more antibacterial activities than pine leaves extract, but generally, both extracts were able to compete with the selected antibiotics of this study.

Key words
Antibacterial, Alcoholic extracts, Juglans regia L, Pinus halepensis Mill, Burn wound infection

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Introduction

The threat of infections caused by drug resistant microorganisms is a global problem,[1-3] so it is essential to carry out research on alternative antimicrobial drugs.[4,5] Burn wound is an ideal environment for the development of drug resistant microorganisms.[6-8] In recent decades, the incidence of drug resistance microorganisms has increased in burn wound infections. Therefore, the study of antibacterial properties of plant extracts is taken into consideration.[9] Walnut leaves are used in traditional medicine due to having various medicinal properties such as anti-diarrhea, antifungal and anti-diabetic effects.[10] Pharmacological effects of walnut leaves have been mentioned anciently and research on walnut leaves is increasing due to its medicinal use.[11] The walnut leaf extract has antimicrobial effect. In addition, it has been shown that walnut leaves help to strengthen the skin, heal the scars and prevent itching and scarring and also, many research have been investigated about wound healing.[12-15] In ancient Iranian medical texts, various parts of pine tree species, especially gums, were used to treat old wounds.[16] In Japanese traditional medicine, pine cones are used as anti-tumor agent for treatment of gastric cancer as an immune system stimulant in people with leukemia. Also, some pine species cones had been used for treatment of some conditions including asthma, bronchitis, and cough in Chinese traditional medicine for many years.[17] The aims of this study were to evaluate the antibacterial effect of walnut and pine leaves alcoholic extracts on pathogenic bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Acinetobacter baumannii, Escherichia coli, Staphylococcus epidermidis,* and *Staphylococcus saprophyticus* isolated from burn wound infections, and compare their effects with the selected antibiotics.

Materials and Methods

Extraction

Walnut and pine leaves were prepared. Pharmacognosy department authenticated the samples. Leaves were air-dried at room temperature, and were ground into powder by a hammer mill and were passed through mesh with 80 sizes. To prepare the extract, 200 g of each powder was soaked in 70% ethanol solution (1:10 ratio) in a closed container and was shacked for 24 hours at dark room.[18] The extracts were filtered through Whatman No 41 filter paper and concentrated under vacuum at 40°C using a rotary machine, and the resulted powder was stored at −80°C until used.[19] The ethanolic extracts of walnut and pine leaves were analyzed using Agilent 7890B gas chromatography coupled to a 5977A series mass spectrometer equipped with a split/splitless injection system and an electron bombardment ionization model, and had MS library for NIST and WILEY.[20] Samples were injected into the GC-MS on a 30 m silica capillary column with internal diameter and film thickness of 0.25 mm and 0.25 µm, respectively. The GC temperature was set to increase from 60°C to 290°C at a rate of 15°C/min and finally held isothermal for 1 min (Split ratio 1:100).[20]

Isolation and identification of bacteria

The assayed microorganisms used in this study were as followed: 1) Local clinical isolates: *Pseudomonas aeruginosa* (n=6), *Staphylococcus aureus* (n=6), *Proteus vulgaris* (n=6), *Acinetobacter baumannii* (n=6), *Escherichia coli* (n=6), *Staphylococcus epidermidis* (n=6) and *Staphylococcus saprophyticus* (n=6). The strains were identified by the use of Biochemical profiles according to the recommendations of the manual of clinical microbiology.[21,22] 2) Reference strains: *P. aeruginosa* PTCC 1430, *S. aureu*s PTCC 1112, *P. vulgaris, A. baumannii* PTCC1855, *E. coli* PTCC1270, *S. epidermidis* PTCC 1114 and *S. saprophyticus* PTCC1440. The clinical isolates and standard strains obtained from Hospital of Tabriz and Microbiology Department of Tabriz Islamic Azad University, respectively.

