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Ayuba Sunday Buru (✉ [sunday.buru@kasu.edu.ng](mailto:sunday.buru@kasu.edu.ng))

Kaduna State University

Vasanthakumari Neela

Universiti Putra Malaysia

Kavitha Mohandas

MAHSA University College

Mallikarjuna Rao Pichika

International Medical University

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## Research Article

**Keywords:** Mode of action, *Cinnamomum impressicostatum*, Methicillin Resistant *Staphylococcus aureus*

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**Kinetics of killing and mode of action of *Cinnamomum impressicostatum* crude stem – bark extract on Methicillin Resistant *Staphylococcus aureus***

Ayuba Sunday Buru\*<sup>1</sup>, Vasanthakumari Neela<sup>2</sup>,  
Kavitha Mohandas<sup>3</sup>, Mallikarjuna Rao Pichika<sup>4</sup>,

<sup>1</sup>Genomic Research Lab, Department of Medical Microbiology and Parasitology,  
Faculty of Basic Clinical Sciences, College of Medicine, Kaduna State University,  
Kaduna, Nigeria

<sup>2</sup>Department of Medical Microbiology and Parasitology,  
Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang  
Selangor, Malaysia

<sup>3</sup>Department of Microbiology, Faculty of Medicine, MAHSA University College,  
Kuala Lumpur, Malaysia.

<sup>4</sup>International Medical University, No.126, Jalan 19/155B, Bukit Jalil, 57000 Kuala  
Lumpur, Malaysia.

Corresponding author\*, e-mail: sunday.buru@kasu.edu.ng

**Abstract**

**Background**

This study evaluated the mode of action of *Cinnamomum impressicostatum* on Methicillin Resistant *Staphylococcus aureus* (MRSA). Cinnamon is one of the most popular spices used by mankind to enhance the flavour of his cuisine. In addition, cinnamon contains medicinally important essential oils in its leaves, stems, twigs, fruits and inner and outer bark. Many species of cinnamon are widely used in traditional medicinal systems around the world for the treatment of a wide variety of infectious diseases including tuberculosis and salmonellosis.

**Methods**

The crude stem – bark water extract of *C.impressicostatum* was generated using sequential soxhlet extraction. The antibacterial activity of the extract was

investigated by performing broth microdilution assays and determination of the Minimum Inhibitory Concentration (MIC). A time kill study was performed to determine the killing efficiency of the extract. Potential bacteriolytic activity of the extract against MRSA was evaluated. Potential summative or synergistic killing effects of the extract when supplemented with 7.5% NaCl was also determined. Leakage of intracellular cytoplasmic components through the bacterial plasma membrane was analysed by determining absorbance at 260 nm. Scanning Electron Microscopy was used to study the damaging effects of the extract on the cell wall of MRSA.

## **Results**

The Soxhlet crude water extract of *C.impressicostatum* stem - bark recorded the highest zone of inhibition (i.e.  $21.0 \pm 1.4$  mm) in disk diffusion assays. Its Minimum Inhibitory Concentration (MIC) against MRSA was  $19.5 \mu\text{g mL}^{-1}$  and its Minimum Bactericidal Concentration was  $39.0 \mu\text{g mL}^{-1}$ . The combinatorial effect of the extract supplemented with 7.5% NaCl resulted in a marked decrease in bacterial growth suggesting enhanced killing with the use of NaCl that may be summative or synergistic in nature. Evidence suggests that treatment with the crude extract causes cytoplasmic leakage, possibly by damage to the bacterial cell wall or cytoplasmic membrane. This was substantiated by post treatment scanning electron microscopic analysis which revealed alterations in cell wall topology, possible damage to the bacterial cell wall and plasma membrane and the presence of vast amounts of cellular debris.

## **Conclusion**

The results of this study indicate that the killing efficacy of *C.impressicostatum* stem - bark extract against MRSA is enhanced by NaCl and that treatment with the plant

extract induces gross and irreversible cellular damage eventually leading to bacteriolysis. The bioactive compound(s) contained in the plant extract thus show immense potential for development into efficacious antibacterial drugs.

**Keywords:** Mode of action, *Cinnamomum impressicostatum*, Methicillin Resistant *Staphylococcus aureus*

## **Introduction**

The development of bacterial resistance to hitherto efficacious antimicrobial agents has surged in the past decade [1,2]. MRSA has been the cause of a wide variety of infectious complications that can be life threatening [3]. It has been implicated as a prevalent hospital-acquired (nosocomial) pathogen causing a substantial burden for health and economies in the world of today [2,4]. Hospital-acquired infections (HAI) are defined as infections acquired in the hospital and which are not in incubationary phase on the patient's admission to hospital. However, we have to bear in mind that a HAI may be caused by a colonising organism which the patient carried on his person before hospital admission [5].

Over recent years, there has been exacerbated interest in the quest for novel antibacterial agents from natural resources, particularly but not exclusively from

plants, and their derivatives. This is because most plants contain a number of secondary metabolites with bioactivity. This includes alkaloids, flavones, phenols, quinones, terpenoids and tannins [6,7]. *Cinnamomum* spp. possess potent antimicrobial activities against a range of micro - organisms. In the ancient world, cinnamon occupied a pre-eminent position and was much sought after; it is often qualified as *sensational cinnamon* and the *spice of life*. It contains medicinally important essential oils in its leaves, stems, twigs, fruits, inner and outer bark. It has enormous valuable pharmacological activities. Much of cinnamon's bioactivity resides in its oil, which is about 90% cinnamaldehyde [8]. Cinnamon as a plant possesses chemo - preventive, antispasmodic, anti - ulcer, choleric, sedative, hypothermic, antifungal, antibacterial, antiviral, antipyretic, lipolytic, antiseptic, anaesthetic, anodyne, cytotoxic, hypolipidemic and antiplatelet properties and also stimulates the immune system. It may be useful in reducing the risk of cardiovascular disease and cancer [9,10]. Most reports on cinnamon reveal its efficacy against drug susceptible strains of micro - organisms, but do not reveal its mode of action (if any) against drug resistant micro - organisms such as MRSA. The repertoire of drugs available for treating refractory MRSA infections is narrowing due to burgeoning resistance to a wide array of drugs. Furthermore, the search for safe alternative drugs from natural sources has intensified due to innate toxicity and side effects in the existing drugs available, such as vancomycin, for treatment of recalcitrant MRSA infections [11]. As such, this study seeks to elucidate the mode of action of *C.impressicostatum* against MRSA.

## **Materials and Methods**

### **Preparation of *C.impressicostatum* crude extracts**

The stem - bark of *C.impressicostatum* was collected and authenticated by Mr. Ahmad Zainudin bin Ibrahim, a taxonomist at the Botanical Gardens Herbarium,

Putrajaya. The voucher specimen (HTBP3152) was deposited in the same herbarium. The plant was washed thoroughly under running tap water, air - dried under shade, powdered using a blender and ground at the Herbal Technology Centre, Forest Research Institute, Malaysia (FRIM). 100g of the powdered plant materials were extracted sequentially using a soxhlet extractor (Favorit<sup>®</sup> apparatus) with 500 mL of solvents of increasing polarity, starting with *n*-Hexane, followed by ethyl acetate, methanol and water. The extracts were then filtered through Whatman<sup>®</sup> filter paper 240 mm (Whatman, England). The filtrates were evaporated using a rotary evaporator (Buchi<sup>®</sup> Rotavapor R-200 and R-215, Switzerland) under reduced pressure at 60°C. The concentrates were then transferred into scintillation vials and placed in a water bath to remove excess solvent and placed in the desiccator to completely dry. The extract was then lyophilised using a freeze dryer (Labconco<sup>®</sup> Freezone 6.0 and 4.5). The crude extracts of hexane, ethyl acetate and methanol were stored at 4°C while the water extract was stored at -80°C until required.

#### **Reference strain**

The reference strain of MRSA (ATCC 700698) used for this study was kindly provided by the Faculty of Medical and Health Science, Department of Medical Microbiology, Universiti Putra Malaysia. The stock culture was stored in Brain Heart Infusion (BHI) Broth (Difco, France) containing 20% glycerol at -80°C until required.

#### **Antibacterial disk diffusion assay**

The antibacterial activities of *C.impressicostatum* crude extracts on clinical isolates were determined using a modified agar disk diffusion method [12,13]. Inocula from frozen stocks of bacteria were subcultured twice on Mueller - Hinton agar. Suitable single colonies taken from these plates were resuspended in Mueller-Hinton broth

and incubated with rotary agitation at 37°C for 1.5 – 2.0 h until the optical density reached the equivalent of a 0.5 McFarland Standard. Using a sterile swab, a loopful of the inoculum was spread onto Mueller - Hinton Agar. Each disk impregnated with the extract was placed on the prepared bacterial culture plates and incubated at 37°C for 18 – 24 h. The disk diffusion assays of extracts and controls (DMSO and commercial antibiotic disks) were carried out in triplicate. The zones of inhibition (mm) were measured and the results were reported as mean  $\pm$  standard deviation (SD).

#### **Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)**

The MIC of the plant water extract against the reference strain of MRSA was determined based on CLSI [14] guidelines and by the modified resazurin method described by Satyajit and colleagues [15]. The assays were performed in triplicate using sterile 96-well microtiter plates carrying twofold serial dilutions of the extract yielding concentrations ranging from 10.0000 mg/mL to 0.0049 mg/mL and standard inoculum strength per well. Plates were then incubated at 37°C for 24 hours. The MIC was taken as the lowest concentration of the extract that yielded no growth of bacteria, as indicated by the lack in colour change in the viability stain, resazurin, from purple to pink or colourless, and it was calculated as the mean of three replicate values.

The MBC was determined by removing 0.1ml aliquots of Mueller - Hinton Broth (MHB) suspension carrying no colour changes (indicating no growth of bacteria) which were then spread plated onto MHA and subsequently incubated at 37°C for

24h. The MBC determinations were carried out in triplicate. MHA plates displaying no bacterial colonies were taken as representative of the minimal bactericidal concentration.

### **Time kill study**

A time kill study was performed to determine the killing efficiency of the active fractions according to the method described by Carson and colleagues, 2002 [16]. The test was performed in duplicate with the turbidity of the bacterial inoculum set at 0.5 McFarland or  $1.5 \times 10^8$  CFU/ml. The extract at concentrations of 1MIC, 2MIC, 4MIC, 8MIC, 16MIC and 32MIC were added to separate tubes that contained  $1.5 \times 10^8$  CFU/ml bacterial suspension. The tubes were then incubated at 37°C in an incubator shaker set at 150 rpm. Hundred microliter samples were taken at 0, 2, 4, 6, 8 and 12 hours post inoculation, neutralised, serially diluted and spread onto MHA plates. The inoculated plates were incubated at 37°C for 18 - 24 h and the emergent colonies were counted thereafter.

