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Bacteriostatic or bactericidal action of four aqueous plant extracts on multi-drug resistant bacteremia and their effect on cells morphology recorded using scanning electron microscopy (SEM)

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Four aqueous traditional plant extracts from rosemary leaves *(Rosmarinus officinalis*), clove buds (*Syzygium aromaticum* [L.] Merr at Perry, Myrtaceae), cinnamon bark (*Cinnamomum zeylanicum*) and ginger rhizomes *(Zingiber officinale*) were tested as natural antibiotics against multi-drug resistant bacteremia isolates: Six Gram negative isolates, viz. *Acinetobacter baumanni/haemolyticus*, *Citrobacter freundii, Escherichia coli*, *E. coli* ESβL*, Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Salmonella* sp. and four Gram positive isolates viz. methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis, Streptococcus pneumoniae* and *Bacillus* sp. were which used to test antibiotic activity. The usage of clove extract (25X) appeared to have maximum antibacterial activity against all tested Gram negative bacteria, when compared with other tested plant extracts at the same concentration. In the case of G^+ve bacteria, tested extracts appeared to have almost the same efficiency. Swabs were taken from inhibition zone resulting from the inhibitory effect of aqueous extracts towards the tested isolates and re-inoculated to detect either the bacteriostatic or bactericidal action. Again, clove extract exhibited the highest effect when compared with other tested against the isolates from bloodstream. Scanning electron microscopy was used to record external morphological changes of the bacterial cells such as appendages, shortness and lyses.

Key words: Aqueous plants extracts, bacteremia, antibiotics, rosemary, clove, cinnamon, ginger, *Escherichia coli*, *Salmonella*, *Acinetobacter, Citrobacter*, *Klebsiella, Pseudomonas*, *Streptococcus, Bacillus, Staphylococcus*.

INTRODUCTION

The spread of drug multiple resistances pathogens are one of the most serious threats to successful treatment of the dangerous and obstinate microbial diseases particularly, which are transported by blood. Bacteremia is one of the most common cases and it spreads mostly in hospitals. The development of antibiotic-resistant strains of bacteria e.g., methicillin-resistant *Staphylococcus aureus* (MRSA) has led to an increase in the incidence of severe bacteremia since the late 1960s (Drago et al., 2007).

Through the ages, essential oils and other extracts of plants have been screened for their potential uses as

alternative remedies for the treatment of many infectious diseases (Majorie, 1999; Tepe et al., 2004) as anti-infective agents (Agunu et al., 2001; Buwa and van Staden, 2006; Fu et al., 2007) and for their antimicrobial activity (Mothana and Lindequist, 2005; Limsuwan et al., 2009).

Many plants extracts contain active components as major source of natural organic compounds (Seenivasan et al., 2006), tannins and phenolic compounds (Ababa et al., 2006). Extracts of some food spices such as soluble arrowroot tea were able to inhibit some intestinal pathogens including *Escherichia coli* 0157:H7 (Aboaba and Efuwape, 2001; Kim and Fung, 2004). These substances are usually found in various parts of the plants like roots, leaves, shoots and bark. Therefore, many plants have become sources of important drugs and the pharmaceutical industries have come to consider traditional medicine as a source of bioactive agents that can be used in the preparation of synthetic medicine (Aboaba et al., 2006).

One of the most important features of all true bacterial cells is their peptidoglycan wall, which is responsible for the rigidity of the cell wall, maintains cell shape, provides physical protection and prevents the cell from bursting in a hypotonic environment (Demchick and Koch, 1996; Beveridge, 1999; Navarre and Schneewind, 1999). The research is designed to select an active plant extract which may be useful in developing new lead compound(s) to combat multi-drug resistant bacteremia isolates instead of antibiotics, which failed in treatments. Subsequently, the bacteriostatic/bactericidal action of tested aqueous extracts was carried out to strengthen the importance of selected plant extract(s) against multi-drug resistant isolates. Finally to confirm the results obtained, SEM was carried out to detect the effect of clove as the best plant extract on changes of cell wall of some genera infected bloodstream.

MATERIALS AND METHODS

Bacterial isolates collections

Nineteen multi-drug resistant bacteria isolates were obtained from a previous study. Isolates were obtained from blood samples of patients suffering from bacteremia. Isolates were identified in the bacteriology laboratory and recorded in hospitalization files in King Khalid University Hospital in Riyadh in a previous study by Alkufeidy et al. (2012). Fifteen isolates of Gram negative bacteria, including *Acinetobacter baumanni/haemolyticus* 2888 and 3106, *Salmonella* sp. 3397 and 4168; *Citrobacter freundii* 2569, *Escherichia coli* ESβL 4838, *Klebsiella pneumoniae* 1100 and *Pseudomonas aeruginosa* 1200; *E. coli* 2462, 2476, 2479, 2501, 2535, 2750 and 2882 and Gram positive bacterial isolates including methicillin-resistant *Staphylococcus aureus* (MRSA) 4182, *Staphylococcus epidermidis* 2509, *Streptococcus pneumoniae* 2675 and *Bacillus* sp. 2566 were used.