Assay for antibacterial activity

The antibacterial activities of extracts were evaluated using agar disk-diffusion[23] micro broth dilution.[24] The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. Bacterial suspensions equivalent to a 0.5 McFarland turbidity were prepared in sterile normal saline solution from clinical and reference isolates.[23-27] A sterile swab dipped into the inoculum tube containing bacterial suspensions and then was cultured on the Müller-Hinton agar (Merck®, Germany). Sterile filter paper disc (6 mm in diameter) were impregnated with walnut and pine leaves Extracts (25, 50, 75 mg ml^{-1}) for 10–15 min and allowed to dry completely for 20–25 min, then evenly placed on the surface of previously inoculated cultures.[23,24] Gentamicin, co-trimoxazole, tetracycline, ciprofloxacin, ceftriaxone and amoxicillin antibiotic discs (Merck®, Germany) were used as positive control and sterile diluent (0.1% peptone water) was negative control for comparison of inhibition zone with sample.[23,24] Plates were incubated at 37°C for 24h, until visible growth of bacteria was evident in control plates. Clearly visible inhibition zones around discs were measured in three directions and averaged. The antibacterial activity was expressed as the diameter of inhibition zone produced by extract against test bacteria.[23,24,28,29]

Determination of MIC and MBC

The broth micro dilution method was performed to determine the MIC and MBC of extracts revealed by the agar diffusion assay.[24] Briefly, MIC and MBC was assayed in the microplate reader, using sterile 96 wells trays. Each well was filled with a total volume of 100 µl containing Müller-Hinton broth (MHB). Different concentrations of the each extract were prepared by serial dilution (dilution by one-half) in MHB. 100 µl of inoculums contains approximately 5×10^5 CFU/ml of test bacteria was added to each well. Negative controls contained non-inoculated medium with extract samples and positive controls wells were prepared with inoculated culture medium with no extracts.[24] Resazurin powder (Sigma-Aldrich) was diluted in distilled water to a final concentration of 1 mg/ml and 10 µl was added to all wells.[24] Microplates were incubated at 37°C for 24 h. The MIC was determined by observing the lowest concentration of extract which would inhibit visible growth of bacteria.[30] For determination of minimum bactericidal concentrations (MBC), 20 µl of the suspension of well before MIC of the extract were cultured on BHI agar using the spread plate technique. After 24 hours of incubation at 37°C, the MBC were evaluated by count the number of bacterial colonies.[24]

Statistical analysis

Each test was repeated in triplicate and all parameters were measured in duplicate. The mean and standard deviation (SD) of the growth inhibition zone diameter in agar disk-diffusion method as well as the MIC and MBC of the extracts, Gentamicin, cotrimoxazole, tetracycline, ciprofloxacin, saftrixone and amoxicillin were determined.[31] Data were analyzed using Statistical Package for Social Sciences for Windows, version 19.0 (SPSS Inc.).

Results

According to GC-MS chromatogram finding of the extracts compounds, walnut leaves contain the major ingredients of Geranyl acetate (30.59%), (E) - 3, 7-dimethyl, 2, 6-Octadienal (3.63%), Geraniol (3.49%), and the pine leaves contains mainly styrene (32.59%), cyclohexanone (3.65%) and decane (3.49%). Details are shown in Tables 1 and Figure 1.

The results of the MICs and MBCs tests showed that walnut leaf extract had the highest effect against clinical isolate of *S. saprophyticus* (MIC: 4.68 ± 2.21 mg ml^{-1} and MBC: 6.25 ± 0.0 mg ml-1). The lowest effect was against clinical isolate of *A. baumannii* (MIC: 18.75±6.84 mg ml-1 and MBC: 27.08 ± 12.28 mg ml^{-1}).

Pine leaf extract had the highest effect against clinical isolate of *S. saprophyticus* (MIC: 6.25 ± 0.0 mg ml^{-1} and a MBC: 9.37 ± 4.41 mg ml^{-1}). The low effect was against clinical isolate of *A. baumannii* (MIC & MBC: 20.83 ± 6.45 mg ml^{-1}) and *P. vulgaris* (MIC: 16.66 ± 6.45 mg ml^{-1} and MBC: 22.91 ± 5.10 mg ml^{-1}). Details are shown in Tables 2.

