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Characterization and inhibition by natural agents of multidrug resistant bacteria isolated from wounds.

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ABSTRACT

Fifty Saudi patients suffering from wounds in many places were analysed microbiologically. Bacterial infections were detected in the all 50 wounds tested. The fifty bacterial pathogens were identified and classified into 4 groups according to isolate number viz. *Staphylococcus aureus*(*S. aureus*) (27 isolates, 54%) >*Pseudomonas aeruginosa*(*P. aeruginosa*) (15 isolates, 30%) >*Eschericidia coli*(*E. coli*) (4 isolates, 8%) >*Streptococcus pyogenes*(*St. pyogenes*) (3 isolates, 6%). *S. aureus* group showed the higher resistance percentage to 17 antibiotics studied, but no one isolate was either completely resistant or completely sensitive to antibiotics. Three organisms were resistant to most antibiotics studied namely: *S. aureus* W13, *P. aeruginosa* W39 and *E. coli* W18. These organisms were inhibited distinctively by either clove oil or potassium alum (5% w/v). A mixture of both natural agents prevented growth of these MDR bacteria within 48-96h.

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INTRODUCTION

Wounds are formed due to microbial infections in a local area of human body; a collection of pus was developed due to formation of a mix of macrophages, killed microbes and dead cells with pain in such area, pyrexia and duration [1]. Wound infections are one of the most common hospital acquired infections and are an important cause of morbidity worldwide [2]. This clearly showed that there is a need to continue research to check the types of bacterial isolates causing wound infections among Saudi patients.

The selection and isolation of multi-drug resistant bacteria (MDR bacteria) from wounds make great challenge for treatment of such wounds; suggestion of treatment protocols for treatment of MDR bacteria is another challenge [3-6]. This makes a need to study the antimicrobial susceptibility profiles for bacteria isolated from wounds and other human systems; selection of MDR bacteria are necessary. To treat them many natural agents are useful. One of them is clove oil which exhibited antimicrobial activity against MDR bacteria [7]. Also potassium alum (Potassium aluminium sulphate) is a non-toxic natural agent and is used for water purification. It decreases the water activity by decreasing free water and increasing bound water around their particles; leading to microbial death [8].

The present work was undertaken to isolate and identify bacteria from wounds. Selection and inhibition by clove and potassium alum of MDR bacteria were also studied.

MATERIALS AND METHODS

Isolation of bacterial isolates:

Fifty bacterial isolates were isolated after streaking of sterile cotton swabs taken from wounds of 50 people admitted to King Fahd Hospital, Riyadh, KSA in the age range 10-60 years old with complaints of puss discharge and foul smelling, on blood agar, brain heart infusion agar (Oxoid). Culture media such as Baird Parker agar, CLED agar, MacConkey agar, Blood agar (all from Oxoid) were used as a confirmatory indicators for growth of the *S. aureus*, *P. aeruginosa*, *E. coli*, *St. pyogenes* bacteria respectively.

Identification of bacterial isolates:

Cultural and morphological criteria were taken after growth of bacterial cultures on their specific media; Gram staining, catalase, urease, nitrate reductase3 and indole production were studied as described previously [9]. Biochemical characteristics and utilization of different carbon sources were studied as described previously [10]. API kits (Biomereux, France) were used as an additional identification criteria.

Antibiotic susceptibility of the obtained bacteria:

Susceptibility of 50 identified bacterial strains to 17 antibiotics were studied as described previously [10]. Antibiotic discs were provided from Promega. Each antibiotic disc contained 25 μg/disc and vancomycin disc contained 4 μg/ml. Discs were put on the surface of BHI agar plates previously seeded with cell suspensions of the tested bacteria. After incubation at 37°C for 48 h, results were taken according to National Committee for Clinical Laboratory Standards [11].

