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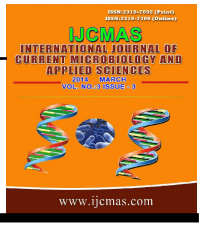

Original Research Article

The Antibacterial effect of Some Natural Bioactive Materials against *Klebsiella pneumoniae* and MRSA

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A B S T R A C T

Two out of 10 tested bioactive plant extracts namely cinnamon and green tea exhibited the highest antibacterial activity against *Klebsiella pneumoniae* and MRSA under test. However the bioactive extracts with antibacterial activity showed a little variation in their activity against the tested bacteria. The methanolic extracts of cinnamon and green tea exhibited a high potency in terms of MIC and MBC values against the growth of *Klebsiella pneumoniae* and MRSA under test. Time-kill curve showed a fast and sharp antibacterial activity against *Klebsiella pneumoniae* and MRSA. Cinnamon and green tea extracts targeted microbial cell membranes and cell walls, and led to structural disorganization of the cell. Methanolic extracts proved to be good candidates for further development as antibacterial agents for infections caused by *Klebsiella pneumoniae* and MRSA and may also oppose the resistance of the conventional used antibacterial agents. Phytochemical analysis of cinnamon extract explored that presence of glycosides, phenol, saponins, tanins and terpenoids and of green tea extract explored the prescence of flavanoids, phenol,steroids and tannins. Electron microscopy examination of Cinnamon and green tea treated cells showed a great variation in the cell structure.

Keywords

Cinnamon; green tea; *Klebsiella pneumonia*; MRSA.

Introduction

In the last several years, the frequency and spectrum of antimicrobial-resistant infections have increased in both the hospital and the community due to the continued use of systemic and topical antimicrobial agents which, in turn, drove the continued search for new agents (Rijnders et al., 2009). In addition, the side effects of over use and misuse of antibiotics can harm vital organs (Bocanegra-Gracia et al., 2009). Most important multidrug-resistant bacteria on the global scale include gram positive (methicillin-resistant *Staphylococcus aureus*, vancomycin resistant enterococci) and gram-negative bacteria (members of enterobacteriaceae producing plasmid-mediated extended spectrum β- lactamase

(ESBL) and others as *Pseudomonas aeruginosa, Mycobacterium tuberculosis* (Sajduda et al., 1998). Nowadays; pharmaceutical companies are developing new antibiotics to replace those that are no longer effective (Silbergeld et al., 2008). Healing with medicinal plants is as old as mankind itself. With the rising prevalence of microorganism showing resistance to antibiotics, there is an urgency to develop new antimicrobial compounds. Since antiquity, plants have been used to treat common infectious diseases. Being nontoxic and easily affordable, there has been resurgence in the consumption and demand for medicinal plants (Jayashree & Maneemegalai, 2008).

Bullerman, 1977; Mabrouk & El-Shayeb, 1980 and Azzouz & Bullerman, 1982 reported that cinnamon inhibited the growth and toxin production of some mould species, with an activity emerging from cinnamic aldehyde and eugenol. Cinnamaldehyde was completely inhibiting both sensitive and resistant strains of *Helicobacter pylori* (Ali et al., 2005). Ouattara et al. (1997) reported that eugenol was shown to have a stronger bactericidal activity against *E. coli* and *K.pneumoniae* than some antibiotics. On the other hand, Nyfors et al. (2004) reported that polyphenols found in tea have been able to inhibit the growth of and/or kill the pathogenic bacteria namely: *Staphylococcus aureus, Salmonella typhi, Salmonella typhimurium, Salmonella enteridis, Shigella flexnieri, Shigella dysenteriae, Streptococcus sobrinus, Lactobacillus rhammosus, Actinomyces viscosus, Listeria monocytogenes, Streptococcus salivarius, Streptococcus mitis* and *Vibrio chlorae.*

Materials and Methods

Microorganisms

Two different bacterial isolates were used throughout the present work; one gram negative *Klebsiella pneumoniae* and the other gram-positive methicillin-resistant *Staphylococcus aureus* were isolated from patients with urinary tract infection among Lebanese community and identified phenotypically as described in Beregy's Manual of determinative bacteriology (Buchanan & Gibbson, 1974).