### **Bacteriolysis**

Bacteriolysis was performed according to the modified method by Ifesan and colleagues [17]. The bioactive fractions of *C.impressicostatum* stem – bark water extract, at concentrations equivalent to the MIC, 2MIC, 3MIC and 4MIC, were added to standard suspensions of MRSA. The control was treated with 1% dimethylsulfoxide. Blanks were set-up corresponding to the dilution of the test agent. Samples were taken immediately after the experiment had been set up at 0 h and also after the test tubes had been incubated in a shaker incubator at 37°C for 8 and 24 h, respectively. The suspensions were mixed by vortexing, and the OD<sub>620nm</sub> was measured to detect cell lysis.

### **Anti - biofilm assay**

The anti - biofilm activity of the bioactive *C.impressicostatum* stem – bark water extract on MRSA was investigated according to the protocol described by Merritt and colleagues [18]. The isolates were grown in tryptic soy broth (TSB) to stationary phase and subsequently diluted 1:100 in TSB. A hundred microlitres of sterile TSB was pipetted into each of the 12 wells of the microtitre plates. Similarly, all the wells were loaded with a hundred microlitres of the bacterial suspension. The plate was covered and sealed with parafilm and incubated at 37°C for 48h. The planktonic bacteria in each microtitre well were removed by briskly but gently shaking off the dish over a wash tray. The wells were washed by submerging the plate first into the water tray and then vigorously shaking out the liquid over the waste tray. The waste water in the tray was replaced when it became cloudy. One hundred and twenty five microlitres of 0.1% crystal violet solution was added to each well and stained for ten minutes at room temperature. The microtitre plate was shaken out over the waste tray to remove the excess crystal violet. The microtitre plate was washed several times and the wash liquid was shaken out. The plate was inverted and vigorously tapped on a paper towel to remove the excess liquid. It was then air - dried. Two hundred microlitres of 95% ethanol was added to each of the wells. The plate was covered and incubated at room temperature for 15 minutes. The contents of each well were mixed briefly by pipetting. One hundred and twenty five microlitres of the crystal violet / ethanol solution was transferred from each well into separate wells in an optically clear flat bottomed 96 - well plate. The optical density of the samples was measured at 570 nm. Duplicate wells containing another set of samples which had formed biofilm were treated with fifty microliters of 100 mg/ml test material and incubated overnight. The contents of the plate were washed thoroughly with tap water. One hundred and twenty five microlitres of crystal violet / ethanol was added

to each well and allowed to stand for 10 minutes. The contents of the wells were transferred into individual cuvettes. The optical density of the samples was read at 570 nm.

### **Salt enhancement of killing efficacy**

The ability of MRSA treated with mixtures of plant extracts and sodium chloride to grow on nutrient agar was investigated according to the method described by Ifesan and colleagues [17]. Suspensions of bacteria ( $1.5 \times 10^8$  CFU mL<sup>-1</sup>) were prepared in tryptic soy broth. They were treated with 0.5MIC, 1MIC and 2MIC of the bioactive *C.impressicostatum* stem – bark water extract and 1% dimethylsulfoxide as a control. In a second group, each concentration of extract was combined with 7.5% NaCl and the control was set up without the extract. The test tubes were incubated in a shaker incubator at 37°C; samples were removed at intervals, serially diluted, streaked onto nutrient agar (NA) and incubated for 24h at 37°C. The numbers of emergent colonies appearing on the NA plates were counted and compared with the control.

### **Loss of 260 nm absorbing materials from within the bacterial cell**

Cytoplasmic and nucleic acid leakage was determined using the modified method described by Oonmett and colleagues, 2006 [19]. MRSA was cultured on TSA plates and incubated overnight. The cells were then harvested and washed with normal saline by centrifugation at 10,000 g for 5 min. After centrifugation, the cells were collected and adjusted to achieve a concentration of approximately  $10^9$  CFU mL<sup>-1</sup>. The inoculum was then mixed with different concentrations of the bioactive *C.impressicostatum* stem – bark water extract at the MIC, 2MIC and 4MIC in the ratio of 1:1 in the test tubes, while the inoculum with 1% dimethylsulfoxide was set up as control. The suspensions were incubated at 37°C for 0, 8, and 24 h. After each

time interval, the suspensions were centrifuged as described above. Concentrations of low molecular - weight metabolites that may leak from cells, including nucleotides and their component structures such as purines and pyrimidines, and amino acids, were determined by reading the absorbance at 260nm using a UV/VIS spectrophotometer.

### **Efflux pump inhibition assay**

The EPI assay or modified MIC - efflux assay was performed based on the method described by Saiful and colleagues, 2006 [20]. Reserpine, a common efflux inhibitor for multidrug - resistant microorganisms including *S.aureus*, used at 50 µg/ml concentration, was used as positive control. *C.impressicostatum* stem – bark extract at 50 µg/ml concentration was added into the bacterial suspension - reserpine mixture. The treatment effect of 50 µg/ml of the extract was compared to the effect of reserpine efflux pump inhibition.

### **Scanning Electron Microscopy**

The effect of *C.impressicostatum* crude stem – bark water extract on the cell - wall of MRSA was studied. Standard bacterial suspensions were treated with 9.7 mg/ml crude extract and incubated at 37°C for 2 hours. The suspensions were then centrifuged at 10,000 rpm for 5 minutes, the supernatants decanted, the cell pellets re - suspended and 0.5 ml of 4% glutaraldehyde added to the cell pellet. The cell pellets were mixed, homogenised and fixed, then refrigerated at 4°C for 24h. The refrigerated cell pellet was allowed to equilibrate to room temperature and was then centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was decanted and the cell pellet was washed three times after a 30 minute interval in - between each wash with 0.1M Sodium cocodylate buffer. The cell pellet was dehydrated briefly

with acetone and then transferred onto a coverslip; it was allowed to dry to critical point and then coated with gold before viewing using a Tabletop SEM TM2000.

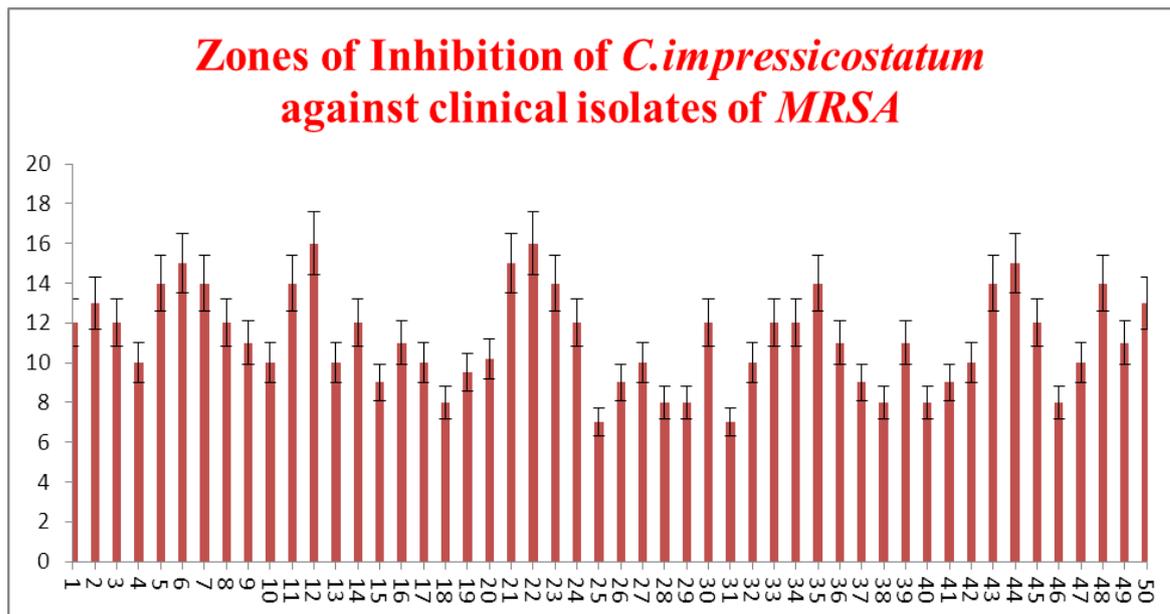
## Results

### Minimum Inhibitory Concentration (MIC)

The MIC of *C.impressicostatum* crude stem – bark water extract against MRSA was determined to be  $19.5 \mu\text{g mL}^{-1}$  and its MBC  $39.0 \mu\text{g mL}^{-1}$

### Antibacterial activity of *C.impressicostatum* against clinical isolates of MRSA

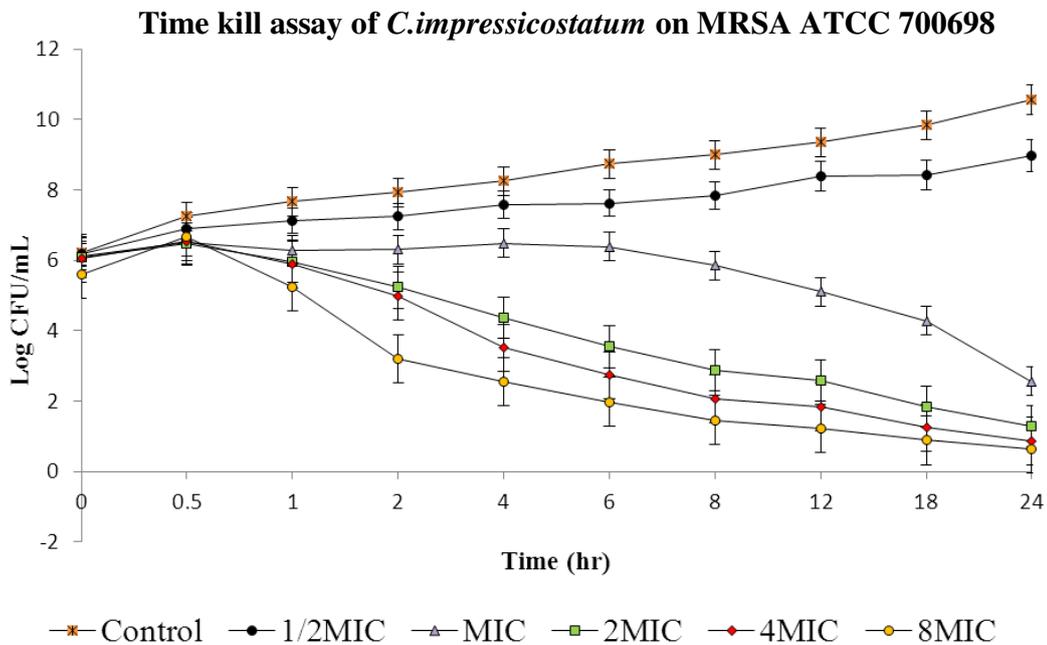
The zones of inhibition generated by *C.impressicostatum* stem bark extract when challenged with 50 clinical isolates of MRSA are shown in Figure 1.0 below. Zones of inhibition (ZI) ranged from 7 – 17 mm.