Inhibition of the multi-drug resistant (MDR) bacteria by clove oil and potassium alum:

Different natural agents were preliminary tested for their inhibitory capability against some MDR bacteria as described previously [12-14] Clove oil and potassium alum (potassium aluminium sulphate) (SomatcoComp.) showed the best inhibitory activity. Either clove oil or potassium alum were prepared. Also mixture of both of them was prepared. A series of test tubes, each containing 10 ml of BHI broth were inoculated by the experimental MDR bacteria (*S. aureus* W13, *P. aeruginosa* W39, *E. coli* W18) (2 x 10^5 CFU/mL for each) and treated with 5% (w/v) sterile liquid nature agent previously mentioned and in another experiment bacterial suspensions were treated with clove oil potassium alum mixture (5% w/v for each). After appropriate time intervals, 1 ml aliquots were withdrawn and analysed for CFU/ml as described previously[15].

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RESULTS

Fifty bacterial isolates were isolated from wound swabs of people admitted to King Fahd Hospital, Saudi National Gaurd. These bacterial isolates were grown on both BHI agar and blood agar (Oxoid) and purified on the same media. These 50 bacterial isolates were characterized regarding cell morphology, Gram staining, catalase reaction. Then they were subjected to identification studies using API kits (Biomereux, France). On the basis of the obtained results about identification and taxonomic criteria such as cell morphology, Gram staining, catalase test and from results obtained API kits containing 20 tests, the 50 bacterial isolates could be grouped in 4 groups as follows (Table 1):

Group 1: This group contained 27 bacterial isolates, all of them were Gram positive cocci, catalase positive, coagulase positive. They gave blue colonies on Baired-Parker agar. According to these results and the ones obtained from API identification kits, the 27 bacterial isolates of this group could be identified as belonging to *S. aureus*. The prevalence of *S. aureus* bacteria was 54% within total bacterial isolates obtained (50 isolates).

Group 2: This group included 15 bacterial isolates, all of them were Gram negative rods shaped bacteria and were catalase positive and urease positive. The results of API kits indicated that the 15 isolates of this groups were strains of *P. aeruginosa*. For confirmation, these isolates grew well on CLED agar and formed green colonies with bluish green diffusible reverse side of colonies. The prevalence of such bacteria was 30% within total bacteria isolated (50 bacterial isolates).

Group 3: This group contained 9 bacterial isolates; all of them were Gram negative rods. They were positive catalase. They grew well on MacConkey agar and formed pink colonies. Additionally the results of API kits and that of possible identification keys showed that these 4 isolates were strains following *E. coli* bacteria. Prevalence of such bacteria was 8% only within 50 ones isolated from wounds.

Group 4: The 3 isolates of this group were of 6% prevalence percentage within 50 isolates obtained from wounds. The 3 bacterial isolates were Gram positive cocci and were catalase positive. Results of API kits referred to *St. pyogenes*. For confirmation, those 3 isolates showed positive anti-streptolysin O (ASO).

The bacterial strains of the 4 groups identified (50 bacterial strains) were bioassayed for their susceptibility to 17 antibiotics. Results are given in Table 2. The percentage of antibiotic resistance were calculated for bacteria belonging to each group. The percentage ratios of resistance of bacteria obtained against 17 antibiotics showed variability; no one bacterial strains was either completely sensitive or completely resistant to the studied antibiotics. Concerning *S. aureus* group, higher resistance percentage (62.96%) was showed against tetracyclin, but lower resistance percentage was showed against both erythromycin and levofloxacin. No resistance was showed against 4 mg/L vancomycin. Methicillin resistant *S. aureus* (MRSA) phenomena was showed by about 40.74% within the 27 strains of *S. aureus*strains of group 1. Regarding *P. aeruginosa* group, higher antibiotic resistance was showed against both cefaclor and methicillin (45.66%); but lower resistance percentage (13.33%) was showed against amoxicillin, ciprofloxacin, levofloxacin and tetracycline. No resistance of *P. aeruginosa* isolates was showed against 4 mg/L vancomycin. The 4 strains of *E. coli* bacteria were resistance against both amoxicillin and cefaclor and were sensityive to amikacin and levofloxacin. The two strains of *St. pyogenes* resistant against amoxicillin, but were sensitive to amikacin, ampicillin, azithromycin, levofloxacin, nitrofurantion, methicillin, gentamycin, erythromycin, ciprofloxacin and ceftazidime. From the antibiotic resistance profiles, it was concluded that 8 strains; 4 strains; 2 strains of *S. aureus; P. aeruginosa; E. coli* were MDR bacteria and were resistant to most studied antibiotics (Table 3).