Chemicals

Medicinal plants were kindly supplied by "Adonic spices", Beirut Lebanon. All the prepared reagents used in the present investigation were provided by Fluka, Germany. The culture media and the antibiotics used were supplied by Oxoid Ltd, United Kingdom. Other used chemicals of laboratory and analytical grade were obtained from recognized chemical suppliers. (Medilic,Beirut).

Inoculum preparation and standardization

Inocula were prepared directly by suspending colonies grown overnight on nutrient agar plate directly in sterile saline solution (0.85%). Suspensions were adjusted by using the Macfarland which corresponds approximately to 1.5x 10^8CFU/ml (Madigan et al., 2006). In some experiments bacterial suspension were diluted, or supplemented with more organisms, as needed to correspond to final inoclum concentrations 1.5x 10^8CFU/ml (Mahon et al., 1998).

Preparation of methanolic extracts

Medicinal plant samples (25g) were extracted with 80%methanol (150ml) by cold maceration for 72hrs. The extract was filtered and the filtrate was concentrated by Rotavapor (R-114) at 60°C to get an oily residue. The extract was stored in labeled sterile screw capped bottle at 4°C till further analysis.

Maintenance of the bioactive extracts

All bioactive extracts were tested for their sterility and stored at 25°C in sterile brown glass containers and placed in dark places to prevent photo-isomerization.

Screening of antibacterial activity of bioactive extracts

The effect of some bioactive extracts (Green tea, garlic, paprika, caraway, onion extract, turmeric, cinnamon, linden, fenugreek, and fennel) was tested against the growth of *Staphylococcus aureus* and *Klebsiella pneumoniae* using the well diffusion method. An inoculum of bacterial suspension (1.5 x 10^8 CFU/ml) equivalent to 0.5 McFarland was prepared, and 25 µl were swabbed over the surface of Müller-Hinton agar plate . A 6mm well was cut in the center of each plate using a sterile cork borer. Twenty five µl of each bioactive extract were pippetted into each well. Plates were placed at 4°C for 1 hr for compound diffusion and then incubated for 24 hrs at 37 °C. Results of the qualitative screening were recorded as average diameter of the inhibition zone surrounding the wells containing the test solution (Falahati et al., 2005). Each experiment was repeated at least three times.

Determination of minimal inhibitory concentration (MIC) of some selected extracts

A series of dilutions of each tested extract was prepared in a 96-well microdilution tray with an initial extract concentration of 100 % and a final concentration of 10%. The cultures of the bacteria under test were diluted in Müller-Hinton broth at a density adjusted to a 0.5 McFarland turbidity. The final inoculum was 1.5 x10^8 CFU/ml of bacterial cultures. After the addition of inocula of bacterial broth, trays were covered, incubated at 37° C for 24 hrs. MICs were determined visually, according to NCCLS guidelines (2000). The experiment was repeated three times. The MIC is the lowest concentration of extract that inhibited the growth of the test strain in the wells by visual reading and by the growth inhibition on macro-plates as described by Ellof (1998).

Determination of minimal bactericidal concentration (MBC) of some selected extracts

The minimum bactericidal concentrations (MBCs) of selected extracts were determined by inoculating the MIC dilution onto Müller- Hinton agar plates and incubated at 37ºC for 18 hrs (Rota et al., 2008). MBCs were determined as the lowest concentration resulting in no growth on subculture. The experiment was repeated three times.

Determination of bacterial time –Kill curve

A time-Kill curve was assessed to investigate the best time for extracts that kills the bacterial vegetative cells. Therefore, the selected extracts that showed a bactericidal effect against the

most promising bacterium under test were used, and survivor (time-kill) curve was plotted. A 16-hrs culture was harvested by centrifugation. The suspension was adjusted using the McFarland standard and was then further diluted in saline 0.85% to achieve approximately 1.5 $x10^8$ CFU/ml. The selected bioactive extracts were added to aliquots of 1 ml Müller-Hinton broth in tubes in water bath at 37ºC in amounts that would achieve bactericidal concentrations for the selected bacteria followed by the addition of 1 ml of the inoculum. Further samples were taken from each tube in order to monitor bacterial growth by measuring the absorbance (optical density) at 600nm wavelength at time intervals (0, 2,4,6,8,12 and 24 hours) and incubated at 37ºC (Yin et al., 2002). The experiment was carried out twice.