The results of disk diffusion test showed that the antibiotic disks of amoxicillin had no effect against *P. aeruginosa* and did not cause the diameter of the inhibition zone. While the of walnut and pine leaves extracts exhibited zone of inhibition diameter of 15.50 ± 0.54 mm and 17 ± 2.8 mm, respectively, both at concentration of 75 mg ml^{-1}. The lowest diameter of the inhibition zone were due to co-trimoxazole (8.66 ± 0.51 mm) and ciprofloxacin (8.83 ± 0.98 mm) against *A. baumannii,* while the diameter of the inhibition zone caused by the extracts were greater than that of these antibiotics. More details are shown in Tables 3.

Discussion

Burn wound infection is one of the most important accidents related to human health.[6,7] Many classes of antimicrobials were used to prevent this type of infections, but unfortunately, but the treatment drug-resistant microorganisms remain as a major unsolved problem.[1,2] The various medicinal plants compounds have been considered as alternative therapies against infections in traditional medicines.[32] The result of this study showed that the walnut and pine leaves extracts had antimicrobial activity against *P. aeruginosa, S. aureus, P. vulgaris, A. baumannii, E. coli, S. epidermidis* and *S. saprophyticus* isolated from burn wound infection. According to these results, the antibacterial effect of walnut leaves extract were more than pine leaf, but generally, both extracts have the ability to compete with the antibiotics of this study. There are very little or incomplete reports regarding that antibacterial activity of walnut and pine leave extract on isolated bacterial burn wound infections. Previous studies have suggested that extracts with more flavones and tannins have more antibacterial effects than other compounds of the extract (34,35) It seems that the antimicrobial activity of the extracts examined in this study are related to the flavonoids, diethyl phthalate (in walnut leaves) and styrene (in pine leaves).[33]

In studies conducted by Ajaiyeoba et al., the antimicrobial potential of various extracts including chloroform, hexane, ethyl acetate and methanol extracts of walnut leaf was investigated on *p. aesogenase, E. coli, S. aureus* and *B. subtilis*, and these results suggested that walnut leaf extracts are effective on these four microorganisms.[34] A study by akan et al. have shown that *A. baumannii* has been resistant to co-trimoxazole, ceftazidime and piperacillin.[35] In the present study, co-trimoxazole and ciprofloxacin produced the lowest diameter of the inhibition zone for *A. baumannii* than the other antibiotics. In current study, the MIC and MBC values showed antimicrobial activity of walnut leaf extract that against *P. aeruginosa, S. aureus, P. vulgaris, A. baumannii, E. coli, S. epidermidis* and *S. saprophyticus*. Jamehdor et al., found that the aqueous extract of walnut leaves had an effect on the growth of *P. aeruginosa*.[36] Rafieian et al. reported that ethanolic extract of walnut leaves has an inhibitory effect on Propionibacterium acnes. The above research concluded that ethanolic extract of walnut leaves has antimicrobial activity against *Salmonella typhimurium, Shigella Dysenteria* and *Listeria monocytogenes*.[18] Olivera et al. found that walnut leaves have both antioxidant and antimicrobial properties and also walnut peel extract is effective against *S. aureus*.[37] One important factor affecting the MIC is the difference in the composition of extracts. The composition of extract is influenced by the geographical location of the plant, season of harvesting, age of plant, growth stage, method of drying, and extraction technique.[24] Prior research has shown that phenolic compounds are found in walnut leaves and are identified as anti-microbial agents.[38] Phenolic compounds exist in many plants, and their microbial effects depend on the location and number of hydroxyl groups on the phenolic ring, and it is claimed that there is a direct relationship between the number of hydroxyl groups and their toxicity on microorganisms.[39] It has also been argued that the potential mechanism for these compounds is to inhibit the enzyme by reacting with sulfidryl groups or a non-specific reaction to the microbial protein.[40] Flavones, flavonoids and flavonols also have a phenolic structure and have antimicrobial activity. Their antimicrobial activity are probably due