Antibiotics sensitivity pattern of isolates

The disk diffusion method was used to detect the pattern of multi-antibiotic resistances of bacteremia isolates. Results were expressed as sensitive (S), intermediate (I) or resistant (R) according to Clinical and Laboratory Standards Institute (CLSI) guidelines. This study used the previous references to determine the isolated bacteria sensitivity to different tested plant extracts using antibiotic vancomycin (VA) as a standard for G^+ve bacteria according to Barry et al. (1986) and antibiotic imipenem (IP) as a standard for G^-ve bacteria according to Primaxin (2009). Vancomycin disks gave ≤ 10 mm for (R), ≥ 11- 14 mm for (I) and ≥ 15 mm for (S) isolates. Imipenem disks hold is interpreted according to the following: ≤4 mm for (R), ≥ 8-15 mm for (I) and ≥16 mm for (S). Antibiotic tests were done in Iso- Sensitive Agar and were carried out at Bacteriology Laboratory in King Khalid University Hospital, Saudi Arabia. Disks of antibiotics (Strength µg/disk) were tested for G^-ve bacteria ring, *Pseudomonas* bacteria ring, Extra G^-ve bacteria ring 1 and 2, as well as for G^+ve bacteria ring, *Staphylococcus* and *Streptococcus* bacteria extra ring. The sensitivity of G^-ve and G^+ve bacteremia isolated was carried out on 32 and 24 antibiotics respectively. The following antibiotics were tested for the sensitivity against G^- bacteremia isolates: Ampicillin $(AMP)_{10\mu g}$, Cephradine $(CE)_{5\mu g}$, Cotrimoxazole $(COT)_{25\mu g}$, Chloramphenicol $(C)_{30\mu g}$, Gentamicin $(GN)_{10\mu g}$, Cefoxitin $(FOX)_{30\mu g}$, Carbenicillin $(CAR)_{100\mu g}$, Piperacillin $(PIP)_{30\mu g}$, ticarcillin $(TIC)_{75\mu g}$, Tobramycin $(TOB)_{10\mu g}$, Amikacin $(AN)_{30\mu g}$, Cefepime $(FEP)_{30\mu g}$, Cefixime $(CFM)_{5\mu g}$, Cetriaxone $(CRO)_{30\mu g}$, Cefuroxime Sodium $(CXM)_{30\mu g}$, amoxicillin/clavulanic acid (AMC) $_{(30\mu g + 10\mu g)}$, Aztreonam (ATM) $_{30\ \mu g}$, Imipenem $(IPM)_{10\mu g}$, Meropenem $(MEM)_{10\mu g}$, Ceftazidime $(CAZ)_{30\mu g}$, Ciprofloxacin $(CIP)_{5\mu g}$, Cefotaxime (CTX), Tazobactam (TZP), Sulphamethoxazole/Trimethoprim (SXT). In case of G^+ bacteremia isolates the following antibiotics were tested for the sensitivity: Erythromycin $(E)_{5\mu g}$, Tetracycline $(TE)_{10\mu g}$, $CE_{30\mu g}$, Clindamycin $(DA)_{2\mu g}$, Penicillin G $(P)_{1unit}$, AMP, Fusidic acid $(FD)_{10unit}$, CXM, Vancomycin $(VA)_{30\mu g}$, CIP, Cefoxitin $(FOX)_{30unit}$, CRO, CTX, GN, Oxacillin $(OX)_{1unit}$, AMC.

Plant samples collections

Leaves of rosemary (*Rosmarinus officinalis* L., Lamiaceae), buds of clove (*Syzygium aromaticum* [L.] Merr at Perry, Myrtaceae), bark of cinnamon (*Cinnamomum zeylanicum,* Lauraceae) and rhizomes of ginger (*Zingiber officinale,* Zingiberaceae) were selected to test their antibacterial activity potentiality towards bacteria isolated from bloodstream. The four plants were purchased from an herbal market in Riyadh City, Saudi Arabia.

Aqueous extraction

Four aqueous traditional plant extracts from rosemary leaves (*R. officinalis*), clove buds (*S. aromaticum* [L.] Merr at Perry, Myrtaceae), cinnamon bark (*C. zeylanicum*) and ginger rhizomes (*Z. officinale*) were air dried and used as natural antibiotics against multi-drug resistant bacteremia isolates. The air-dried parts of each plant were grinded into powdery form using a sterile electric grinder.

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The extractions of the water-soluble ingredients were carried out as follow: 25 g of each of the grounded parts were extracted by successive soaking for 24 h using 100 mL of distilled water in a 250 mL sterile conical flask. It was filtered through six layers of muslin cloth and centrifuged at 160 rpm. The supernatant was concentrated to 25x the original volume, to magnify efficiency of the extracts. The concentrated extracts were sterilized by autoclaving for 20 min at 15 pounds pressure and refrigerated at 4°C prior to use.

Antibacterial activity of aqueous plant extracts

The efficiency of antibacterial activity of aqueous plant extracts was determined by using the agar well diffusion method on Mueller-Hinton (MH) agar medium plates (Mueller and Hinton, 1941). Half milliliter volume of the suspensions at exactly 0.5 McFarland standards were prepared, to obtain an equivalent approximate density of bacteria $1x10^8$ colony forming unit (cfu) in 1 ml, they were spread over the plates. Hundred microlitres of the different plant extracts (25x) was aliquoted into respective wells in a well (11 mm diameter) of the medium. Sterile distilled water (100 µL) was used as a control. Diffusion plates were incubated at 37°C for 24 h.

Evaluation of the antibacterial potential of aqueous plant extracts

Evaluation of the zone of inhibition resulting from the effects of tested extracts on the bacteremia isolates, as sensitive (S), intermediate (I) or resistant (R), was estimated according to vancomycin (VA) 10 µg/disks which was used as a standard for G^+ve bacteria. Imipenem (IP) 10µg/disk was used as a standard for G^-ve bacteria.

Bacteriostatic/bactericidal aspect after exposure to plant extracts

The first experiment was carried out on solid media. The resulting clear zone of inhibition was swabbed using a sterile cotton swab and used to inoculate new plates containing nutrient agar-oxide (NAO) medium to determine if extracts were bacteriostatic (BS) or bactericidal (BC) in nature.

The second experiment was carried out in broth media. Isolated bacteria were cultivated in nutrient broth-oxide (NBO), and the clove extract instead of distilled water, for 24 h at 37°C. A sterile cotton swab was dipped in the bacterial suspension and used to streak the inoculations on NAO plates. Plates were incubated at 37°C for 24 h. Distilled water was used to prepare NBO in control treatment.

Experimental tests *in vitro*

The experimental tests in the present research were conducted according to method described in National Committee for Clinical Laboratory Standards (NCCLS) (2002) and Clinical and Laboratory Standards Institute (CLSI) (2012).

Morphological changes of bacterial cells treated with clove extract

Scanning electron microscopy (SEM) was chosen to examine the morphological changes of the following isolates: (G-ve) *A. baumanni/haemolyticus* 3106*, E. coli* 2479, *E. coli* ESβL 4838, *K. pneumoniae* 1100*, P. aeruginosa* 1200 and *Salmonella* sp. 3397, and G^+ve MRSA 4182, *S. epidermidis* 2509, *S. pneumoniae* 2675 and *Bacillus* sp. 2566 as bacteria, cultured in media containing clove extract. Control treatments were conducted using NBO with distilled water.

In the central lab in King Saud University, the cultures were fixed with agraded series of ethanol, allowed to dry and then coated with a thin layer of gold as a conducting material using the vacuum evaporation machine (Fine Coat Ion Sputter JCF-1100), at 1200 V, following Afrikian et al*.* (1973) method. Bacterial cell morphology was observed under a SEM (JEOL JSM-6360LV SEM) at magnification of 15,000x.