Screening for testing the antimicrobial combinations of the promising extracts with antibiotics against *Klebsiella pneumoniae* and MRSA

Seven antibiotic discs were selected. one was used for both gram-negative and gram-positive bacteria;ciprofloxacin (CIP).Two antibiotic discs for gram-negative bacteria were used: gentamycin(GM)and ciproflaxin(CIP). The discs used for gram-positive bacteria were cefoxitin (FOX), oxacillin ,(OX) ,ceftriaxone (CRO) and vancomycin (VA) (Abd-El Aal et al., 2007).A McFarland 0.5 standard bacterial suspension was swabbed on the top of the solidified Müller- Hinton agar plates and allowed to dry for 10 min. The discs combined with bioactive extract were placed on the inoculated agar by pressing slightly. The plates were placed at 4ºC for 1 hr for compound diffusion and then incubated for 24 hrs at 37ºC (Abd-El Aal et al., 2007). Zone of inhibition were recorded in millimeters and the experiment was repeated three times.

Phytochemical analysis (Sibi et al.,2013).

Test for Flavonoids (Ammonia test)

1 ml of the extract was taken in the test tube and ammonia solution was added (1:5) followed by the addition of conc.sulphuric acid. Appearance of yellow color and its disappearance on standing indicates the positive test for flavonoids.

Test for Glycosides (Keller Kiliani test)

5 ml of each extract was added one at a time with 2 ml of glacial acetic acid which was followed by the addition of few drops of ferric chloride solution and 1 ml of conc. sulphuric acid. Formation of brown ring at interface confirms the presence of glycosides.

Test for Phenols (Ferric chloride test)

The plant extract (0.5ml) was added with few drops of neutral ferric chloride (0.5%) solution. Formation of dark green color indicates the presence of the phenolic compounds.

Test for Saponins (Froth test)

1 ml of the extract was taken in a test tube and distilled water (2 ml) was added to it. The test tube was then kept in boiling water bath for boiling and was shaken vigorously. Existence of froth formation during warming confirms the presence of saponins.

Test for Steroids: (Libermann - Burchard's test)

Acetic anhydride (2ml) was added to 0.5ml of the bioactive plant extract and

then followed by the addition of 2 ml conc. sulphuric acid slowly along the sides of the test tube. Change of colour from violet to blue or green indicates the presence of steroids.

Test for Tannins (Ferric chloride test):

The plant extract (1ml) was added to 5 ml distilled water, boiled, then cooled down and 0.1% ferric chloride solution was added. Appearance of brownish green or blue black coloration confirms the presence of tannins.

Test for Terpenoids (Salkowski test)

The plant extract (5ml) was added in test tubes containing 2 ml chloroform, followed by the addition of 3 ml of conc. sulfuric acid. Formation of reddish brown layer at the junction of two solutions confirms the presence of terpenoids.

Transmission Electron Microscopy (TEM)

On the basis of MIC, MBC values and Time-Kill curve data, *Staphylococcus aureus* was incubated with cinnamon extract (300µl/ml) and green tea extract (200µl/ml) and *Klebsiella pneumonia* was treated with cinnamon extract (500µl/ml) and green tea extract (300µl/ml) respectively for 24 hrs. Freshly taken samples were fixed using a universal electron microscope fixative. Series dehydration steps were followed using ethyl alcohol and propylene oxide. The samples was then embedded in labeled beam capsules and polymerized. Thin sections of cells exposed to extracts were cut using LKB 2209-180 ultra-microtome and stained with a saturated solution of urinyl acetate for half hour and lead acetate for 2 min (McDowell and Trump, 1976). The procedure was applied to control cells not exposed to extracts and to extract-exposed cells. Electron Micrographs were taken using a Transmission Electron Microscope (JEM-100 CX Joel), at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

Results and Discussion

In a comparative study to differentiate the antibacterial effect between some bioactive plants extracts, screening experiments were done. Two out of ten plant extracts tested (green tea extract and cinnamon) showed a wide variation of antibacterial activity against the growth of *Klebsiella pneumoniae* with average zones of 27 and 19 mm respectively. On the other hand, the other tested extracts (caraway, fennel, garlic, fenugreek, onion, paprika, turmeric and linden extracts) showed no evidence for antibacterial activity against *Klebsiella pneumoniae* under test.Fig.1,plate1. However; six out of ten plant extracts (green tea,cinnamon, garlic,onion,parika and fennel extract) inhibited the growth of *MRSA* under test with average inhibition zones of 35,25,16,15.15 and 14mm respectively. On the other hand, the other tested extracts (caraway, turmeric, feneugreek and tilia extracts) did not show any antibacterial activity against *MRSA* under test. Fig. 2,plate 2.