**Figure 1.0 Antibacterial activity of 50 clinical isolates tested against *C.impressicostatum* crude stem bark water extract (zone diameters are expressed in mm  $\pm$  standard deviation)**

### Time kill assay

The results of the time - kill assay are shown in Figure 3.0. Bacterial growth was stable at time zero, but within 2 - 4 h of MRSA being treated with the MIC and 8MIC of crude stem - bark water extract of *C.impressicostatum*, there was at least a two log - fold decrease in the number of viable cells. Treatment with the MIC and 2MIC caused at least a two log - fold reduction in the cell count after 2h, whilst treatment with 1/2MIC caused an insignificant reduction in cell count as compared to the control after 24h.



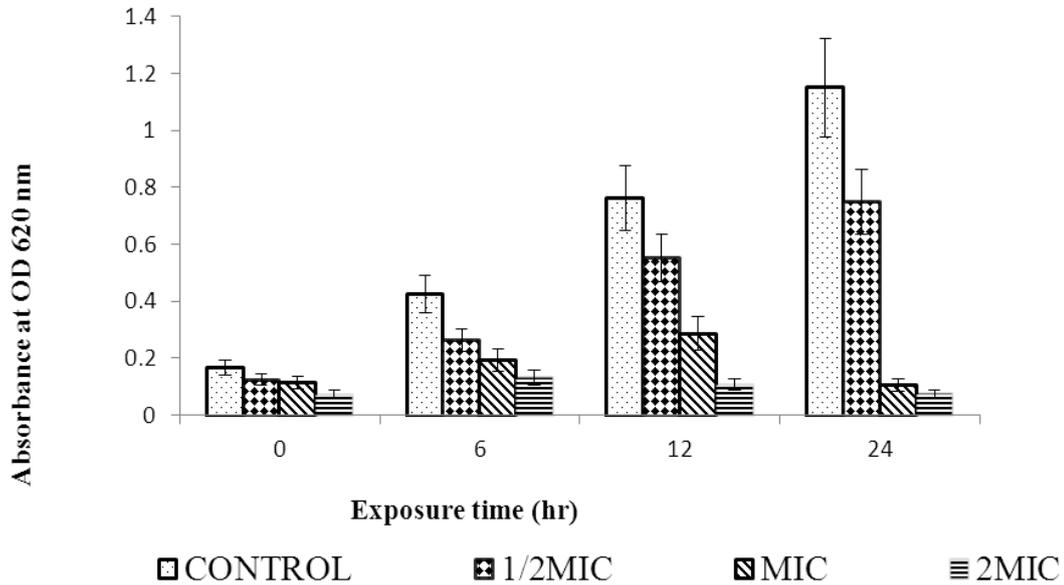
**FIGURE 3.0.** The kinetics of the killing curves after treatment of MRSA ATCC 700698 with the soxhlet crude stem – bark water extract of *C.impressicostatum*. Each symbol denotes mean  $\pm$  standard deviation.

## **Bacteriolysis**

The bacteriolytic activity of the soxhlet crude stem – bark water extract of *C.impressicostatum* on MRSA is presented in Figure 4.0. There was a significant decrease in the optical density as compared to the control

6 hours after treatment with 2MIC of extract; for the treatment with 1MIC of extract, a significant decrease was noticed after 12 hours, whilst for the treatment with ½MIC of extract, there was no significant difference at time 0 as compared to the control. Whilst there were increases in OD with increasing length of exposure and incubation for the controls and 1/2MIC treatments, suggesting growth of the bacterium, decreases in OD were observed for the MIC treatment between 12 and 24 hours of incubation, suggesting bacteriolysis, whilst for the 2MIC treatments, small decrements in OD were observed between 6, 12 and 24 hours of incubation, again suggestive of bacteriolysis.

### Bacteriolytic activity of *C. impressicostatum* on MRSA ATCC 700698

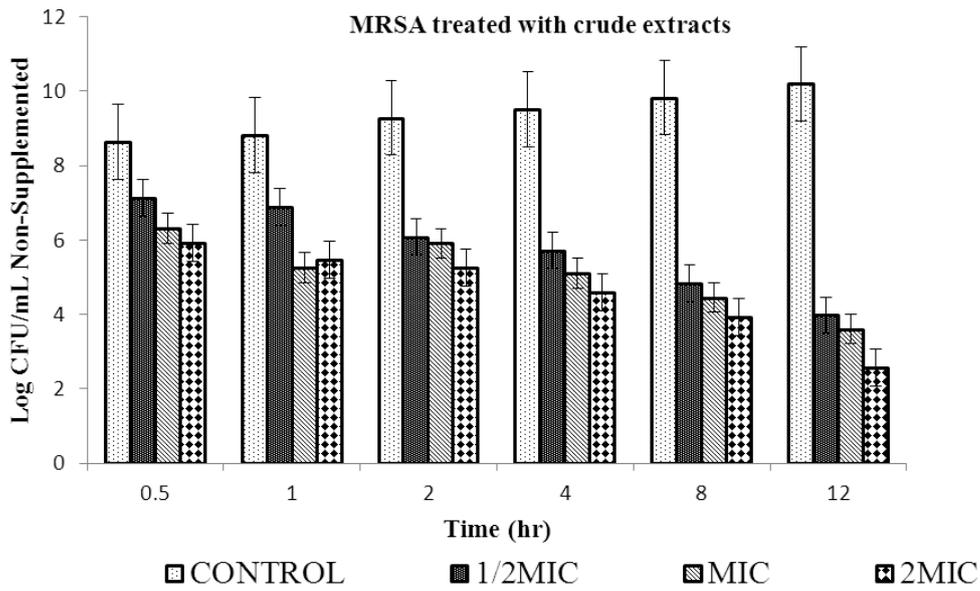


**FIGURE 4.0.** The bacteriolytic activity of MRSA ATCC 700698 treated with different concentrations of *C.impressicostatum* soxhlet crude stem – bark water extract. The control was treated with 1% DMSO. The data are expressed as means  $\pm$  standard deviation

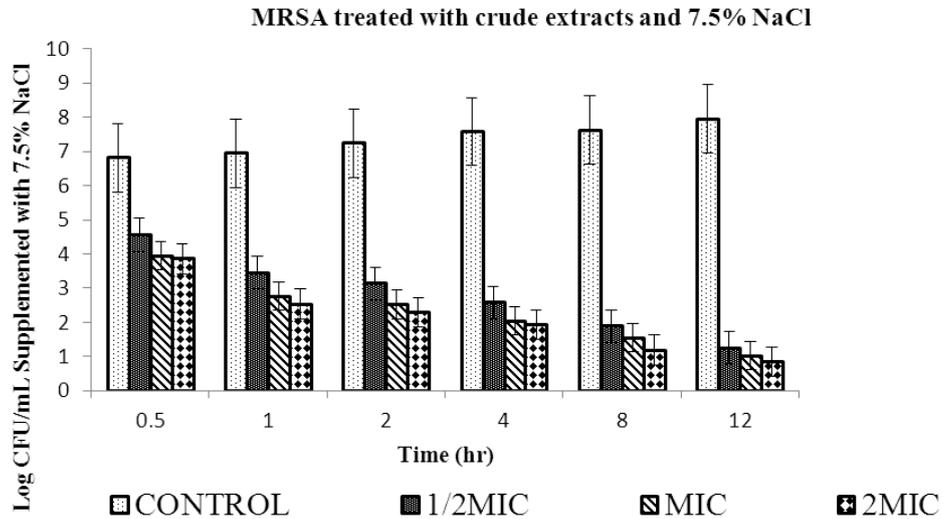
#### **Salt enhancement of killing efficacy**

Figures 5.0 (A) and (B) below clearly show that viable colony counts decrease with increasing lengths of incubation and also with increasing concentrations of *C. impressicostatum* crude extracts. Supplementing *C.impressicostatum* crude extracts with sodium chloride caused a general reduction in viable colony counts as compared to treatment with non-supplemented crude extracts.

(A)



(B)



**FIGURE 5.0.** (A) MRSA treated with 1/2MIC, MIC and 2MIC of *C.impressicostatum* crude stem – bark extract with increasing lengths of exposure; (B) MRSA treated with 1/2MIC, MIC and 2MIC of *C.impressicostatum* crude stem – bark extract supplemented with 7.5% NaCl, with increasing lengths of exposure. The data are expressed as means  $\pm$  standard deviation

### Loss of 260 nm absorbing material from within the bacterial cell

Leakage through the bacterial cytoplasmic membrane was analysed by determining absorbance at 260 nm (Figure 6.0). There was a marked increase in the OD at 8 h relative to the control, however, at 24 h, lower OD was recorded for all the treatments. The increase in the OD measurement at 8 h is suggestive of cytoplasmic membrane damage resulting in the leakage of guanine and cytosine into the medium, which were subsequently detected spectrophotometrically at 260 nm.

### Loss of 260 nm absorbing material from within MRSA ATCC 700698 upon treatment with *C.impressicostatum* crude stem – bark water extract

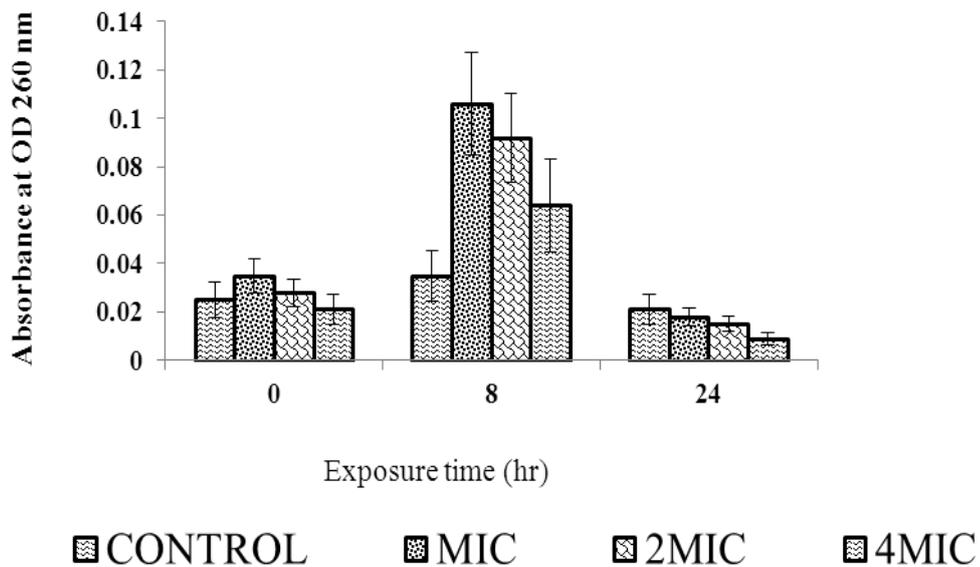
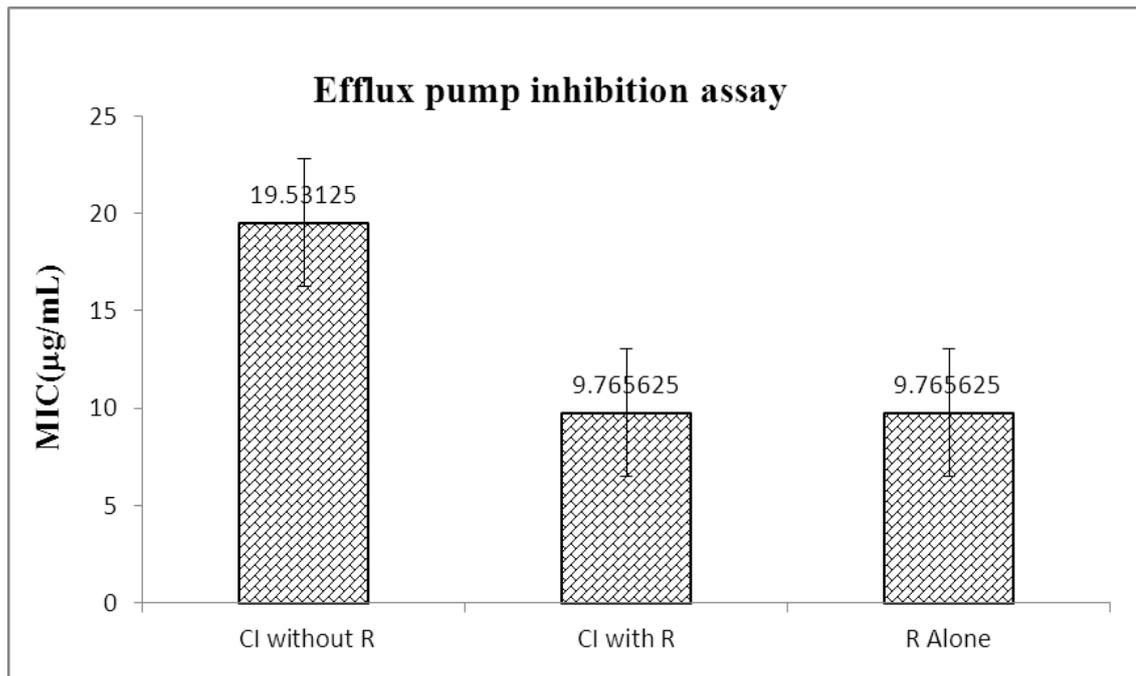