In an endeavour to decrease bacterial infections of wounds, different natural agents were bioassayed for their inhibitory activity against MDR bacteria obtained herein (data not shown); only clove oil and potassium alum solution at 5% w/v inhibited some MDR bacteria. Results are given in Figures 1, 2, 3. The MDR *S. aureus* W13 (control cells) grew rapidly and log CFU/mL of treated cells with both clove oil and potassium alum decreased by 1-2 log cycles within 96 h and difference between control cells and treated ones was 5-6 by cycles after 96 h of incubation (Figure 1).

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Table 1: Growth of bacteria, their numbers and codes; and their prevalence within total bacteria isolated (50 isolates) from wounds.

Bacterial group number	The identified bacteria	Number of isolates and their codes	(%) prevalence percentage
1	*S. aureus*	(27 isolates) W1, W4, W7, W8, W11, W13, W14, W20, W22, W23, W24, W25, W30, W31, W33, W35, W36, W37, W38, W40, W41, W42, W45, W47, W48, W49, W50	54%
2	*P. aeruginosa*	(16 isolates) W2, W3, W5, W6, W9, W12, W15, W16, W17, W26, W32, W39, W43, W44, W46	30%
3	*E. coli*	(4 isolates) W18, W19, W28, W29	8%
4	*St. pyogenes*	(3 isolates)W21, W28, W34	6%

Table 2: Percentage values of antibiotic resistance within total identified bacteria and isolated from wounds.

Bacterial group	Amikacin	Amoxicillin	Ampicillin	Azithromycin	Cefactor	Cefotaxime	Cefotaxime	Ceftazidime	Ceftriazone	Ciprofloxacine	Erythromycin	Gentamycin	Methicillin	Nitrofurantion	Levofloaxcin	Tetracyclin	Vancomycn
S. aureus	14/27 (51.85)	12/27 (44.44)	15/27 (55.55)	12/24 (44.44)	6/27 (29.62)	12/27 (44.44)	10/27 (32.09)	16/27 (59.25)	15/27 (55.55)	7/27 (25.92)	5/27 (18.51)	6/27 (22.22)	11/27 (40.74)	10/27 (37.03)	5/27 (18.51)	17/27 (62.96)	0/27 (0)
P. aeruginosa	0/15 (0)	2/15 (13.33)	6/15 (40)	5/15 (33.33)	7/15 (46.66)	3/15 (20)	3/15 (20)	5/15 (33.33)	5/15 (33.33)	2/15 (13.33)	4/15 (26.66)	6/15 (40)	7/15 (45.66)	3/15 (20)	2/15 (13.33)	2/15 (13.33)	15/15 (100)
E. coli	0/4 (0)	4/4 (100)	3/4 (75)	1/4 (25)	4/4 (100)	3/4 (75)	2/4 (50)	2/4 (50)	2/4 (50)	2/4 (50)	3/4 (75)	2/4 (50)	2/4 (50)	2/4 (50)	0/4 (0)	2/4 (50)	4/4 (100)
St. pyogenes	0/2 (0)	2/2 (100)	0/2 (0)	0/2 (0)	1/2 (50)	0/2 (0)	0/2 (0)	0/2 (0)	1/2 (50)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	1/2 (50)	2/2 (100)

Cells of *P. aeruginosa* without treatments multiplied rapidly and increased 3 log cycles within 96 h, however in treated samples no growth was showed; difference between growth values of control and treated samples with either clove oil or potassium alum was about 5 log cycles (Figure 2).

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Table 3: Multidrug resistant bacteria isolated from wounds.

Bacterial group	Multidrug resistant isolates Isolate no. and code
S. aureus	W4, W8, W13, W20, W36, W45, W47, W50
P. aeruginosa	W3, W12, W39, W46
E. coli	W18, W28

* Bacterial strains resistant to most studied antibiotics.