Overall; cinnamaldehyde was the predominant active compound found in cinnamon oil (Baratta et al., 1998 and Simic et al., 2004). Matan et al. (2006) reported that cinnamon oil was not harmful when consumed in food products and it inhibited the growth of molds, yeast and bacteria. Ouattara et al. (1997) reported that eugenol was shown to have a

stronger bactericidal activity against *E. coli* and *K.pneumoniae* than some antibiotics**.** Similarly , Akgül & Kıvanç (1989) reported that four spices (cumin, cinnamon, cloves, and fennel) were shown to have an inhibitory effect against *S. aureus*, which is an important pathogen in food poisoning. Nyfors et al. (2004) reported that polyphenols found in tea have been able to inhibit the growth of and/or kill the following pathogenic bacteria: *Staphylococcus aureus, Salmonella typhi, Salmonella typhimurium, Salmonella enteridis, Shigella flexnieri, Shigella dysenteriae, Streptococcus sobrinus, Lactobacillus rhammosus, Actinomyces viscosus, Listeria monocytogenes, Streptococcus salivarius, Streptococcus mitis* and *Vibrio chlorae.*

The most promising bioactive plant extracts were green tea and cinnamon with MIC values ≤ 500µl/ml. The present study showed that over all high potency in terms of MIC values was exhibited by the selected bioactive plant extracts (cinnamon and green tea extracts) against the entire tested bacterial strains. MIC values of the extracts were in the following order: cinnamon (500µl/ml) > green tea (300 µl/ml) against the growth of *klebsiella pneumoniae* (Plate.3a, b). Whereas MIC values of the tested extracts against the growth of MRSA were cinnamon (300µl/ml) > green tea (200 µl/ml)(Plate3; c,d) . Roccaro et al. (2004) reported that MICs of green tea against *staphlococcus areus, Staphlococcus epidermis* and *Probionibacterium acnes* were 1.25, 0.625 and 1.25mg/ml respectively. A wide variation in the MIC (9.089-94.61 mg/ml) of tea extract against different bacterial strains; *S.typhimurium* (94.61 mg/ml) > *S. typhi* Ty2a (91.98mg/ml) > *S.typhi* (79.56 mg/ml) > *E. coli* (88.30 mg/ml)> *Y. enterocolitica* (47.30 mg/ml) > *S. dysenteriae*(9.09 mg/ml) . Variations were also demonstrated in the MICs of different tea extracts against *S. dysenteriae* in the order of : organic solvent green tea extract 3.3 mg/ml < boiled green tea extract 6.27 mg/ml < black tea extract 9.09 mg/ml (Tiwari et al., 2005).

MBC determination was based on the MIC , where the most promising plant extracts under test (cinnamon and green tea) showed bactericidal effect against *Klebsiella pneumonia* with MBC values of 500µl/ml and 300µl/ml, and MBC values of 300µl/ml and 200µl/ml with gram-positive (MRSA) respectively (Plates 3; a, b, c and d).

The time kill curve study showed that cinnamon and green tea exhibited a good and rapid bactericidal effect within 6-8 hrs for *Klebsiella pneumoniae* and within 2-4hrs for MRSA**.** (Fig 3 & 4). Masatomo & Kazuko (2004) reported that a time kill study analysis showed that the survival of resting cells decreased immediately and rapidly with catechins, and the survival rate was < 1% after 4 hrs. However, a few colonies still survived after 24 hrs of culturing.

In the present investigation, a comparative study was done to evaluate the antibacterial activity of the most promising bioactive extracts (cinnamon and green tea) against *Klebsiella pneumoniae* and MRSA versus commonly used antibiotics alone and in combined with the extracts. Seven antibiotic discs were selected**,** one was used for both gram-negative and gram-positive bacteria; ciprofloxacin (CIP).Two antibiotic discs for gram-negative bacteria were used: gentamycin (GM)and ciproflaxin(CIP). The discs used for gram-positive bacteria