RESULTS

The sensitivity of G^-ve and G^+ve bacteremia isolates were carried out with 24 and 16 antibiotics respectively and summarized in Tables 1 and 2. G^-ve isolates showed differentiation for their resistant (R) response, ranging from one antibiotic in the case of *P.aeruginosa* 1200 and *Salmonella* sp*.* 3397, up to 14 antibiotics as in the case of *E. coli* ESβL 4838.

G^+ve *S. pneumoniae* displayed sensitivity (S) responses towards all tested antibiotics but it was resistant (R) to only CN. *Bacillus* sp*.* isolate showed sensitivity response towards all tested antibiotics but it was resistant to 3 antibiotics (P, AMP and CRO). *S. epidermidis* and MRSA4182 isolates showed (R) response against 9 and14 out of 16 antibiotics, respectively (Table 2).

Evaluation of the antibacterial potential of plant extracts is done in Table 1 and revealed that all tested G^-ve multi-drug resistant bacteremia isolates were (S) towards clove extract treatment. The different efficiency effect of rosemary, ginger, cinnamon and clove extracts are shown in bacteremia isolates to be 26.7, 53.0, 66.7 and 100%, respectively.

In the case of G^+ve bacteremia isolates, high effect of clove, ginger and cinnamon extracts appeared towards tested isolates. Whereas, the intermediate effect of rosemary extract was shown in *S. pneumoniae*, and high effect was showed in all other tested bacteria (Table 2). Re-inoculated bacterial cells from zone of inhibition on NAO was taken as criteria to detect bacteriostatic (BS) and bactericidal (BC) aspect of plant extracts towards G-ve and G^+ve isolates from bloodstream (Tables 1 and 2). Results indicated that clove, rosemary, ginger and cinnamon extracts gave BC effect 33.3, 22.2, 25 and 23.1% against G^-ve isolates respectively. Clove, rosemary and cinnamon extracts revealed BC effect against MRSA4182, while all tested extracts displayed BS towards the rest of G^+ve isolates*.*

In the case of evaluation of the BS/BC after exposure to clove extract in NBO, results indicated that all tested bacteria did not grow after treatment for 18 h after streaked on NBO plates, except the *Bacillus* sp. 2566 which had growth.

To detect the influence of clove extract on change of ultrastructure cell wall of bloodstream isolates by using SEM, short rods of untreated control *Acinetobacter baumanni/haemolyticus*3106 can clearly observed

Table 1. Sensitivity pattern and bacteriostatic/bactericidal (BS/BC) action of Gram negative (G⁻ve) bacteremia isolates to different plant extracts as compared to traditional antibiotics.

G⁻ve bacteria/No. of specimen	*Resistance to antibiotics	**Plant extract							
		Clove		Rosemary		Ginger		Cinnamon	
		Inhibition zone mm	BC/BS action	Inhibition zone (mm)	BC/BS action	Inhibition zone (mm)	BC/BS action	Inhibition zone (mm)	BC/BS action
A. baumanni/ haemolyticus 2888	AMP, CE, COT, C, FOX, CFM, CRO, CXM, AMC, ATM, CTX	22* S**	BC	0.0 R	NT	12 R	BS	25 S	BS
*A. baumanni/ haemolyticus*3106	AMP, CE, C, FOX, FEP, CFM, CRO, CXM, ATM, CTX ,	35 S	BC	34 S	BC	29 S	BS	32 S	BS
Citrobacter freundii 2569	AMP, CE, C, FOX, PIP, AMC	17 S	BS	0.0 R	NT	12 R	BS	22 S	BS
Escherichia coli 2462	AMP, CE, COT, C, CAR, PIP, TIC, SXT	22 S	BS	18 S	BS	12 R	BS	12 R	BS
E. coli 2476	AMP, CE, COT, C, CAR, PIP, TIC, TOB, AN, SXT	25 S	BC	12 R	BS	17 S	BS	20 S	BS
E. coli 2479	AMP, CE, CAR, PIP, TIC, AN	35 S	BS	25 S	BS	22 S	BS	15 I	BS
E. coli 2501	AMP, CE, COT, C, CAR, PIP, TIC, TOB, CIP	32 S	BS	12 R	BC	22 S	BS	2 R	BC
E. coli 2535	AMP, CE, COT, C, GN, FOX, CAR, PIP, TIC, TOB, CIP	21 S	BS	0.0 R	***NT	18 S	BS	18 S	BS
E. coli 2750	AMP, CE, COT, C, FOX, CAR, PIP, TIC, AMC, CIP, TZP	19 S	BS	0.0 R	NT	0.0 R	NT	23 S	BS
E. coli 2882	AMP, CE, COT, C, CAR, PIP, TIC, AMC, TZP, SXT	25 S	BS	13 R	BS	22 S	BS	19 S	BS
E. coli ESβL 4838	AMP, CE, COT, CAR, PIP, TIC, FEP, CFM, CRO, CXM, AMC, ATM, CAZ, CTX	25 S	BC	12 R	BS	2 R	BC	35 S	BS
Klebsiella pneumoniae 1100	AMP, CE, C, FOX, CXM,SXT	22 S	BC	0.0 R	NT	0.0 R	NT	0.0 R	NT
Pseudomonas aeruginosa 1200	CTX	25 S	BS	14 I	BS	0.0 R	NT	0.0 R	NT
Salmonella sp. 3397	AMP	28 S	BS	0.0 R	NT	18 S	BC	25 S	BC
Salmonella sp. 4168	AMP, CFM, CXM, CAZ, CTX	27 S	BS	20 S	BS	17 S	BC	27 S	BC
% Efficiency - % BC		100	33.3	26.7	22.2	53.3	25	66.7	23.1

*Mean diameter of three replicates rounded to the nearest mm. R-resistant, I-intermediate, S- susceptible (NCCLS, 2002). ** According to CLSI (2012) where imipenem (IPM) 10 µg/disks gave ≤13 mm for resistant (R), ≥ 14-15 mm for intermediate (I) and ≥16 mm for sensitive (S), as a standard for G⁻ve bacteria. ***NT: not tested to detect BC and BS, exhibited resistance to plant extract, where no inhibition zone appeared.