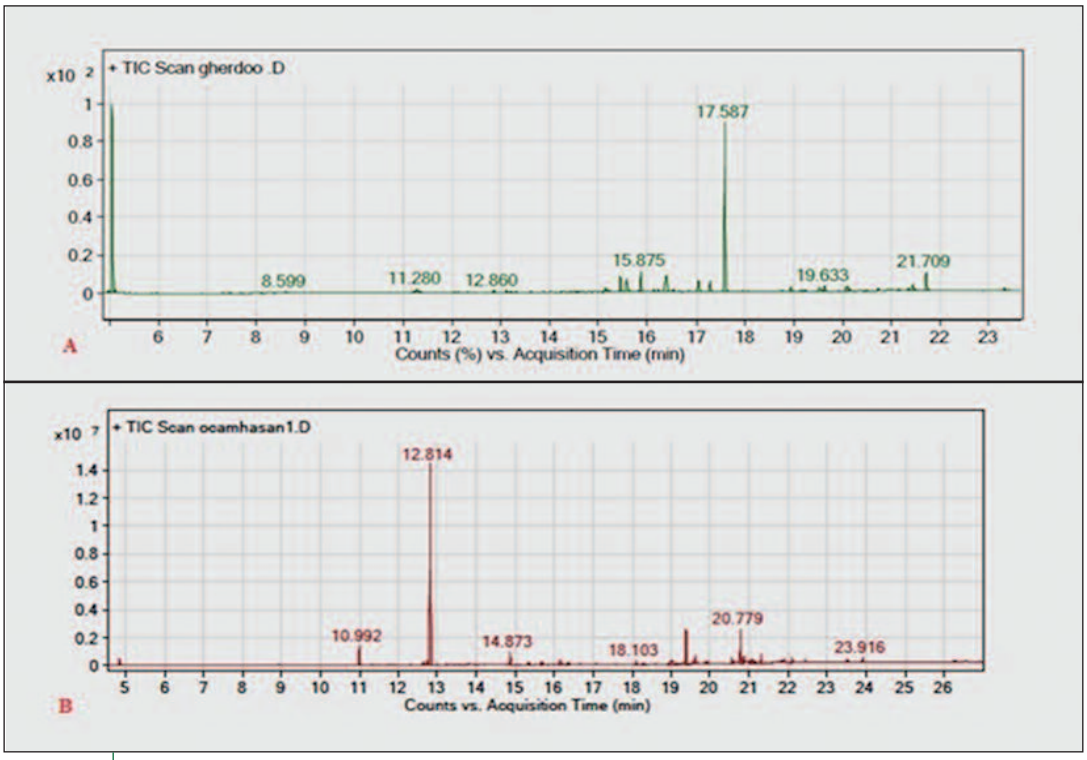

Figure 1 GC-MS Chromatogram of ethanolic extract of the *Walnut* (A) and *Pine* (B) leaf.

Table 1 Main phytochemicals identified in the ethanolic extracts of the *Walnut* and *Pine* leaf

No	Compound name	Formula	Retention time	Percent
	Walnut leaf			
1	Geranyl acetate	$C_{12}H_{20}O_2$	17.58	30.59
2	Diethyl Phthalate	$C_{12}H_{14}O_4$	19.53	2.78
3	Geraniol	$C_{10}H_{18}O$	15.57	3.49
4	(E)-3,7-dimethyl, 2,6-Octadienal	$C_{10}H_{16}O$	15.87	3.63
5	Citral	$C_{10}H_{16}O$	15.44	2.66
6	Neric acid	$C_{10}H_{16}O_2$	16.53	0.63
7	Linalool	$C_{10}H_{18}O$	13.99	0.30
8	Epoxy-linalooloxide	$C_{10}H_{18}O_3$	16.13	1.1
9	Furanoid	$C_{10}H_{18}O_2$	12.86	0.93
10	5-Hydroxymethylfurfural	$C_6H_6O_3$	15.14	1.61
11	Geranic acid	$C_{10}H_{16}O_2$	17.05	3.10
12	2,7-Octadiene-1,6-diol, 2,6-dimethyl	$C_{10}H_{18}O_2$	19.87	2.37
	pine leaf			
13	Styrene	C_8H_8	18.56	32.59
14	Cyclohexanone	$C_6H_{10}O$	19.63	3.65
15	Decane	$C_{10}H_{22}$	17.56	3.49
16	Hexanone	$C_{10}H_{14}$	17.65	3.25
17	Dodecanone	$C_{12}H_{26}$	16.44	2.01
18	Tetradecane	$C_{14}H_{28}$	18.56	0.25
19	Hexadecane	$C_{14}H_{34}$	14.20	1.8
20	Tetradecanoiceacid	$C_{14}H_{25}$	17.5	1.23
21	Octadecane	$C_{18}H_{36}$	14.52	0.68
22	Hexadecanoiceacid	$C_{10}H_{20}O_2$	17.12	1.3