FIGURE 6.0. MRSA optical density (OD) measurements at 260 nm upon treatment with different concentrations of *C.impressicostatum* crude extracts. 1% DMSO was used as control. The data are expressed as means of  $\pm$  standard deviation

### **Efflux pump inhibition assay**

The efflux pump mechanism has been recognised as an important mediator of antibiotic resistance in bacteria, especially in MRSA. This study was carried out to determine whether *C.impressicostatum* crude extract possesses efflux pump inhibitory activity. Results indicated no significant reduction in the MIC value when comparing Reserpine (an efflux pump inhibitor) alone and *C.impressicostatum* extract with Reserpine. This indicates that *C.impressicostatum* extract does not exert any significant anti – efflux activity on MRSA. If it had exerted anti – efflux activity on MRSA, the MIC in the ‘CI with R’ treatment is likely to have been lower than the treatment ‘R Alone’. The result is shown in Figure 7.0 and is suggestive that *C.impressicostatum* crude extract is antagonistic to the effects of Reserpine, and may actually enhance extrusion of metabolites from the bacterial cell.

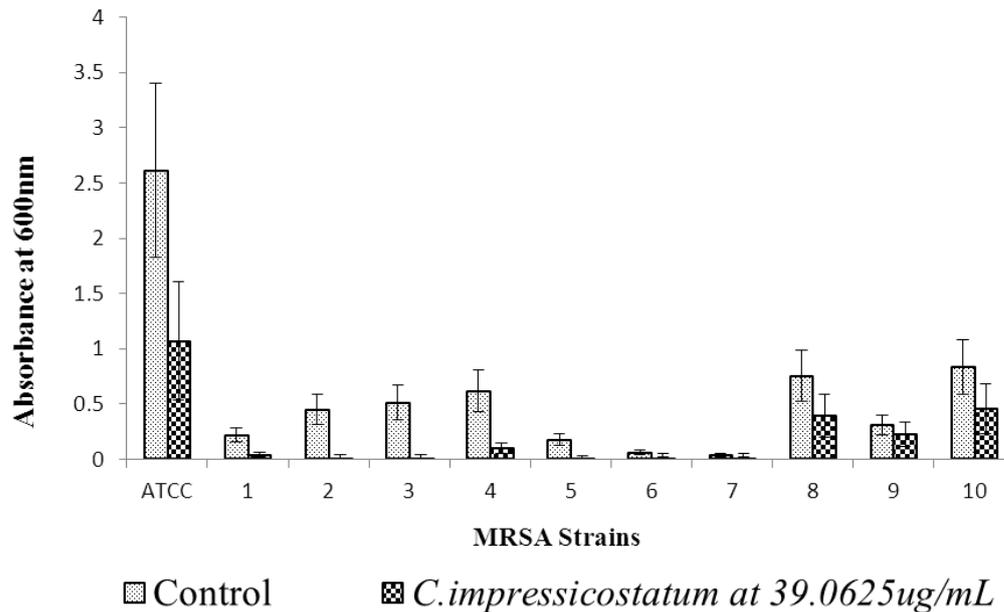


**Figure 7.0. Efflux pump inhibition assay :** The first bar, *C.impressicostatum* extract (CI) without Reserpine (CI without R), the second bar, *C.impressicostatum* extract with Reserpine (CI with R) and the third bar, Reserpine alone (R alone). The MIC values obtained when the plant extract was tested in combination with Reserpine and when Reserpine was tested alone were the same, an indication that *C.impressicostatum* crude extract does not possess any significant efflux pump inhibitory activity, but rather, it may be antagonistic to the effects of Reserpine, and may actually enhance extrusion of metabolites from the bacterial cell.

#### **Biofilm formation assay**

The effect of *C.impressicostatum* crude stem – bark water extract on biofilm formation of ATCC and clinical strains of MRSA were evaluated. Results showed a decrease in optical density of the treatments as compared to the control, an indication that *C.impressicostatum* extract exerts anti – biofilm activity on MRSA (Figure 8.0).

**Figure 8 The Effect of *C.impressicostatum* stem bark extract on Biofilm formation by MRSA strain ATCC 700698 and Clinical Strains of MRSA**



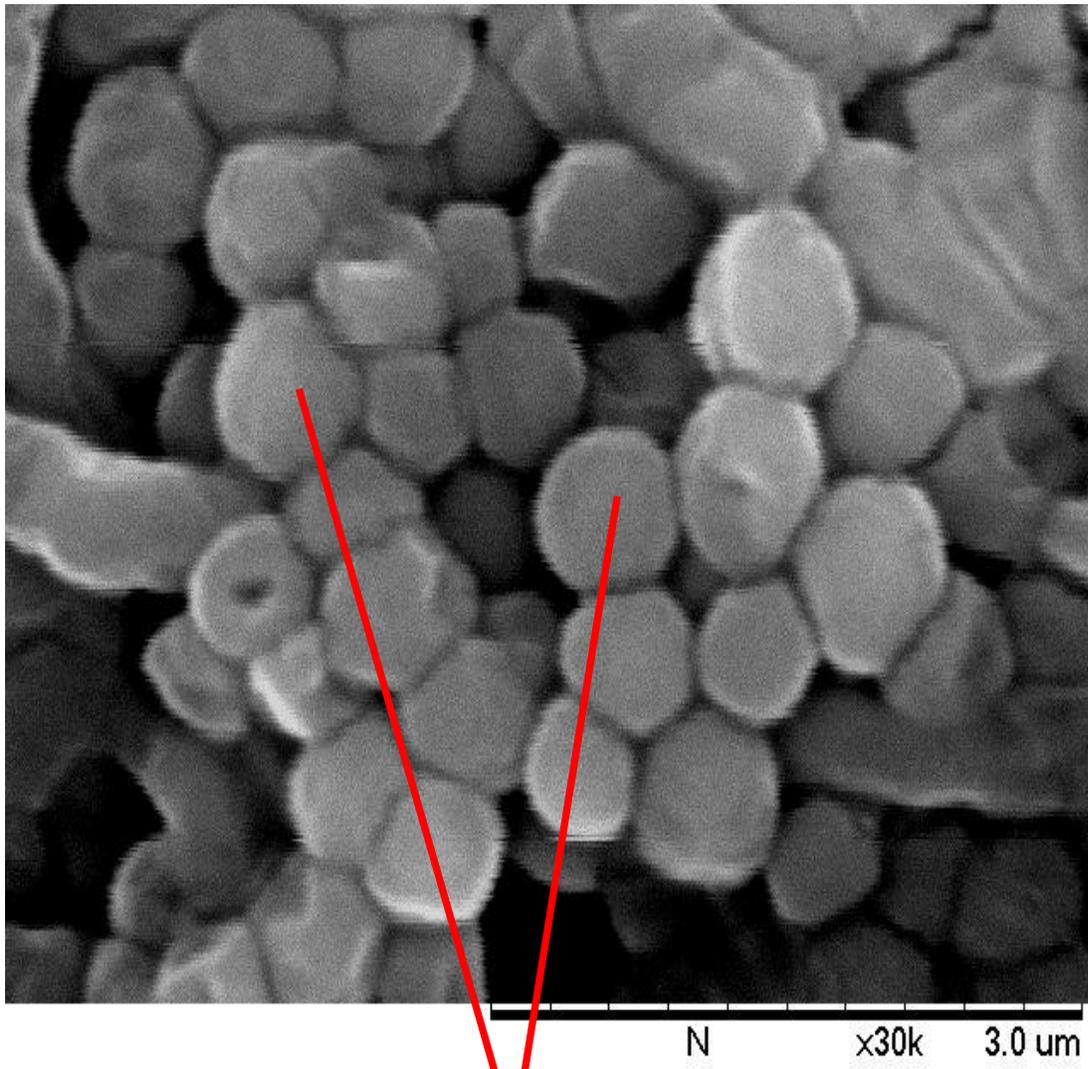
**Figure 8.0. Results showing a decrease in absorbance and biofilm formation when comparing control MRSA strains without treatment with crude plant extract and MRSA strains treated with 39.0625  $\mu$ g/mL of *C.impressicostatum* crude extract.**

### Scanning electron microscopy

Morphological changes observed in MRSA external structure after treatment with a sub - inhibitory concentration of crude *C.impressicostatum* stem - bark extract are shown in Figure 9.0. Post - treatment changes included roughening of the surface of the cell wall, elongated and pleomorphic forms, all possibly indicative of cell wall and / or plasma membrane damage and the presence of vast amounts of granular and amorphous debris, possibly resulting from cellular lysis.