Table 2. Sensitivity pattern and bacteriostatic/bactericidal (BS/BC) action of Gram positive (G^+ve) bacteremia isolates to different plant extracts as compared to traditional antibiotics.

G^+ve bacteria/no. of Specimen	*Resistance to antibiotics	**Plant extract							
		Clove		Rosemary		Ginger		Cinnamon	
		Inhibition zone mm	BC/BS action	Inhibition zone (mm)	BC/BS action	Inhibition zone (mm)	BC/BS action	Inhibition zone (mm)	BC/BS action
Staphylococcus aureus (MRSA) 4182	E, TE, CE, DA, P, AMP, FD, CXM, CIP, FOX, CRO, CTX, GN, AMC,	35* S**	*BC	24 S	BS	19 S	BC	27 S	BC
Staph. epidermidis 2509	E, DA, P, AMP, CXM, CIP, CRO, OX, AMC	35 S	*BS	37 S	BS	27 S	BS	29 S	BS
Streptococcus pneumoniae 2675	GN	33 S	BS	12 I	BS	22 S	BS	20 S	BS
Bacillus sp. 2566	P, AMP, CRO	35 S	BS	22 S	BS	22 S	BS	25 S	BS
% Efficiency - % BC		100	25	75	0.0	100	25	100	25

*Mean diameter of three replicates rounded to the nearest mm. R-resistant, I-intermediate, S- sensitive (NCCLS, 2002): ** According to CLSI, (2012) where vancomycin (VA) 10µg/disks gave ≤ 10 mm for resistant (R), ≥ 11- 14 mm for intermediate (I) and ≥ 15 mm for sensitive (S) isolates as a standard for G^+ve bacteria.

(Figure 1a), while after treated with clove extract, it showed a proportional shortness of cells (Figure 1b). Shortness and decreasing number of *E. coli* 2479 cells were observed after exposure to clove extract (Figure 2b), as compared of untreated control cells straight rods which occurred singly (Figure 2a).

E. coli ESBL 4838 showed straight rods occurring singly (Figure 3a); and the cells after treatment showed some shortness with some deformities in the external shape of cell (Figure 3b). Cells of untreated *K. pneumoniae* 1100 showed straight rods arranged singly (Figure 4a); but the cells after being exposed to clove extract, showed full lyses of bacterial cell wall (Figure 4b).

Before treatment, *P. aeruginosa* 1200 showed straight rods or slightly curved rods (Figure 5a); but the micrograph of cells after treated showed some deformities in the external shape of cell and decreased the number of cells (Figure 5b).

Untreated control *Salmonella* sp. 3397 showed straight rods cells (Figure 6a); but the treated one showed some shortness of cells and full lyses in others (Figure 6b).

None treated MRSA showed spherical cells in irregular clusters (Figure 7a); bacterial cells after treatment showed some appendages, some deformities in the external shape of cell and lyses of bacterial cell wall (Figure 7b). The other micrograph (Figure 7c) for treated bacterial cells showed full lyses of bacterial cell wall.

Micrograph of none treated *S. epidermidis* 2509 showed spherical cells in irregular clusters (Figure 8a); but after treatment with clove extract, it showed an appendages on bacterial cell wall, littleness of the cell diameter and decreasing number of bacterial cells (Figure 8b).

The SE micrograph of non-treated *S. pneumonia* 2675 showed spherical cells, usually, they are seen as pairs of cocci (diplococci) (Figure 9a). Bacterial cells after treatment with clove extract, showed no change that indicate the cells did not lyses after treatment (Figure 9b and c).

The SE micrograph of non-treated *Bacillus* sp.2566 showed road-shaped and straight cells (Figure 10a). The bacterial cells after treatment showed no change either in external shape or number of bacterial cells (Figure 10b).

DISCUSSION

The aqueous extracts of clove**,** rosemary, ginger and cinnamon were selected for their antibacterial activity against multi-drug resistance bacteremia isolates. All tested aqueous plants extracts were effective against all tested G^+ve bacteria.

The clove aqueous extract was effective against all tested G^-ve beside G^+ve bacteria. While the rest plants extracts were different in their

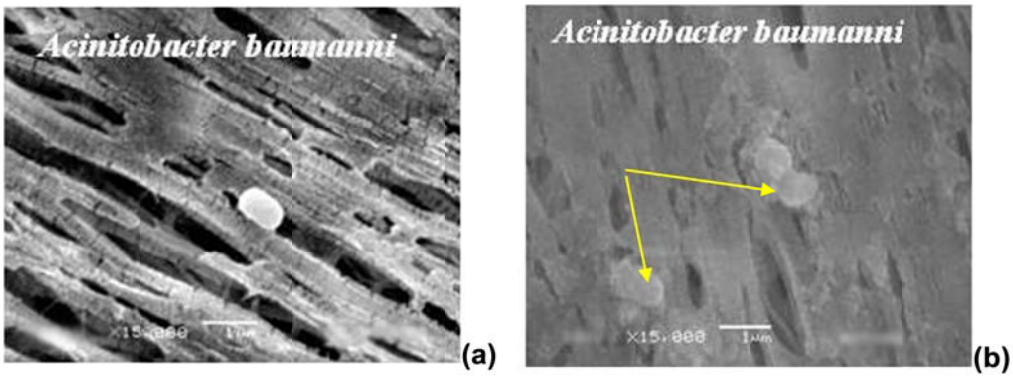

Figure 1. Scanning electron microscope observations of *Acinetobacter baumanni/haemolyticus* 3106 from blood stream mounted on filter paper, (a) Untreated control cells showed rods, spherical in the stationary phase of growth; (b) bacterial cells after exposure to aqueous clove extract, indicates a beginning cellular disruption and proportional shortness of cells. Magnification 15,000x.