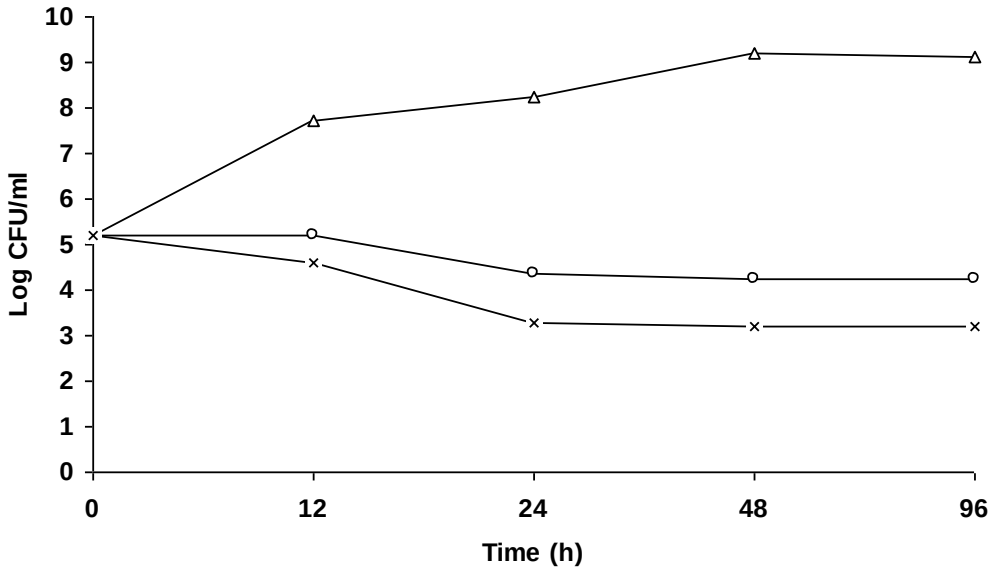

Figure 1:Effect of both clove oil (5) and alum on growth of MDR *S. aureus* W13.Δ, o, x; control, treated cells with clove oil (5); with alum solution respectively.

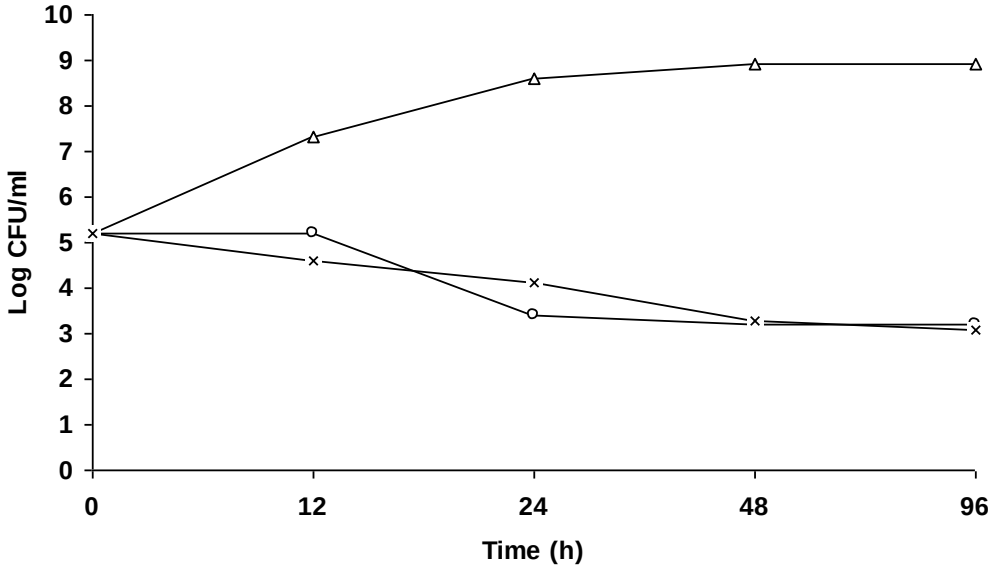

Figure 2: Inhibition of *P. aeruginosa* W39 by two natural agents. Δ, cells without treatment; o, x cells treated with clove oil, potassium alum respectively.