to the combination of extracellular proteins or the formation of a complex with the cell wall or membrane disruption of the microorganisms.[33] Tannins are also a group of phenolic compounds that are found in walnut leaves, their antimicrobial effect is related to inhibiting germicidal adhesion and also inhibiting enzymatic activity and transfusion protein.[41] Kim et al. has compared the antibacterial effects of pine needles extract on human skin pathogens. It has been concluded that they have an antibacterial effect on *E. coli, S.aureus* and *P. acnes*.[42] Hawford et al., found that pine coniferous material had a inhibitory effect on *S. aureus*.[43] Batisa et al., have also confirmed the inhibitory effect of pinewood gum on *S. aureus* and a number of gram-negative bacteria.[44] Antibacterial properties of pine leaf are due to the presence of monoterpene and dipropenoid,[45] as well as chemical compounds in the pine leaf include limonene, tannin, flavonoids, resins, most and terpenoves.[46] The findings of this study showed that the walnut leaves extract had more antibacterial activities than pine leaves against the bacteria studied in this study. However, this antibacterial activity on pathogenic bacteria has only been tested *in vitro*, and further research is required to confirm these antibacterial activities *in vivo*.

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Conflict of interest

The authors confirm that this article content has no conflict of interest.

Table 2 MICs and MBCs of *Walnut* and *Pine leaf* extract against standard and clinically isolated bacteria (mean±SD)

Bacteria	*Walnut leaf*		*Pine leaf*	
	MIC (mg ml-1)	MBC (mg ml^{-1})	MIC (mg ml^{-1})	MBC (mg ml^{-1})
P. aeruginosa^{S}	6.25±0.0	6.25±0.0	3.12±0.0	3.12±0.0
P. aeruginosa^{C}	11.45±2.55	16.66±6.45	7.29±2.55	7.29±2.55
A. baumannii^{S}	12.50±0.0	25.00±0.0	12.50±0.0	12.50±0.0
A. baumannii^{C}	18.75±6.84	27.08±12.28	20.83±6.45	20.83±6.45
E. coli^{S}	6.25±0.0	6.25±0.0	12.50±0.0	12.50±0.0
E. coli^{C}	10.41±3.22	16.66±6.45	16.66±6.45	20.83±6.45
P. vulgaris^{S}	6.25±0.0	6.25±0.0	12.50±0.0	12.50±0.0
P. vulgaris^{C}	9.37±3.42	14.58±5.10	16.66±6.45	22.91±5.10
S. aureus^{S}	3.12±0.0	4.68±2.21	6.25±0.0	6.25±0.0
S. aureus^{C}	5.72±1.27	9.37±3.42	9.37±3.42	11.45±2.55
S. epidermidis^{S}	3.12±0.0	4.68±2.21	6.25±0.0	9.37±4.41
S. epidermidis^{C}	5.72±1.20	8.33±3.22	9.37±4.42	14.58±5.10
S. saprophyticus^{S}	6.25±5.01	9.37±3.40	8.33±3.22	12.50±0.0
S. saprophyticus^{C}	4.68±2.21	6.25±0.0	6.25±0.0	9.37±4.41

S: standard isolate, C: clinical isolate

Table 3 Diameter of inhibition zone (mm) of pine and walnut leaves and antibiotic disks extracts tested against standard and clinically isolated bacteria