Fig.1 Effect of some plant extracts on the growth of *Klebsiella pneumoniae*

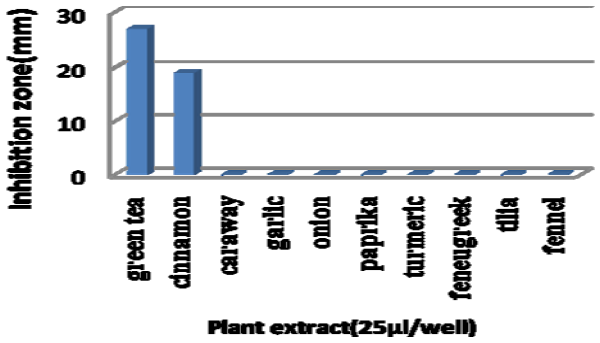

Fig.2 Effect of some plant extracts on the growth of *MRSA*

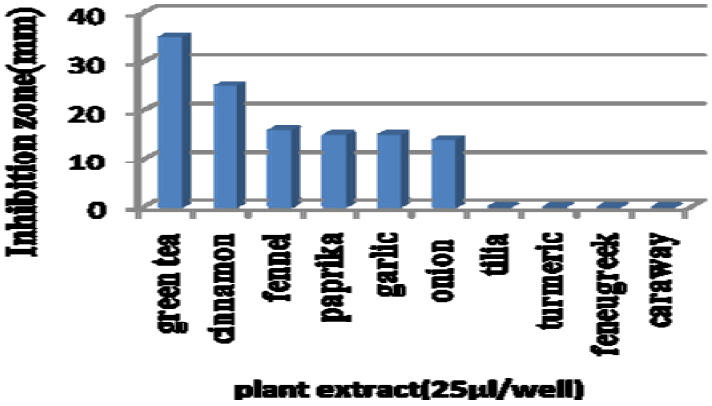

Plate.1 Well diffusion test of *Klebsiella pneumoniae* against :cinnamon extract(a), green tea extract(b), caraway extract(c), garlic extract(d), turmeric extract(e),fenugreek extract(f), fennel(g) , onion(h) , paprika(i) and tilia(j)

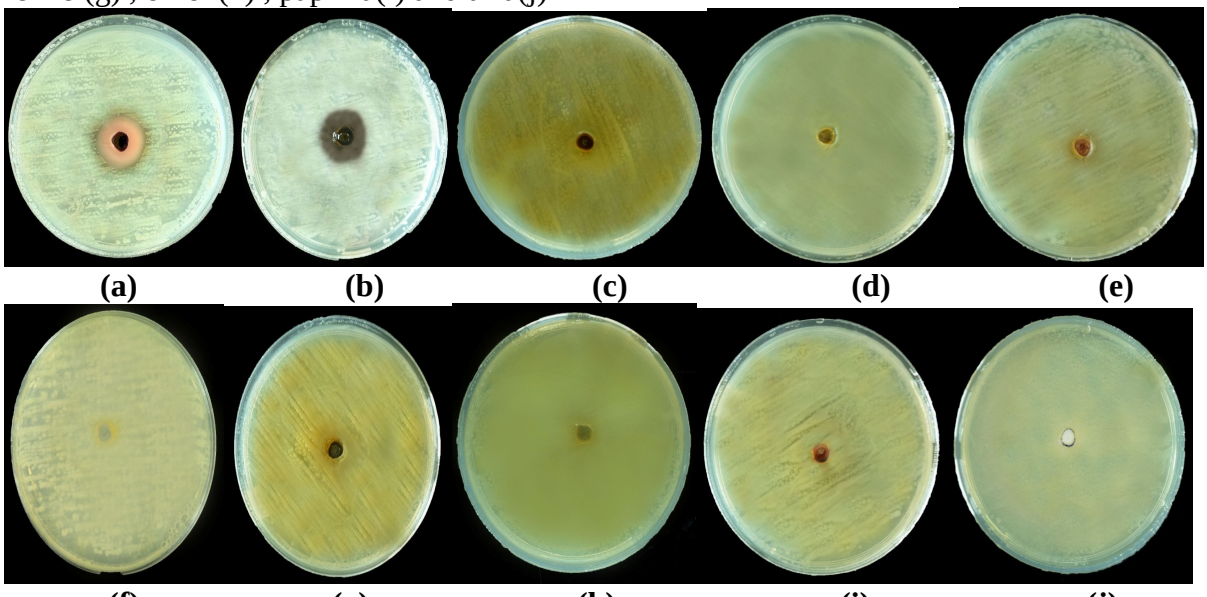

Plate.2 Well diffusion test of MRSA against : cinnamon extract(a), green tea extract(b), caraway extract(c), garlic extract(d), turmeric extract(e), fenugreek extract(f) , fennel(g) , onion(h), paprika(i) and tilia(j)