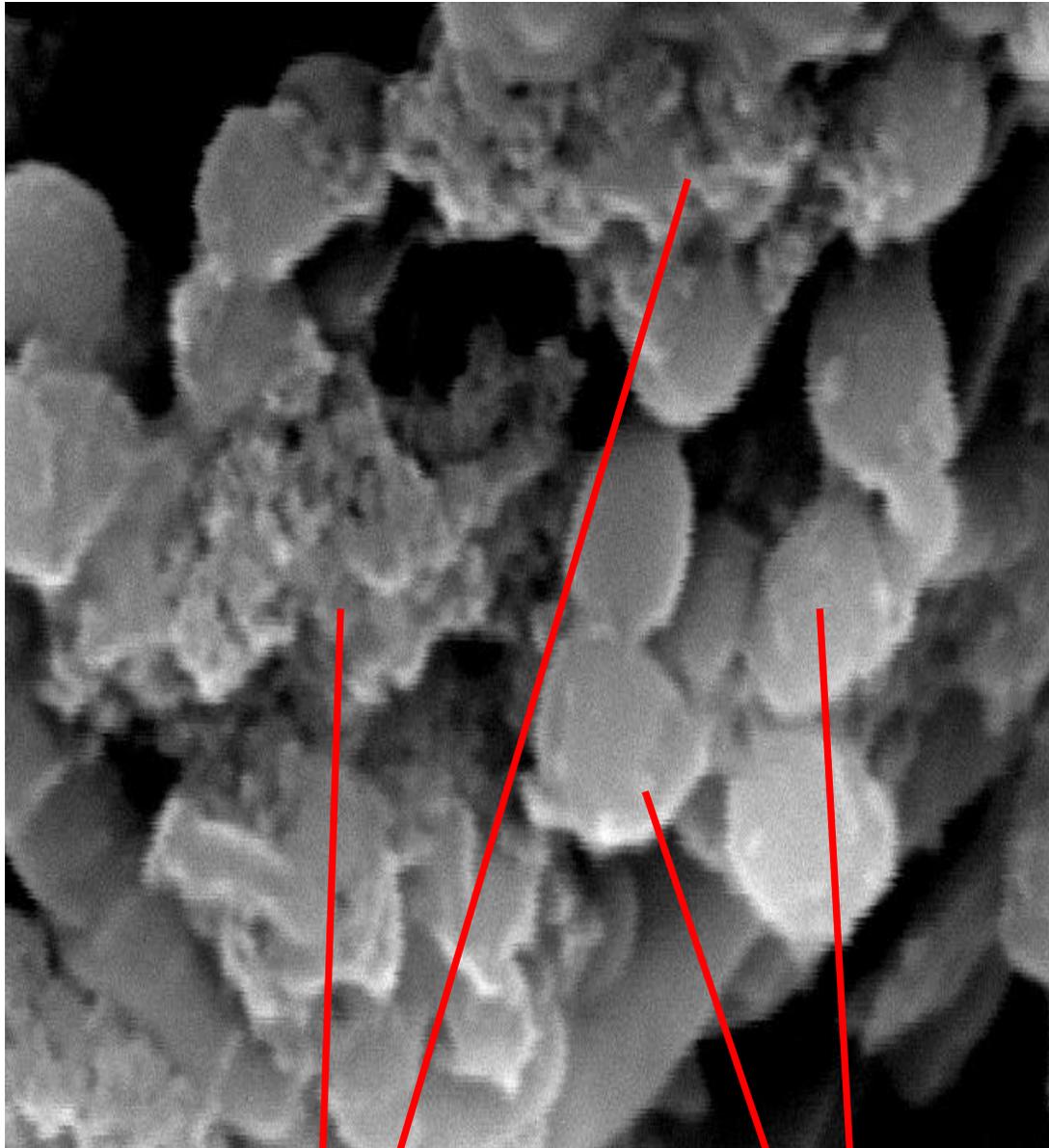
**FIGURE 9.0. (A) Control: Scanning electron micrograph of MRSA treated with 1% DMSO WITHOUT *C.impressicostatum* crude extract. (B) - (D) scanning electron micrographs of MRSA treated for 2 h with 9.7µg/mL sub-inhibitory concentration of crude *C.impressicostatum* stem – bark extract**

(A) Control



**Smooth surfaced MRSA cells**

(B)

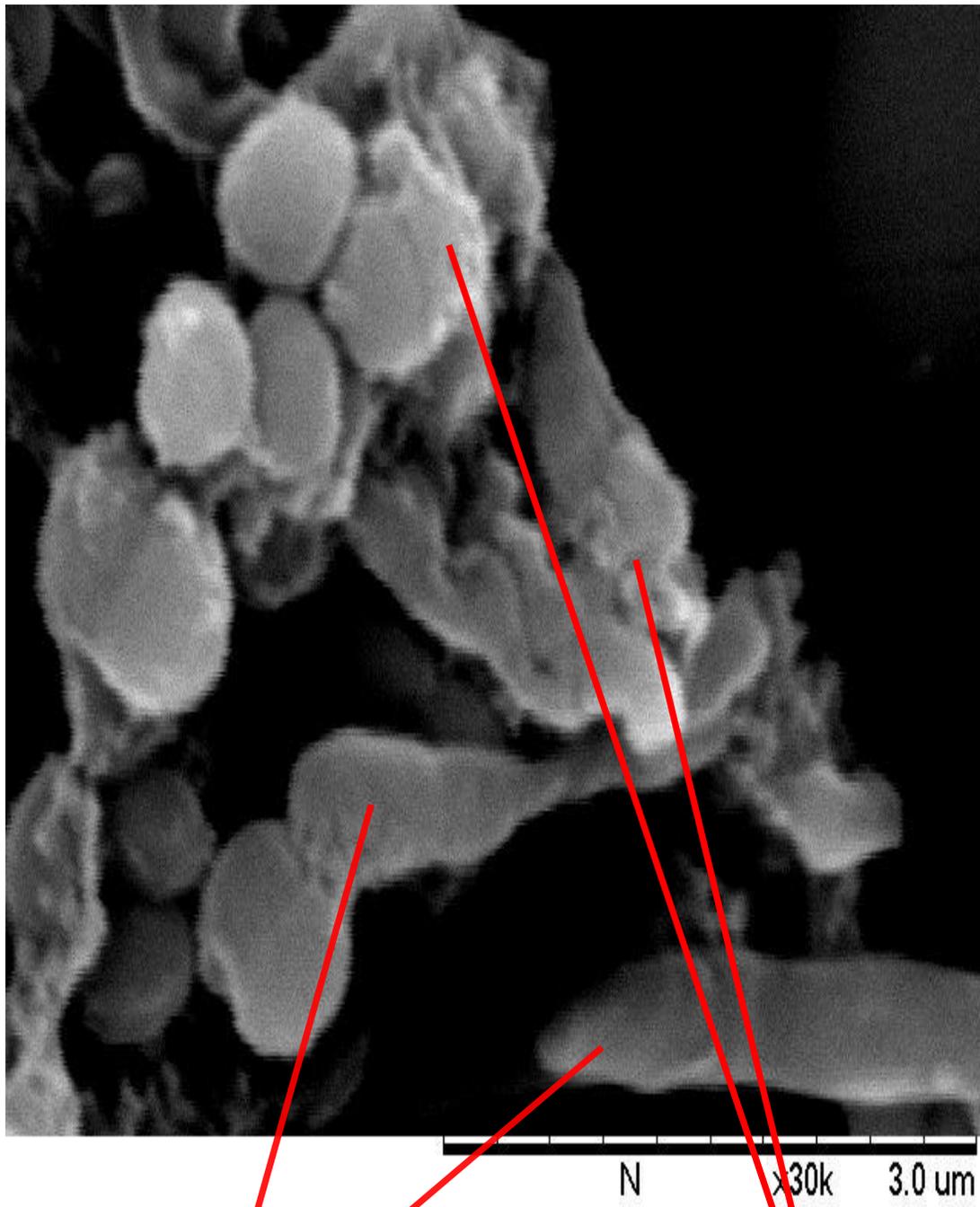


N x30k 3.0 um

**Vast amounts of granular debris**

**Rough surfaced MRSA cells**

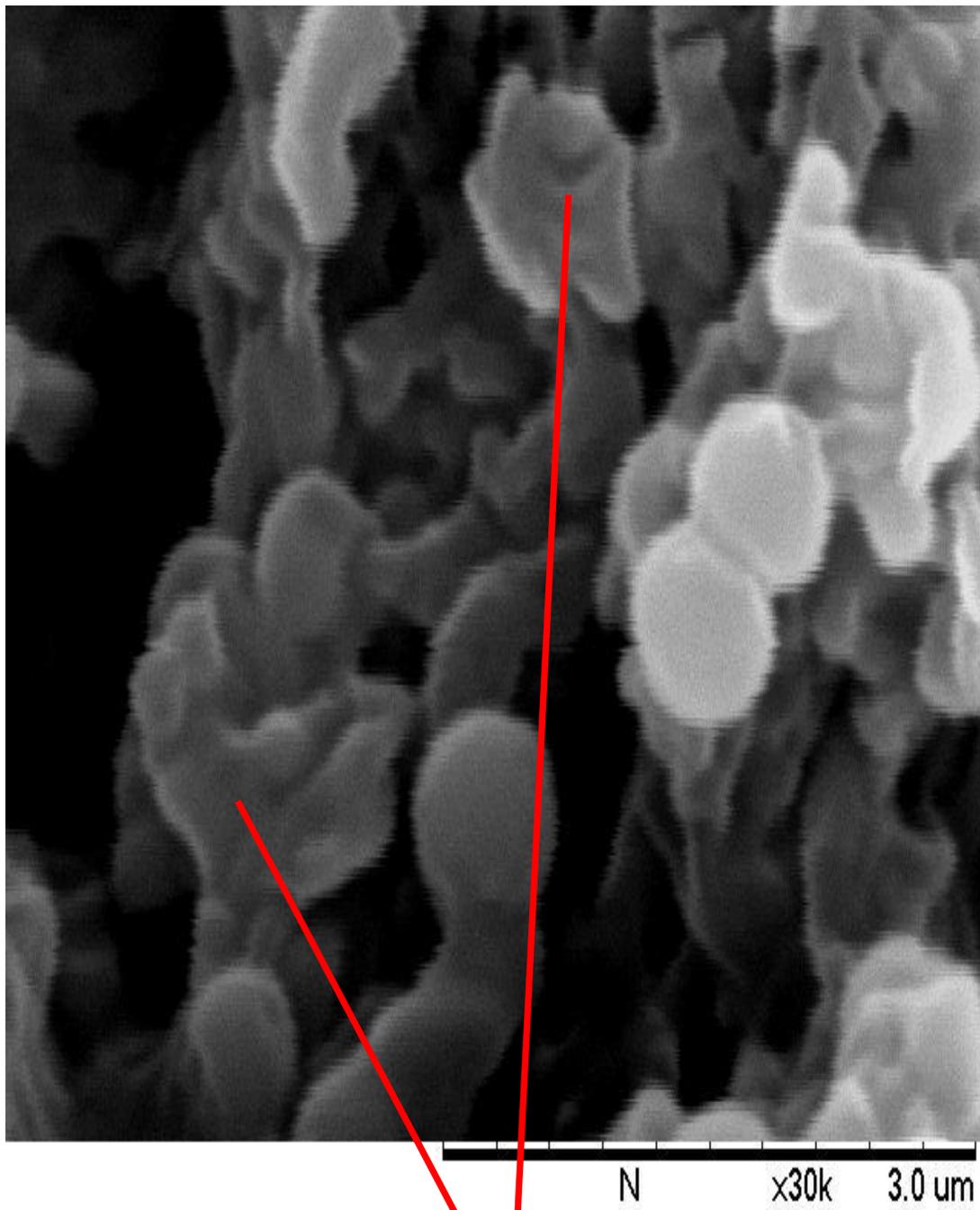
(C)