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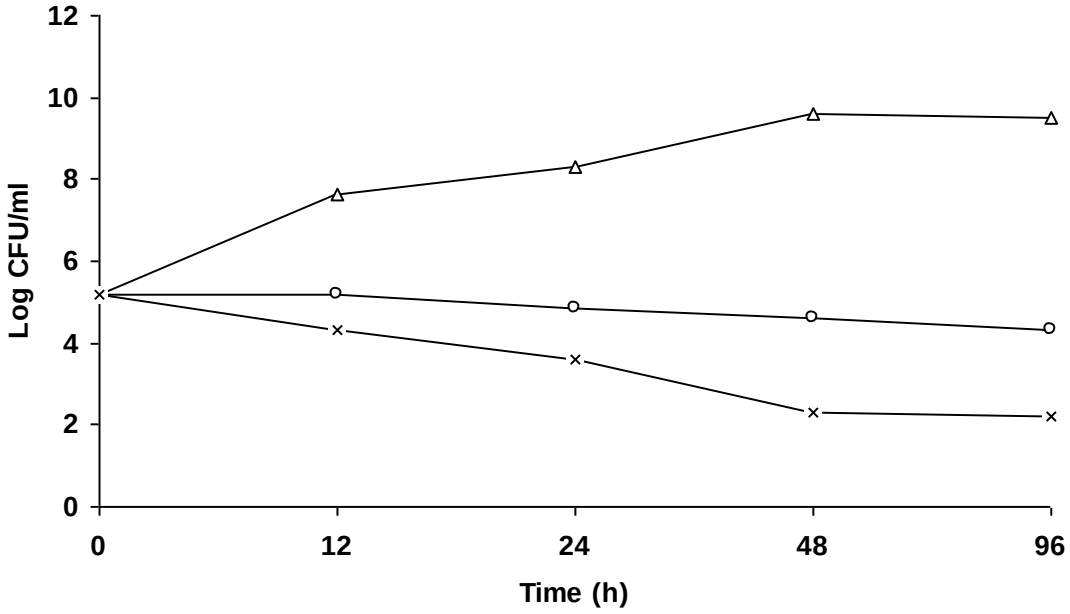

Figure 3: Inhibition of *E. coli* W18 by two natural agents. (Δ), cells without treatment; (o) cell treated with 5% clove oil, (x) cells treated with 5% potassium alum respectively.