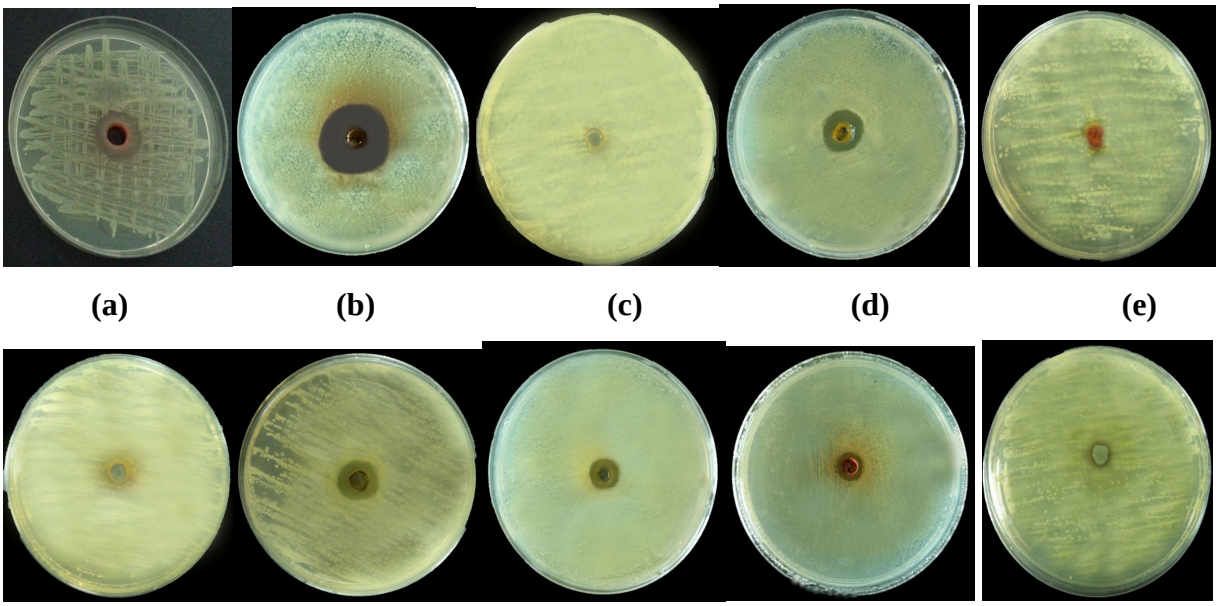

Plate.3 Minimum bactericidal concentration of cinnamon and green tea against *Klebsiella pneumoniae* (a,b) and *MRSA* (c,d): cinnamon(a,c) and green tea (b,d)

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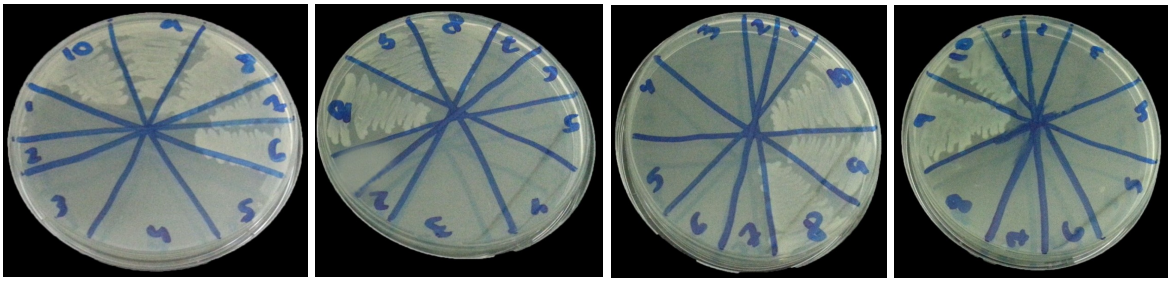


Fig.3 Time kill curve of *Klebsiella pneumoniae* treated with green tea and cinnamon