**Grossly elongated cells**

**Disintegrating cells**

(D)



**Pleomorphic forms**

## Discussion

The importance and significance of the quest for antibacterial agents from natural resources for use as therapeutics against multidrug - resistant micro - organisms cannot be underestimated. MRSA is a significant cause of complicated hospital and community acquired infections. In the USA alone, antibiotic resistance has been shown to be implicated in about 70% of hospital-acquired infections [21]. Cinnamon has been shown to possess antibacterial activity against a wide range of micro - organisms as shown in preliminary screening using the modified disk diffusion assay and its spectrum of bioactivity includes MRSA (unpublished data). This study aims to elucidate the mechanism of antibacterial action of *C.impressicostatum* against MRSA in view to its further development as a therapeutic agent against infections caused by this pathogen. Antibacterial chemotherapeutic agents utilise various complex mechanisms to inhibit the growth of bacteria. These include inhibition of cell wall biosynthesis, disruption of cell membranes, inhibition of nucleic acid and protein synthesis, inhibition of nucleic acid metabolism [22] and drug efflux pumps [23]. The results shown in Figure 4 suggest that treatment of MRSA with *C.impressicostatum* crude stem – bark extract induces bacteriolysis, possibly via release of membrane or cell wall bound autolytic enzymes [16]. The release of these autolytic enzymes may have been induced by the crude plant extract either damaging or loosening the structure of the bacterial membrane or cell wall. Activation of these enzymes may have weakened the cell wall exposing the cytoplasmic membrane to the external medium with consequent rupture of the cytoplasmic membrane due to osmotic pressure. Alternatively, one or more components in *C.impressicostatum* stem - bark extract may be interacting with the surface components of MRSA, eg cell wall peptidoglycan or membrane proteins, causing perturbations in the cell wall and / or cytoplasmic membrane structure, eventually leading to bacteriolysis. As such, induction of bacteriolysis must be one mechanism of action of the bioactive compound(s) contained in *C.impressicostatum* extracts. In fact, in support of this hypothesis, antibacterial phenolic

compound(s), which this extract has been found to be particularly rich in, have been reported in the literature to exert bacteriolytic effects [24].

The inhibitory effect of salt on most microorganisms has been discussed by various sources [25, 26]. Salt basically exerts a desiccative effect on microorganisms, which may kill, if present excessively. However, *S.aureus* has the ability to grow in the presence of high salt concentrations [25, 27]. As such, *S.aureus* is categorised as halo - tolerant and this property can be explained by its ability to accumulate osmo - protective molecules such as choline, glycine, betaine and L-proline [28]. Treatment of MRSA with *C.impressicostatum* stem – bark extract in the presence of 7.5% NaCl (Figure 5) resulted in an enhanced killing effect that may be summative or synergistic in nature. One hypothesis that could explain this phenomenon is the leaching of bacterial cell wall bound autolytic enzymes by the combinatorial action of salt and extract. This would ultimately cause autolysis of bacterial cells.

The marked increase in OD 260 nm readings after 8 h exposure to *C.impressicostatum* stem - bark water extract is suggestive of heavy cytoplasmic leakage and release of intracellular bacterial components into the medium, particularly guanine and cytosine (Figure 6). This may be an indication of gross and irreversible cytoplasmic membrane and cell wall damage. The subsequent reduction in OD 260 nm readings observed after 24 h may be due to degradation of the released compounds. The permeabilisation effect of the cytoplasmic membrane of MRSA to small cellular components as a result of exposure to *C.impressicostatum* extract may be due to membrane depolarisation, caused by uncoupling of the electron transport chain, thus affecting membrane energetics and causing leakage of cytoplasmic components [29]. Alternatively, if the mode of action of *C.impressicostatum* extract is to cause damage to the bacterial cell wall, the resultant exposure of the cytoplasmic membrane to the external medium may have caused rupture due to osmotic pressure with concurrent leakage of its intracellular components.

Analysis of SEM images of MRSA post - treatment with *C.impressicostatum* crude stem – bark extract suggests alterations in cell wall topology and damage to the bacterial cell wall. In addition, vast amounts of what apparently is cellular debris was present (Figure 9) suggestive of cell lysis or fragmentation. Thus, clearly one mode of *C.impressicostatum* bioactivity possibly resides in the induction of either internal or external mechanisms of cell lysis, fragmentation and / or apoptosis.

Time - kill determinations were performed to assess the viability of MRSA when treated with *C.impressicostatum* stem - bark extract. Standard bacterial suspensions were exposed over time periods of 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h to concentrations of ½MIC, 1xMIC, 2xMIC, 4xMIC and 8xMIC of *C.impressicostatum* extract. The decrease in the number of viable cells was first observed after thirty minutes, but was significant after two hours when challenged with 4xMIC and 8xMIC of extract. The interactions of the hydrophobic components of the bacterial cell wall are believed to play a critical role in antimicrobial susceptibility and resistance [30]. There are no in - depth studies on record that assess the mechanism of antibacterial action of *Cinnamomum* species. Based on our studies, we can assume that the mechanism of action involves the nature of its constituents. Its effect in reducing the number of viable bacterial cells is an indication of growth inhibition and killing of MRSA at different stages of bacterial growth, particularly during exponential and stationary phase.

The efflux pump mechanisms used by most micro - organisms are known to be complex transport proteins which play functional roles in extrusion of toxic substances or compounds such as clinically known antibiotics from the inside of cells to the external environment. These complex proteins are known to be found in both the Gram positive and the Gram negative bacteria and also in eukaryotic organisms [31]. There are specific pumps for different types of substrates. These pumps may transport a wide range of structurally different compounds including antibiotics of different multiple classes. These pumps are also associated with multiple

drug resistance (MDR). In the prokaryotic kingdom, there exists five major families of efflux transporters: [31] they include MATE (multidrug and toxic efflux), ABC (ATP binding cassette), RND (resistance – nodulation - division), SMR (small multidrug resistance) and MF (major facilitator). In all these systems, the proton motive force is utilised as an energy source, with the exception of the ABC family, which exports substrates by ATP hydrolysis. In phagocytic cells, the accumulation of antibiotics is modulated by these pumps and plays a very significant role in trans - epithelial transport of these drugs [31]. The efflux pump mechanism has been recognised to mediate drug resistance and is an important provider of antibiotic resistance in bacteria, especially in MRSA. Results of this study suggest that MRSA carries efflux pumps, a fact supported by Hiramastu and colleagues [32].

Reserpine alone had a stronger inhibitory effect on MRSA than treatment with *C. impressicostatum* stem – bark extract. At the very least, a summative effect was expected with the combinatorial treatment of Reserpine and *C. impressicostatum* stem – bark extract. However, this was not observed. The effect of the combinatorial treatment was equivalent to treatment with Reserpine alone. The latter suggests that Reserpine and *C. impressicostatum* stem – bark extract in the combinatorial treatment may be acting antagonistically to nullify individual antibacterial effects. It may be suggested that *C. impressicostatum* stem – bark extract can reverse the activity of the efflux pump inhibitor Reserpine and its mode of action may be to actually activate the transmembrane transporters of MRSA and increase the rate of extrusion of metabolites from the bacterial cell.

Biofilms are known to comprise of surface - associated microbes that produce an extracellular matrix of complex extracellular polysaccharides. The bacterial cells form biofilms, swarm and engage in pili – mediated twitching. Although each of these group behaviours is extensively studied on its own, little is known about the relationships among these behaviours [33]. Biofilm formation is one of the most important virulence traits utilised by *S. aureus* to form extensive

colonies on damaged tissues and implanted biomaterials [34]. Many nosocomial pathogenic bacteria are the cause of acute and chronic infections due to their ability to form biofilms [35,36]. Although biofilm - forming properties have been well demonstrated by members of the *Staphylococcus* genus such as *S.epidermidis* and *S.aureus*, they are less well studied in the more recently evolved Methicillin - Resistant *Staphylococcus aureus* (MRSA), which has developed from several different clonal lineages of Methicillin-Susceptible *S. aureus* strains via acquisition of certain genetic mobile elements called *Staphylococcal Chromosomal Cassette mec* (SCC*mec*). The ability of MRSA to produce biofilms has resulted in difficulties in understanding its high clonal diversity, including its enhanced propensity to spread and cause opportunistic human infections in various parts of the world. The initial bacterial monolayer that sticks to a polymeric surface changes to a common biofilm that includes bacteria and an extracellular slime substance. The proliferation of the bacteria and the formation of the slime results in a higher resistance to antibiotics because drugs are prevented from reaching the bacteria that are protected by the biofilm slime [37]. Many studies have concluded that the formation of the biofilm is caused by adherence at late stages of bacterial growth. In this process, the organisms stick to each other through polysaccharide intercellular adhesion (PIA), which is synthesised by products of the *icaADBC* operon [38]. The biofilm complex structures are known to be inherently resistant to most antimicrobial challenge and make them very difficult to eradicate from the environment and infected host [34]; there is clearly a need for novel antimicrobial agents with new mechanisms of action against microbial biofilms. The results of this study clearly indicate that *C. impressicostatum* stem – bark extract possesses anti – biofilm activity against MRSA. It is postulated that a component(s) of *C. impressicostatum* stem – bark extract may be inhibiting one or more elements of the *icaADBC* operon in MRSA that enables biofilm formation (unpublished data).

## Conclusions

The exposure of MRSA to *C.impressicostatum* crude stem - bark water extract caused cellular distortions, alterations in cell wall topology and gross damage to the bacterial cell wall. As the time kill study against MRSA treated with plant extract demonstrated dose dependent growth inhibitory activity, coupled with the MBC assay that demonstrated the killing effect of the plant component(s), we speculate that one or more components in *C. impressicostatum* stem – bark extract interacts with the peptidoglycan layer, lipoteichoic and wall teichoic acids, coupled with possible extraction of membrane lipids and lipoteichoic acids, possibly triggering secondary responses in the bacterial cells, resulting in the observed inhibition and death of the bacterium. The plant extract may also be inducing the release of membrane and / or cell wall bound autolytic enzymes, resulting in autolysis.

The extensive cell wall damage observed when MRSA was treated with *C.impressicostatum* stem - bark extract may due to the fact that the bioactive molecule(s) in the plant extract have similar effects to the beta - lactam antibiotics in inhibiting correct cell wall biosynthesis and architecture. Alternatively, the cell wall damage may be due either directly or indirectly to the interplay of apoptotic mechanisms induced upon exposure to the plant extract.