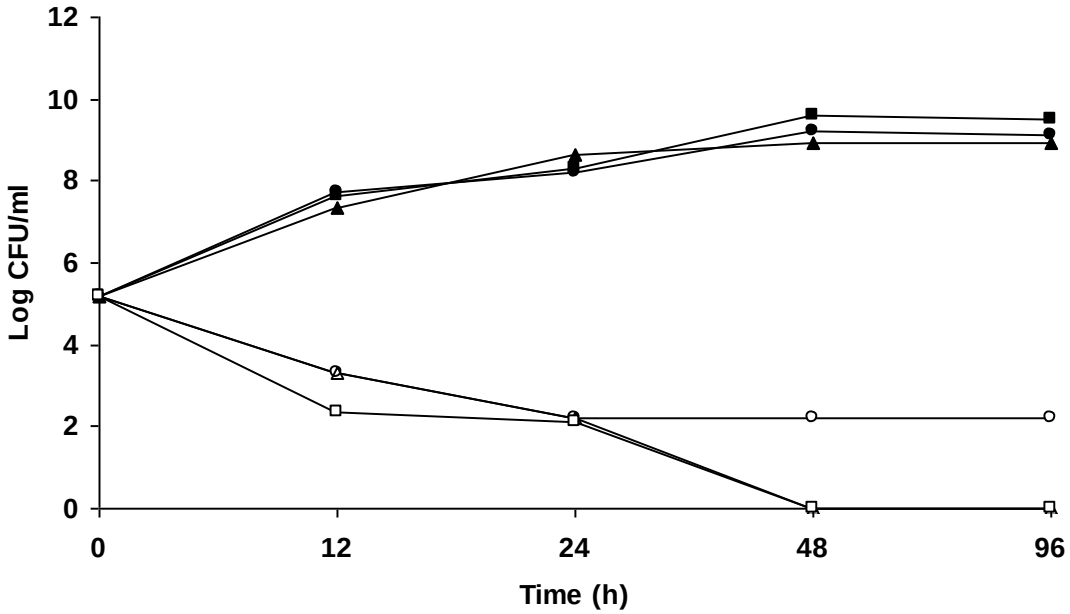

Figure 4: Inhibition of three MDR bacteria by clove oil –potassium alum mixture (5%-5% w/v).•& o, control & treated cells of S. aureus W13; ▲&Δ, control & treated cells of P. aeruginosa W39; ■&□, control & treated cells of *E. coli* W18.

Addition of clove oil and potassium alum to cell suspensions of *E. coli* W18 resulted in decrease of growth values by 1-3 log cycles; growth of *E. coli* W18 cells without treatment increased from 2 x 10^5 CFU/mL to 5.1 x 10^9 CFU/mL (Figure 3).

Mixture of both clove oil-potassium alum, 5% w/v of each was prepared. Addition of this mixture to cell suspensions of the MDR bacteria resulted in reduction of growth of *S. aureus* W13 by 80% and prevented growth of both *P. aeruginosa*W39 and *E. coli* W18 within 48h; indicating on distinctive inhibiting activity of clove oil-potassium alum mixture.

DISCUSSION

The prevalence of bacterial pathogens within Saudi patients suffering from wounds were 100% as all wounds tested showed bacterial growth. Latter work in this respect showed that bacterial pathogens prevalence within wounds was 80-100% [16, 17].

All fifty bacterial pathogens obtained were characterized by phenotypic and biological criteria [10, 18]. The identified bacteria were differentiated into groups which were arranged in the differentiated into groups which were arranged in the following descending order according to isolates number in each group viz. *S. aureus* (27 isolates) >*P aeruginosa* (15 isolates) >*E. coli* (4 isolates) >*St.pyogenes* (3 isolates). Latter published work showed that the identified bacteria herein were common contaminants of wound [19]. *S. aureus* strains were reported previously to be the more prevalent organisms in wound of yellow pus samples; followed by *P. aeruginosa* in wounds of blue green pus samples. However, *E. coli* and other many bacterial pathogens were reported previously to be secondary infections of wounds due to decrease in health care of patients. They could be derived from dusts or human microflora [16].

The antibiotics resistance percentage were 54%; 30%, 8%, 6% within bacteria belonging to *S. aureus; P. aeruginosa; E. coli; St. pyogenes* respectively. These results are in conform with published previously [20]. The resistance of bacteria to antibiotics are due to thickening of outer membrane protein, secretion of enzymes that could degrade antibiotics, modification of site(s) receptors and are mostly due to genetic factors [21, 22].

The selection of multi-drug resistance bacteria in this study makes an interest to inhibit them by natural agents. Many agents were tried and only clove oil and natural alum inhibited these bacteria. Alum (aluminium potassium sulphate) has recommended as category I active ingredient in mouth or skin washes by FDA [23]. Also clove extract was reported to inhibit bacterial and fungal pathogens [7, 8].

Growth of *S. aureus* W18, *P. aeruginosa* W39 and *E. coli* W18 was prevented after almost 48-96 h of incubation in broths containing both clove oil and potassium alum (5% w/v for each). Potassium alum decreases free water of wounds and limits metabolic activity of bacterial pathogen [23]. Clove oil contains phenolic compounds, galic acid and eugenol which acts distinctively in synergistic effect with potassium alum which denaure protein and react with cell membrane phospholipids changing the permeability of cell membrane leading to cell death [24, 25].

Further work will be necessary to study the effect of both clove oil and potassium alum solution *in vivo*.

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