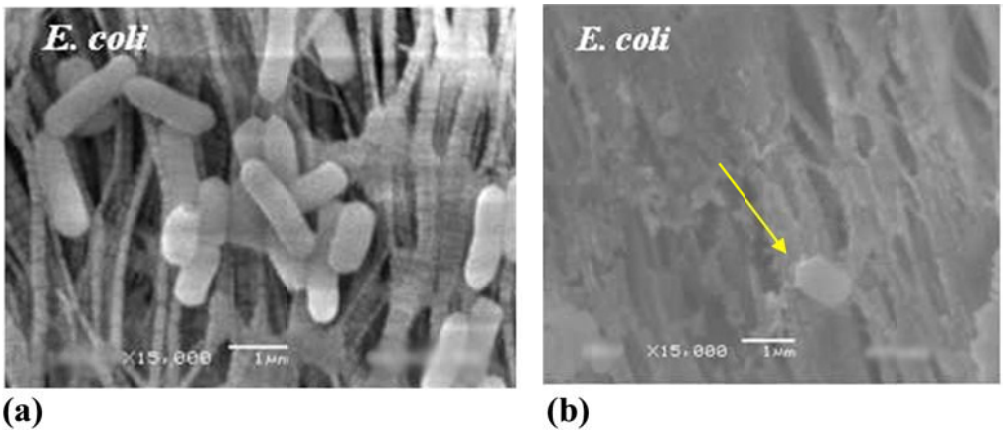

Figure 2. Scanning electron microscope observations of *Escherichia coli* 2479 from blood stream mounted on filter paper, (a) Untreated control bacterial cells showed straight rods occurring singly; (b) bacterial cells after exposure to aqueous clove extract, indicates a cell size reduction and decreasing number of cells. Magnification 15,000x.

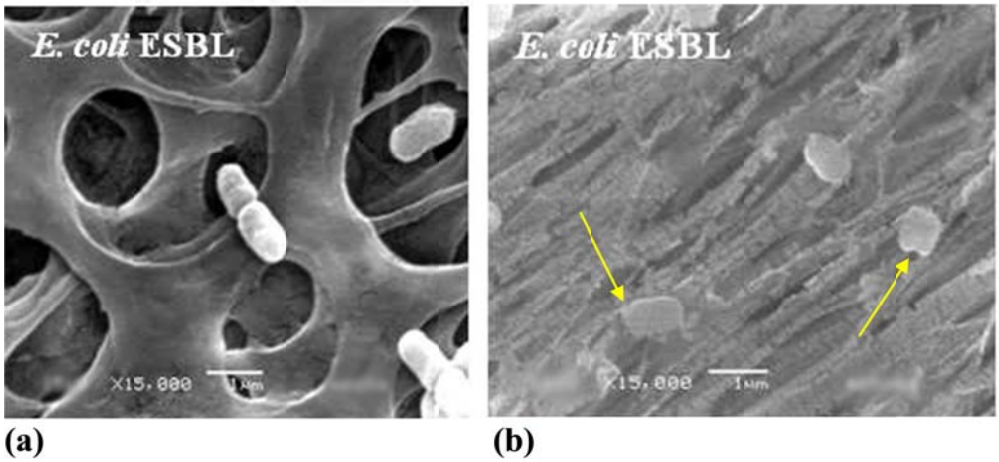

Figure 3. Scanning electron microscope observations of *Escherichia coli* ESβL 4838 from blood stream mounted on filter paper, (a) Untreated control bacterial cells showed straight rods occur singly; (b) bacterial cells after exposure to aqueous clove extract, indicates a shortness of cell with some deformities in the external shape of cell. Magnification 15,000x.

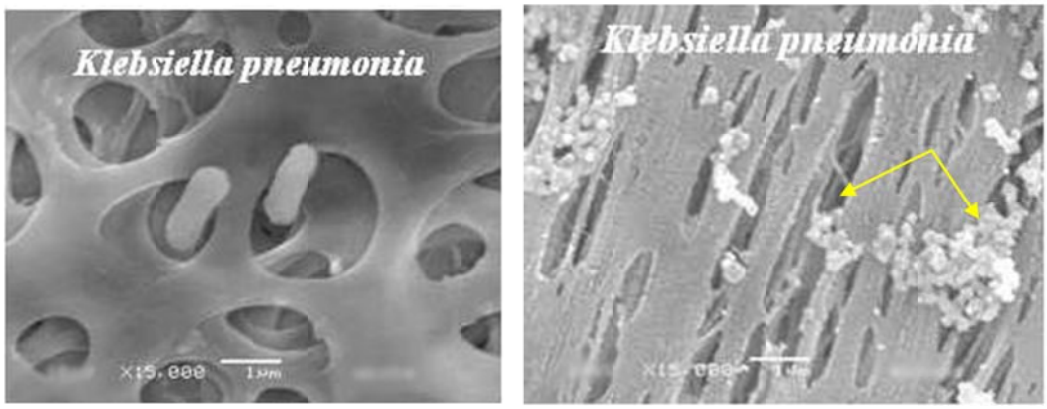

Figure 4. Scanning electron microscope observations of *Klebsiella pneumoniae* 1100 from blood stream mounted on filter paper, (a) Untreated control bacterial cells showed straight rods arranged singly; (b) bacterial cells after exposure to aqueous clove extract, indicates full lyses of bacterial cell wall. Magnification 15,000x.

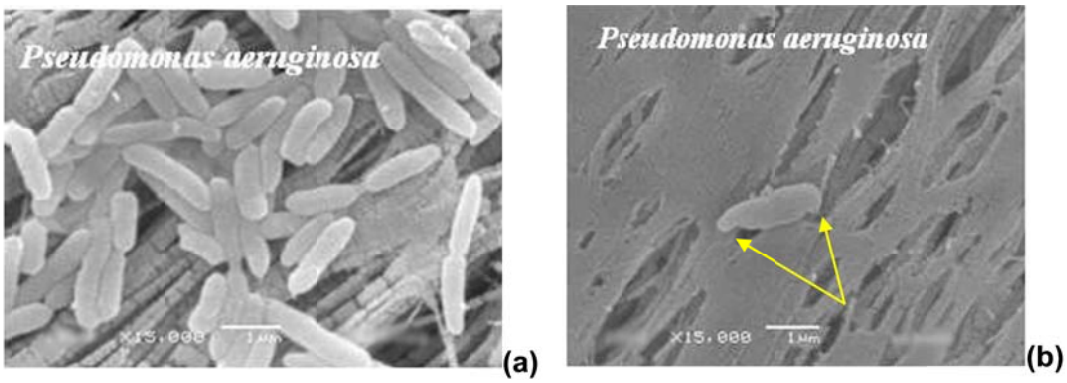

Figure 5. Scanning electron microscope observations of *Pseudomonas aeruginosa* 1200 from blood stream mounted on filter paper, (a) Untreated control bacterial cells showed straight rods or slightly curved rods; (b) bacterial cells after exposure to aqueous clove extract, indicates deformities in the external shape of cell and decreasing number of cells. Magnification 15,000x.