Bacteria	*Walnut leaf* extract			*Pine leaf* extract		
	25 mg ml^{-1}	50 mg ml^{-1}	75 mg ml^{-1}	25 mg ml^{-1}	50 mg ml^{-1}	75 mg ml^{-1}
P.aeruginosa^{S}	13±0.0	16±0.0	19.50±0.70	15±0.0	18±0.70	22±1.4
P.aeruginosa^{C}	10.50±0.54	13.50±0.54	15.50±0.54	11.50±2.1	14.16±2.48	17±2.8
A.baumannii^{S}	10.50±0.70	14.50±0.70	18±0.0	11±1.41	14±0.0	16.50±0.70
A.baumannii^{C}	9.16±0.75	11.66±1.03	14.16±0.72	9±0.89	11.66±1.50	15.50±1.64
E.coli^{S}	13±0.0	17±0.0	21±0.0	13.5±0.70	16.50±0.70	19.50±0.70
E.coli^{C}	9.83±0.75	12.16±1.16	15.16±1.94	10.83±0.98	14.16±0.75	15.66±0.51
P.vulgaris^{S}	13.50±0.70	17.50±0.70	23±0.0	13±0.0	16±1.4	19±0.0
P. vulgaris^{C}	10±0.89	12.50±1.22	17±0.63	12.50±0.70	14±0.89	15.50±0.83
S. aureus^{S}	16±0.0	19±0.0	23±0.0	12.50±0.70	15±0.0	18±0.0
S. aureus^{C}	11.83±1.47	14±2.58	17±0.0	9.83±0.75	12.33±0.51	16±0.63
S.epidermidis^{S}	15±1.41	18±0.0	20±0.70	13.50±0.70	15.50±0.70	19±0.0
S.epidermidis^{C}	12±0.89	13.83±0.98	16.50±2.12	10±0.63	12.33±0.51	16±0.63
S. saprophyticus^{S}	16.50±0.70	20±0.0	23.50±0.70	13±0.0	16±0.0	18.50±0.70
S. saprophyticus^{C}	12.33±0.51	14.16±0.98	17±0.63	10.16±0.75	12.16±0.70	15.83±0.75
Antibiotic disks						
Bacteria	**Tetracycline**	**Amoxicillin**	**Co-trimoxazole**	**Gentamicin**	**Ciprofloxacin**	**Ceftriaxone**
P.aeruginosa^{S}	15±0.0	0.0±0.0	11.50±0.70	14±0.0	20.50±2.12	17.50±0.70
P.aeruginosa^{C}	10.66±1.21	0.0±0.0	9±0.89	10.16±1.83	16.50±2.94	13.50±3.20
A.baumannii^{S}	16±1.4	14.50±2.1	11±0.0	15.50±0.70	11±1.41	18±0.0
A.baumannii^{C}	12.16±2.78	9.66±0.51	8.66±0.51	11.33±1.36	8.83±0.98	13.16±1.94
E.coli^{S}	18±0.0	17±0.0	27±0.0	17±1.4	21.50±0.70	16.50±2.12
E.coli^{C}	12.50±2.73	10.66±1.36	22.16±3.18	12±1.89	18.83±1.60	13±2.28
P.vulgaris^{S}	18.50±2.12	14.50±2.1	13.50±2.12	16±1.41	15.50±2.22	17±1.41
P. vulgaris^{C}	12.83±2.04	10.33±1.2	10.16±1.16	12.83±1.72	12.50±2.25	12.16±2.40
S. aureus^{S}	20±0.0	22.50±2.12	15±1.41	18.50±0.70	25.50±0.70	23±1.41
S. aureus^{C}	13.83±1.32	16±1.41	9.32±0.81	13.66±2.42	18.83±4.16	15.66±3.50
S.epidermidis^{S}	19.50±0.70	22±1.41	15.50±.070	15±0.0	22.50±3.5	21±0.0
S.epidermidis^{C}	11.83±2.04	16.83±1.47	11.83±0.98	12±0.0	18.16±2.13	16.33±2.58
S.saprophyticus^{S}	19±0.0	23±0.0	16±0.0	18±0.0	23±1.41	19.50±0.70
S.saprophyticus^{C}	13.16±0.75	15.66±2.65	11.33±1.86	15±0.63	21.16±0.70	15.83±1.60

Περίληψη

Τίτλος

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Λέξεις κλειδιά

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