*C.impressicostatum* stem - bark extract in combination with NaCl yielded enhanced bacterial cell death possibly via summative or synergistic mechanisms. Empirical analysis also suggests that treatment of MRSA with extracts leads to heavy cytoplasmic leakage of intracellular bacterial components into the medium. SEM images of MRSA post - treatment with *C.impressicostatum* crude extracts indicated alterations in cell wall topology, damage to the bacterial cell wall and possibly also to the cytoplasmic membrane and also the presence of vast amounts of cellular debris, thereby elucidating its mode of action as bacteriolysis triggered via internal or external mechanisms.

## Competing interests

The author(s) declare that they have no financial and / or non - financial competing interests.

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# Figures

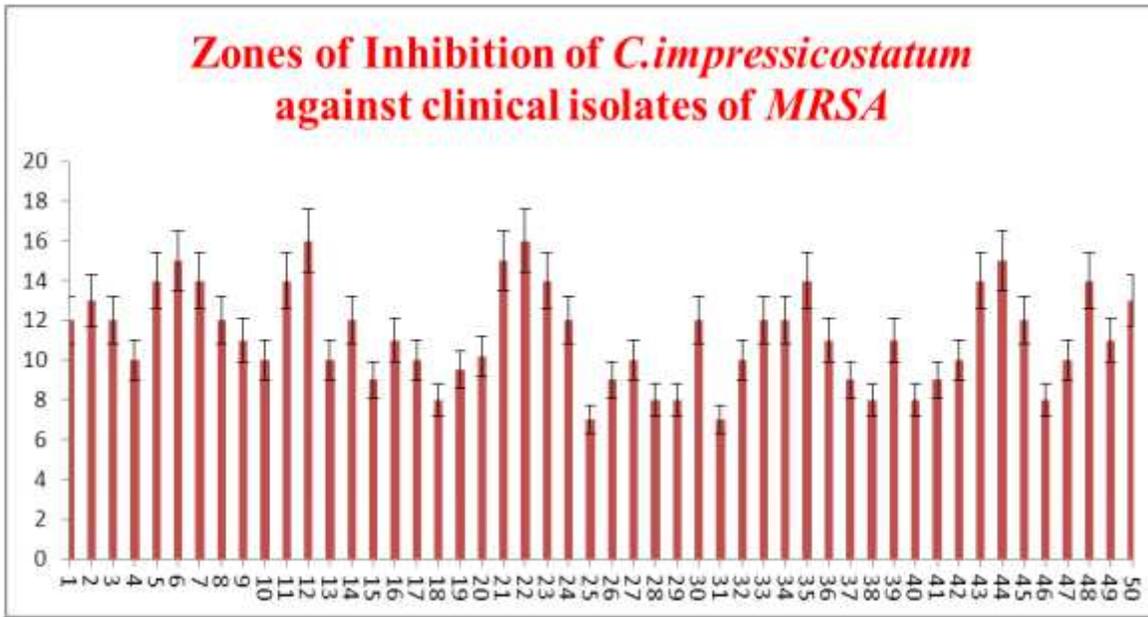


Figure 1

Antibacterial activity of 50 clinical isolates tested against *C. impressicostatum* crude stem bark water extract (zone diameters are expressed in mm  $\pm$  standard deviation)

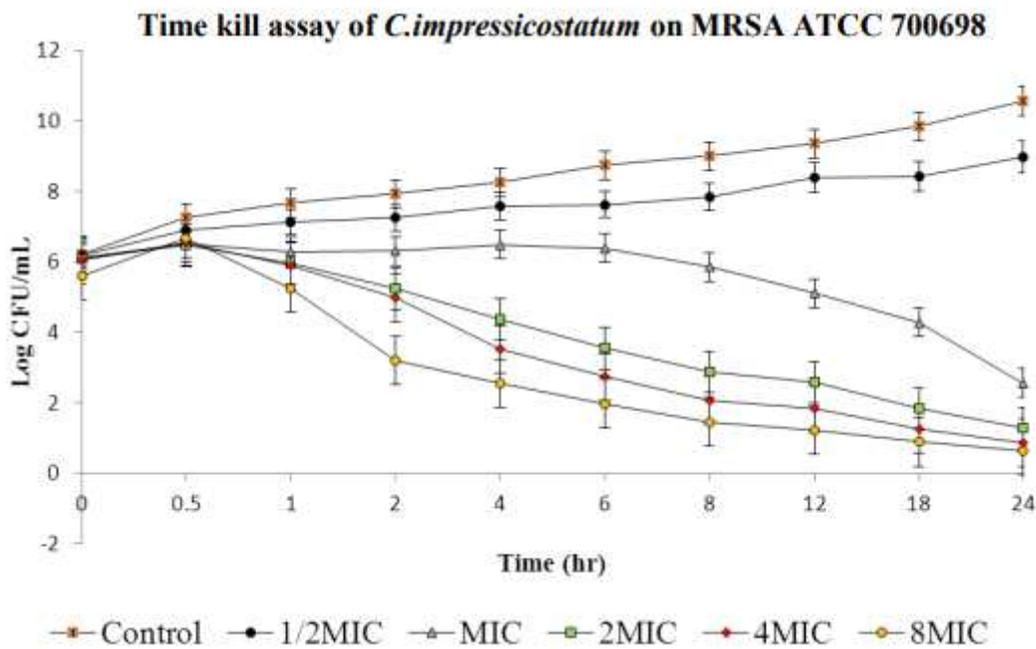
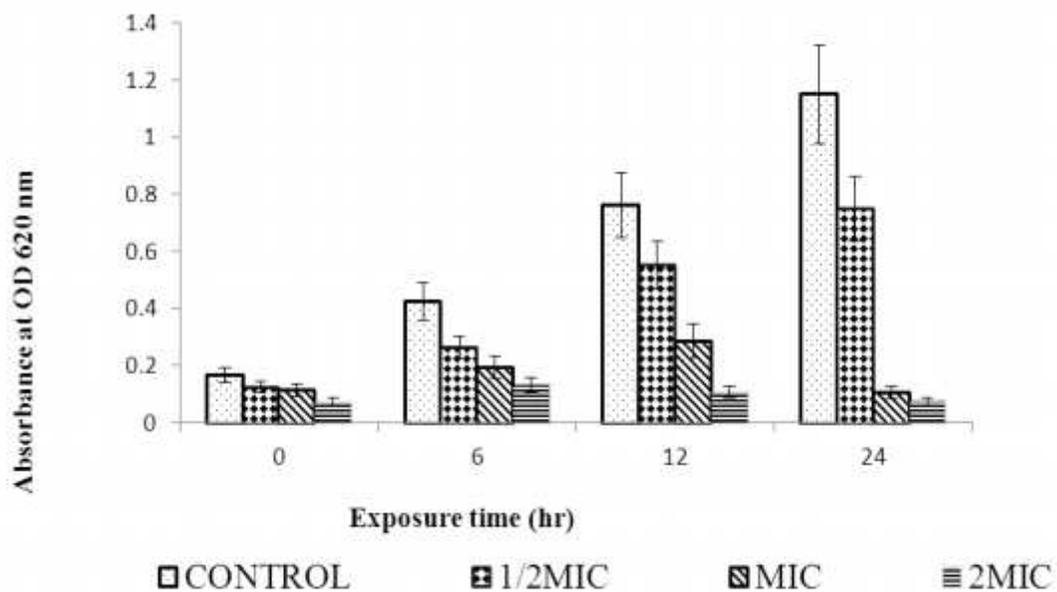


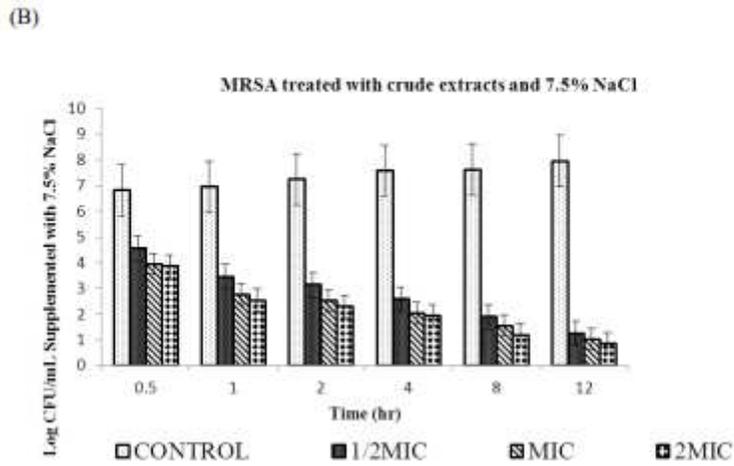
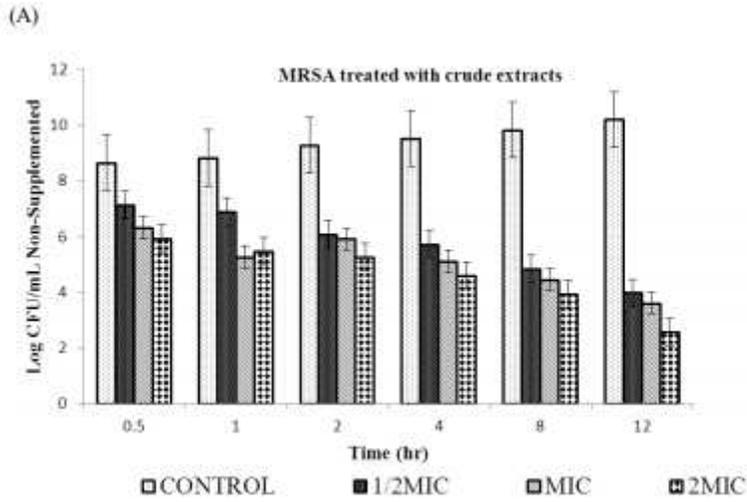
Figure 2

The kinetics of the killing curves after treatment of MRSA ATCC 700698 with the soxhlet crude stem – bark water extract of *C. impressicostatum*. Each symbol denotes mean  $\pm$  standard deviation.