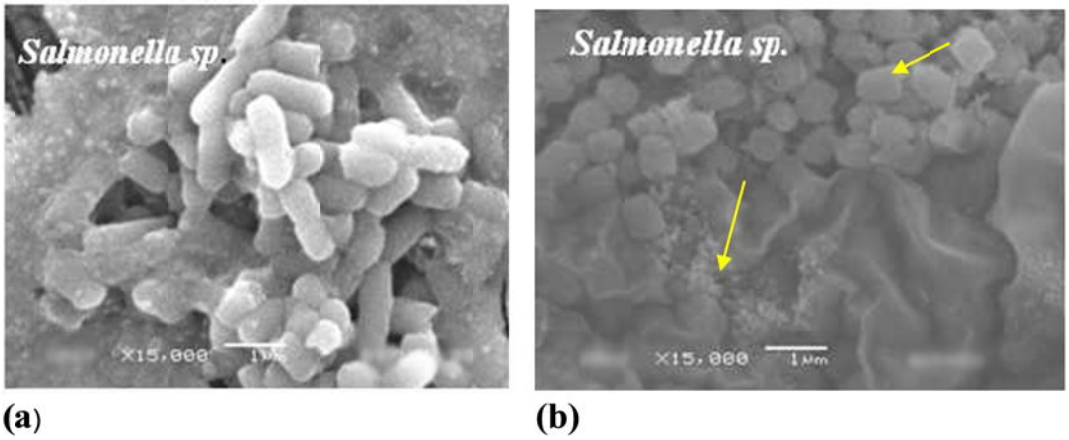

Figure 6. Scanning electron microscope observations of *Salmonella* sp. 3397 from blood stream mounted on filter paper, (a) Untreated control indicated clustered bacteria and straight rods cells; (b) bacterial cells after exposure to aqueous clove extract, indicated a decrease in cell size and full lyses in others. Magnification 15,000x.

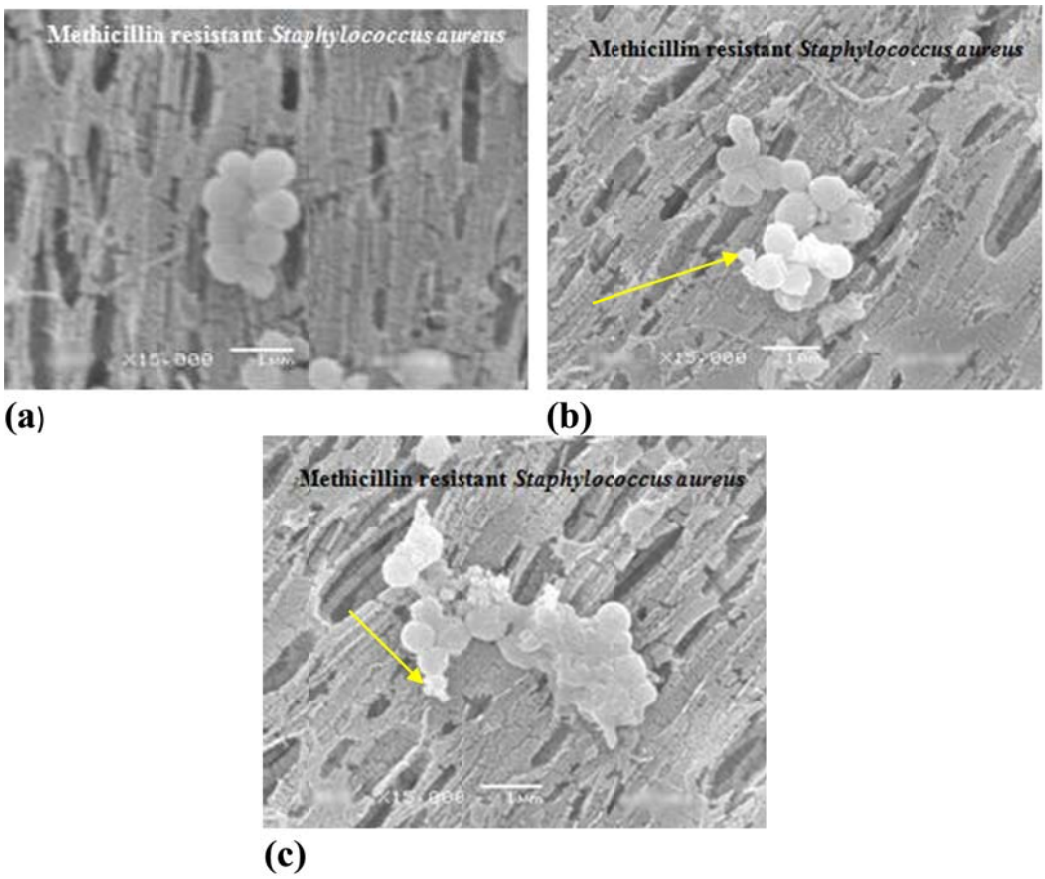

Figure 7. Scanning electron microscope observations of Methicillin Resistant Staphylococcus aureus 4182 (MRSA) from blood stream mounted on filter paper. (a) Untreated control bacterial cells appeared spherical in irregular clusters; (b) bacterial cells after exposure to aqueous clove extract, showed appendages on bacterial cell wall,, (c) some deformities in the external shape of cell and lyses of bacterial cell wall, which show full lyses of bacterial cell wall. Magnification 15,000x.

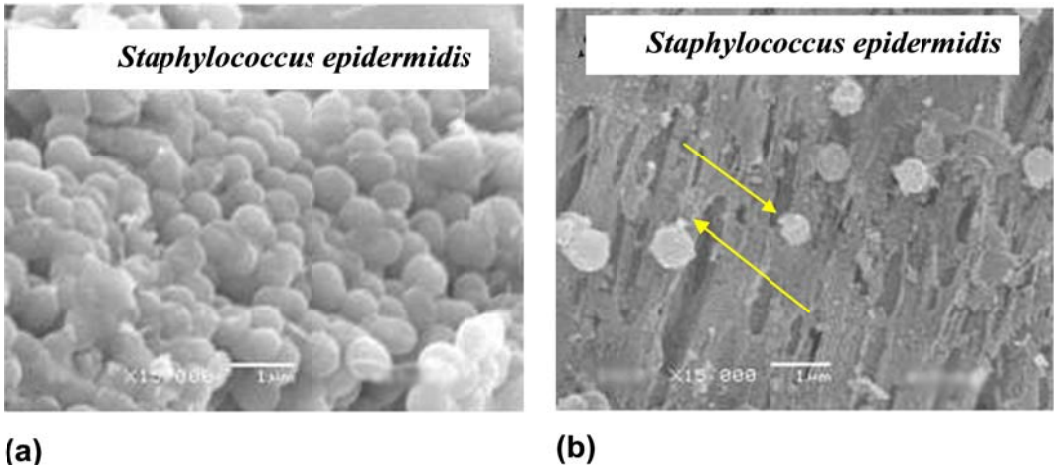

Figure 8. Scanning electron microscope observations of Staphylococcus epidermidis 2509 from blood stream mounted on filter paper, (a) Untreated control bacterial cells before exposure to aqueous clove extract showed spherical cells in irregular clusters; (b) bacterial cells after exposure to aqueous clove extract, appear as appendages on bacterial cell wall, showed cellular disruption and release of intra-cellular materials, have reduction in cell diameter and decreased number of bacterial cells. Magnification 15,000x.

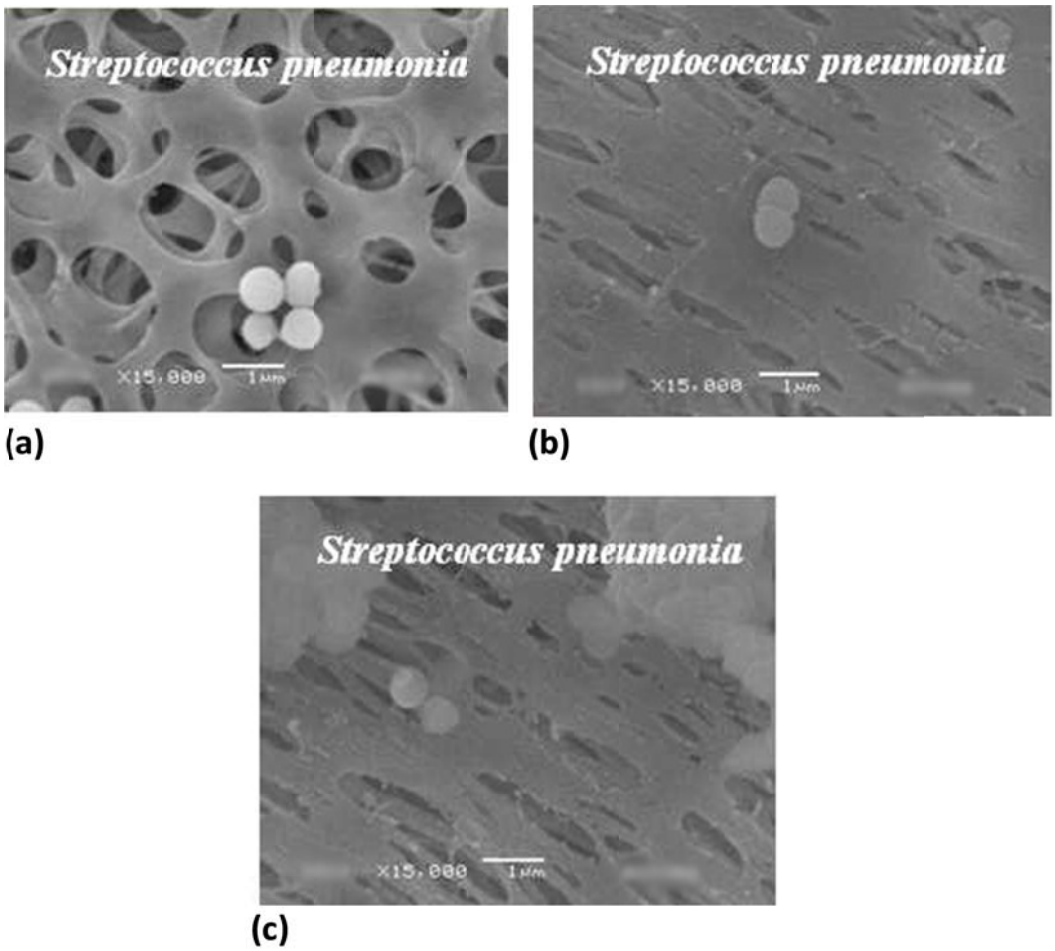

Figure 9. Scanning electron microscope observations of *Streptococcus pneumoniae* 2675 from blood stream mounted on filter paper, (a) Untreated control cells appeared spherical usually, they are seen as pairs of cocci (diplococci); (b) and (c) bacterial cells after exposure to aqueous clove extract, showed no change, which indicate the cells did not lyses after treatment. Magnification 15,000x.

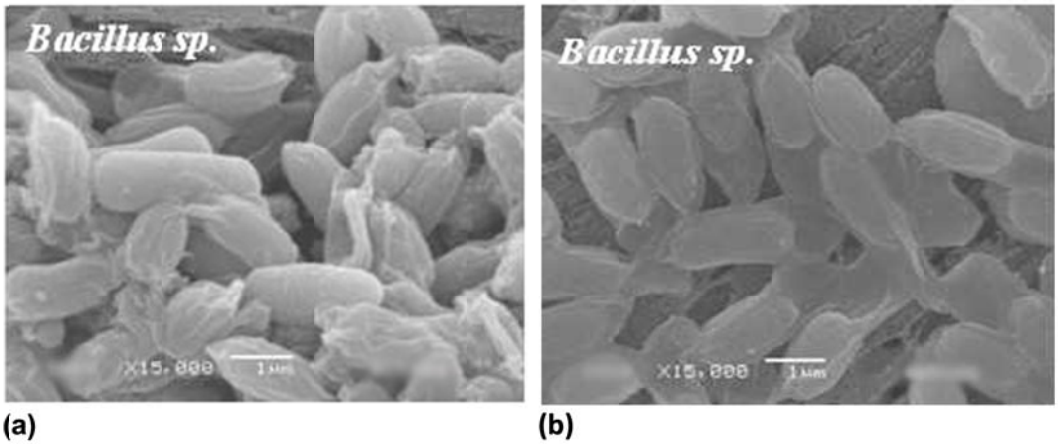

Figure 10. Scanning electron microscope observations of *Bacillus* sp. 2566 from blood stream mounted on filter paper, (a) Untreated control bacterial cells appeared road-shaped and straight cells; (b) the bacterial cells after exposure to aqueous clove extract, showed no change either in external shape or number of bacterial cells. Magnification 15,000x.