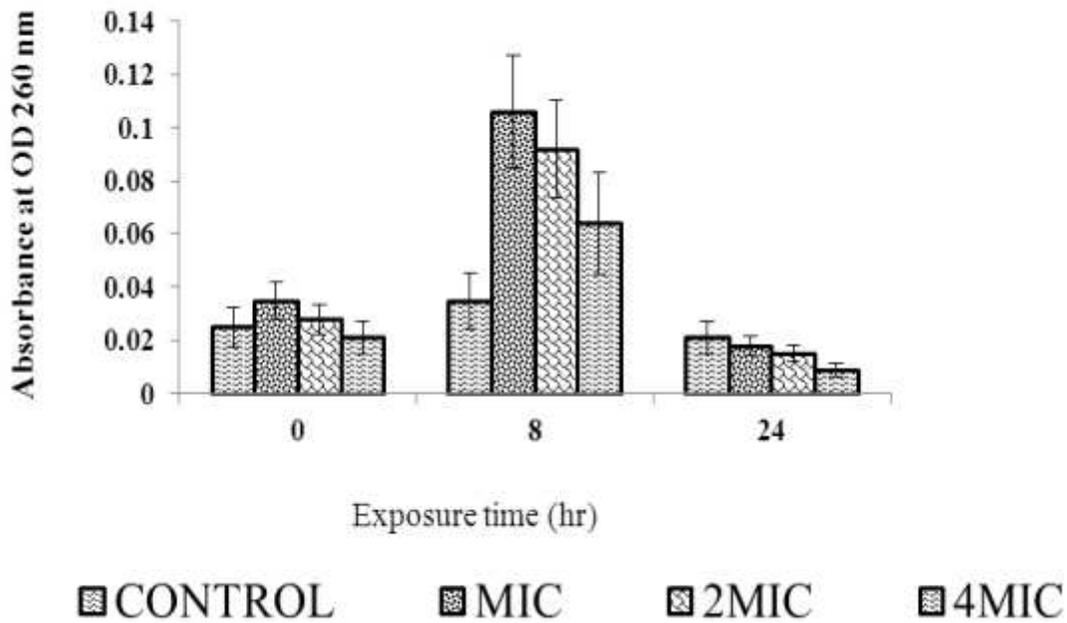
**Figure 3**

The bacteriolytic activity of MRSA ATCC 700698 treated with different concentrations of *C. impressicostatum* soxhlet crude stem – bark water extract. The control was treated with 1% DMSO. The data are expressed as means  $\pm$  standard deviation



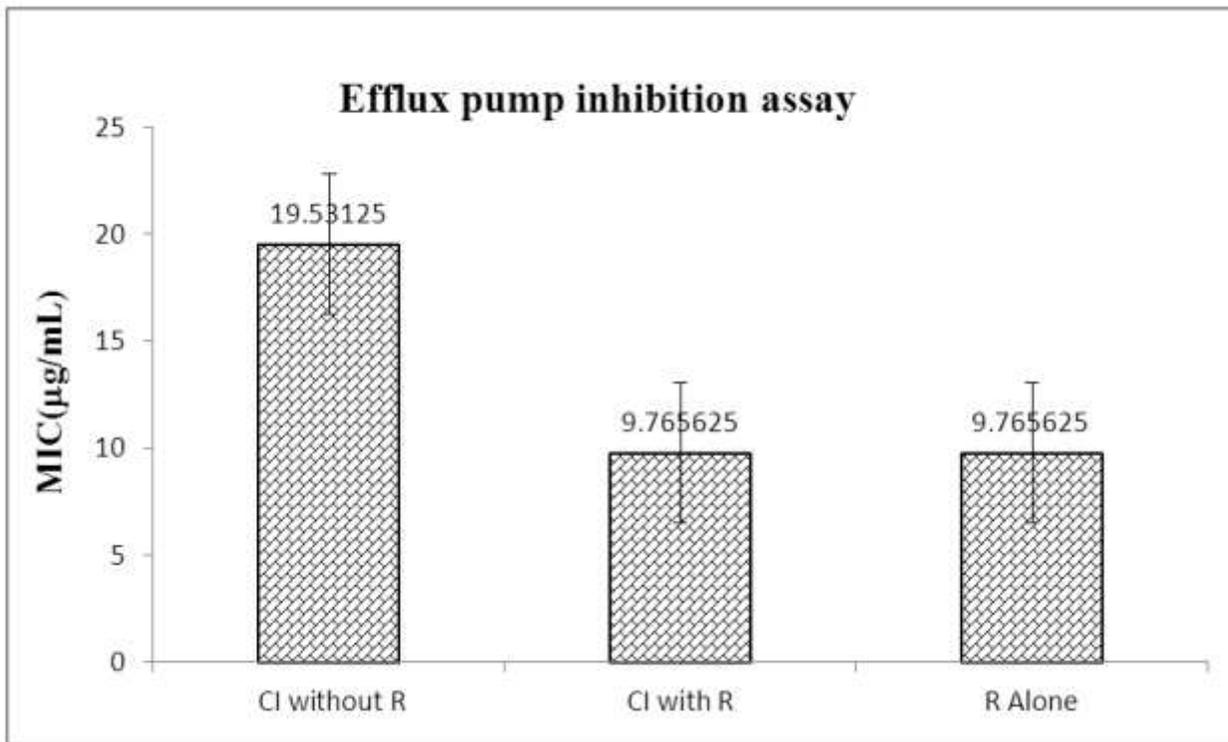
**Figure 4**

(A) MRSA treated with 1/2MIC, MIC and 2MIC of *C.impressicostatum* crude stem – bark extract with increasing lengths of exposure; (B) MRSA treated with 1/2MIC, MIC and 2MIC of *C.impressicostatum* crude stem – bark extract supplemented with 7.5% NaCl, with increasing lengths of exposure. The data are expressed as means  $\pm$  standard deviation



**Figure 5**

MRSA optical density (OD) measurements at 260 nm upon treatment with different concentrations of *C. impressicostatum* crude extracts. 1% DMSO was used as control. The data are expressed as means of  $\pm$  standard deviation



**Figure 6**

Efflux pump inhibition assay : The first bar, *C.impressicostatum* extract (CI) without Reserpine (CI without R), the second bar, *C.impressicostatum* extract with Reserpine (CI with R) and the third bar, Reserpine alone (R alone). The MIC values obtained when the plant extract was tested in combination with Reserpine and when Reserpine was tested alone were the same, an indication that *C.impressicostatum* crude extract does not possess any significant efflux pump inhibitory activity, but rather, it may be antagonistic to the effects of Reserpine, and may actually enhance extrusion of metabolites from the bacterial cell.

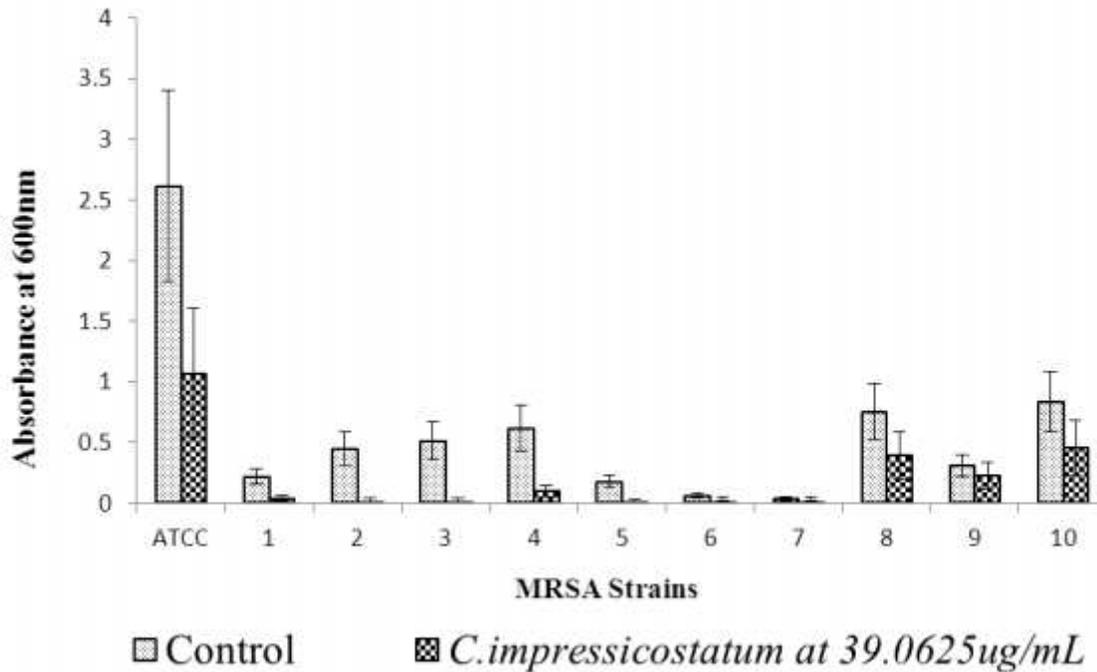
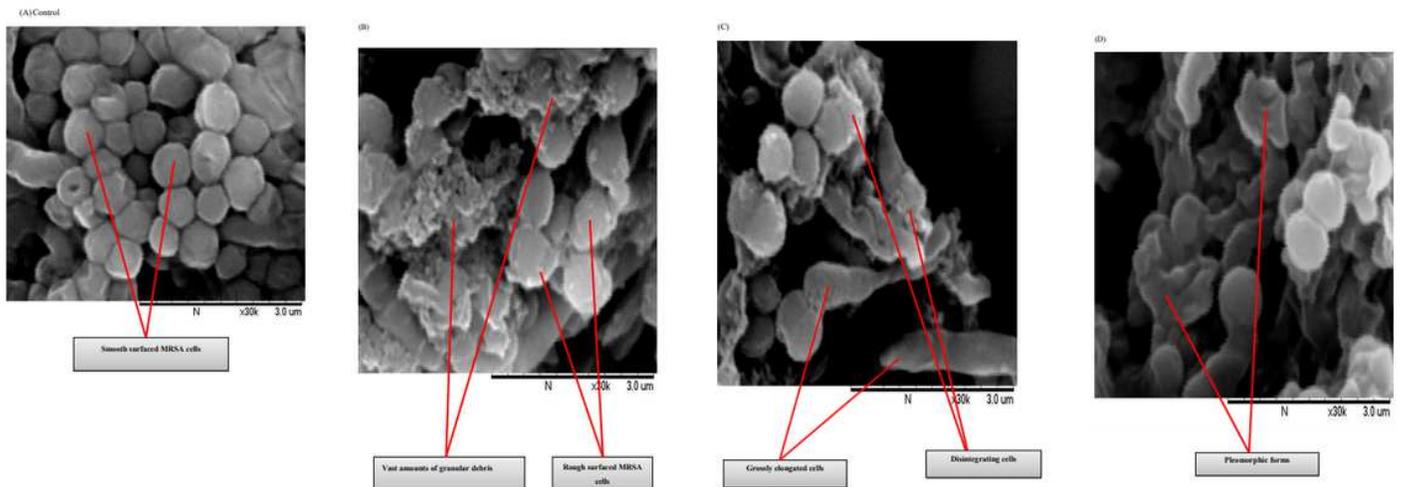


Figure 7

Results showing a decrease in absorbance and biofilm formation when comparing control MRSA strains without treatment with crude plant extract and MRSA strains treated with 39.0625 µg/mL of *C.impressicostatum* crude extract.



## Figure 8

(A) Control: Scanning electron micrograph of MRSA treated with 1% DMSO WITHOUT *C.impressicostatum* crude extract. (B) - (D) scanning electron micrographs of MRSA treated for 2 h with 9.7µg/mL sub-inhibitory concentration of crude *C.impressicostatum* stem – bark extract