antibacterial activity against tested G⁻ve bacteria. On the contrary was the result obtained by Jigna et al. (2006) who revealed that the aqueous extracts of 12 plants each belonging to different families were inactive but methanol extracts were more active against G⁺ve bacteria than G⁻ ve bacteria. The Twelve plants were *Abutilon indicum* L., *Acorous calamus* L., *Ammania baccifera* L., *Argyrea nervosa* Burm. F., *Bauhinia variegata* L., *Crataeva religiosa* Forst., *Hedychium spicatum* L., *Holarrhena antidysenterica* L., *Piper nigrum* L., *Plumbago zeylanica* L., *Psoralea corylifolia* L., *Saussurea lappa* Costus. The most susceptible bacteria were *K. pneumoniae* and the most resistant bacteria was *E. coli*. *B. variegata* L. exhibited remarkable antibacterial activity. This may be due to general physiological differences in the cell wall membrane constitution of G⁻ve and G⁺ve bacteria. Suffredini et al. (2006) and Joy et al. (2007) confirmed that the G⁻ve bacteria, due to their external lipopolysaccharide structure, were more resistant, which can explain why G⁺ve bacteria were more sensitive to studied plant extracts than G⁻ve bacteria.

Although the activity of aqueous rosemary leaves extract on bacterial growth indicated lowest effectiveness of tested plant extracts, where most tested G⁻ve isolates were (R) to the extract, whereas the rest of tested isolates exhibit (S) response towards the extract. But still the aqueous extract revealed activity against some G⁻ve of multi-drug resistance bacteremia isolates, where Nascimento et al. (2000) and Fu et al. (2007) reported that the effectiveness of rosemary extract may be due to its chemical composition: flavonoids, phenolic acids, essential oils and diterpenes.

The findings of the present study revealed that aqueous extract of ginger rhizomes may contain potent antibacterial property against about half of multi-drug resistance tested G⁻ve isolates, as well as all tested G⁺ve isolates. Bhargava et al. (2012) submitted that the antimicrobial activity of the ginger extracts (ethanol and methanol) was exhibited against *E. coli, P. aeruginosa, S. aureus* and *Enterococcus faecalis.* While only the following isolates of G⁻ve bacteria: isolates of *E. coli* 2462 and 2501, *K. pneumoniae*1100 and *P. aeruginosa*1200 showed (R) to cinnamon bark extract, but the rest of tested multi-drug resistance isolates either G⁻ve or G⁺ve bacteria were (S) to the aqueous extract. But the previous studies suggested that the antibacterial activity of cinnamon was probably due to their major component, cinnamaldehyde and their properties could be multiple (Tabaka et al., 1999; Blumenthal, 1998).

The present results cleared that aqueous clove extract was able to inhibit all multi-drug resistance isolates as compared to cinnamon, ginger and rosemary extracts. This result agreed with that of Nascimento et al. (2000) who showed that the highest antimicrobial potentials were observed for the extracts of clove. The antimicrobial activity of the clove may due to chemical composition of its dried buds which was detected as: essential oils could be associated with eugenol, the main component of clove oil; and the other components as flavonoids and tannins (Fu et al., 2007). The mechanism of inhibition activity may be regarded to be presence of tannin by producing hydrogen bonds with proteins, which converted its structure and lead to blockage of the protein synthesis in bacteria, and tannins considered as a phenolic compounds of plants which have anti oxidative effects (Makoto et al., 1995).

Study on bacterial sensitivity towards antibiotic(s) after exposure to clove plant extract, did not achieve the goal of experiment, which was designed to detect the loss of antibiotic resistance by antibacterial agent. While results obtained by Shoeib and Al-Obiri (2014) were considered to be the first addressing the efficiency of ozone as antibacterial agent in destroying the resistance of bacterial vaginosis to some antibiotics, and subsequently restoring the capacity of these antibiotics in treatment again.

The present results exhibited death of all tested isolates after treatment, either G⁻ve bacteria or G⁺ve bacteria except *Bacillus* sp. *Bacillus* sp. showed change in sensitivity to some tested antibiotics according to clearance zone measured after the treatment, for the following antibiotics E, TE, CE, DA and VA were measured before the exposure, as 35, 32, 30, 33 and 35 mm, it was decreased after treatment as 26, 16, 27, 19 and 20 mm respectively, but still (S) response.

The current results suggest that clove extract act as antibacterial agent, which led the vegetative cells of *Bacillus* sp. converted to spores form, in addition the spore staining strengthened this finding. Thus, re-inoculating the bacterial suspension on NAO to test the sensitivity towards antibiotics, showed decreased in zone of inhibition which explains that the spores germinated and cells acquired resistance from their exposure to clove, subsequently to tested antibiotics. Nikolaev (2004) confirmed that the extracellular compounds of bacterial spores involved in their adaptation to unfavorable environmental conditions, including bactericidal concentrations of toxic substances (oxidants, phenols, and heavy metals) and antibiotics.

The scanning electron micrograph showed the modification of the external shape of cells after treatment by aqueous clove's buds extract. These modifications were developed from shortness, decreasing number of cells, appendages, some deformities in the external shape of cell, up to lyses of some cells walls even full lyses.

Due to bacterial cells containing a high concentration of dissolved solutes, there was a considerable turgor pressure to develop about 2 atmospheres in a bacterium. This is roughly the same as pressure in an automobile tire (Madigan et al., 2012). Lyses of cell walls result of two groups of enzymes, hydrolyses and syntheses, have to combine to allow the insertion of new subunits into the murein net. The action of these enzymes must be well

coordinated to guarantee growth of the stress-bearing sacculus without risking bacteriolysis (Höltje, 1998).

Our conclusion have strengthened that aqueous extracts especially clove has potential antibacterial agent(s), representative of bactericidal action, decreasing number of cells as well as have capability to change cell wall from deformities up to full lyses towards bacteremia isolates which revealed multi-drug resistance. Consequently aqueous extract of clove may be used in the future as natural therapeutics for its efficacy and low side effects, for diseases caused by multi-drug resistance bacteria such as bacteremia, which led to the failure of antibiotics in their elimination.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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