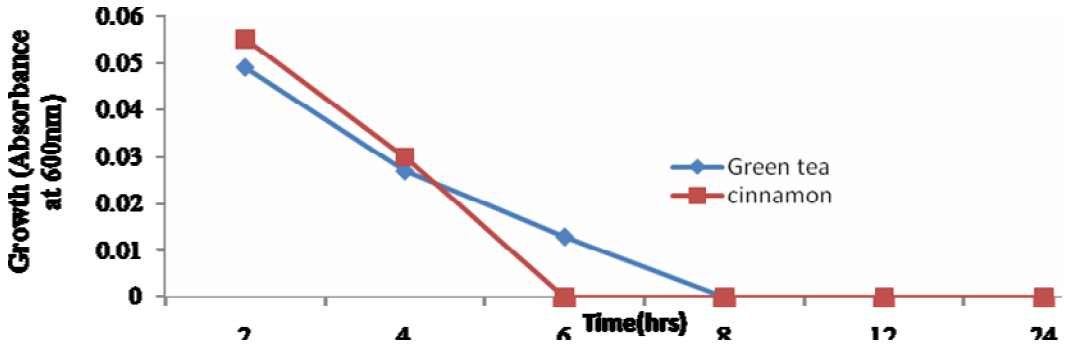


Fig.4 Time kill curve of MRSA treated with green tea and cinnamon

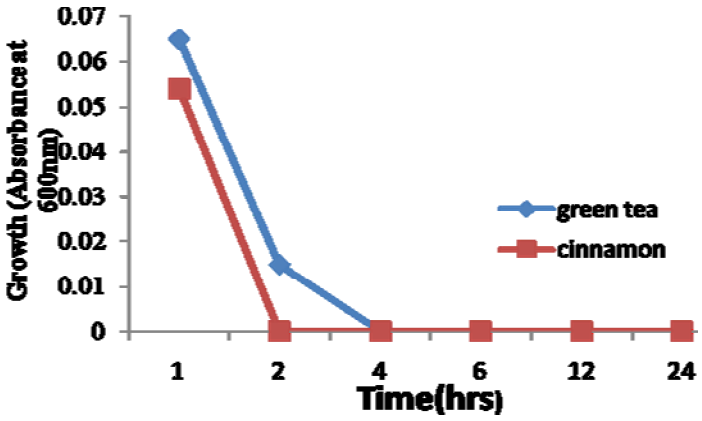


Fig.5 The effect of cinnamon, green tea and their combined action with Antibiotics against *Klebsiella pneumoniae*

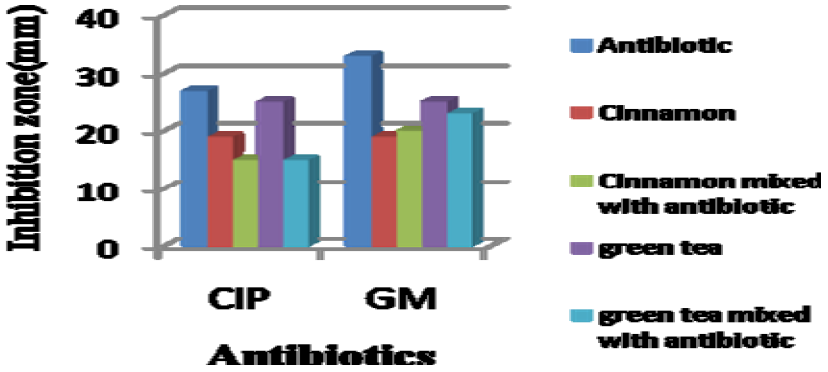


Fig.6 The effect of cinnamon, green tea and their combined action with antibiotics against *MRSA*

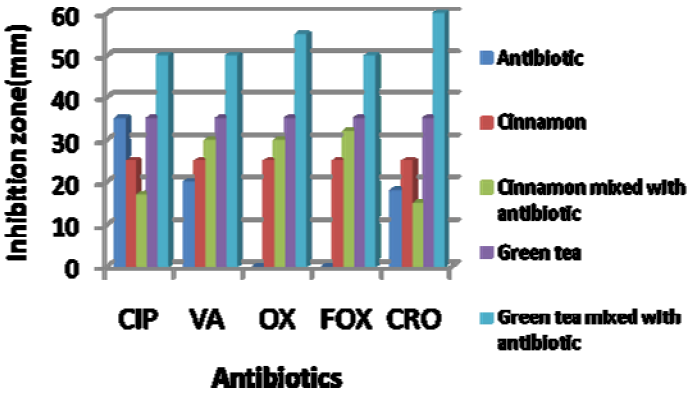
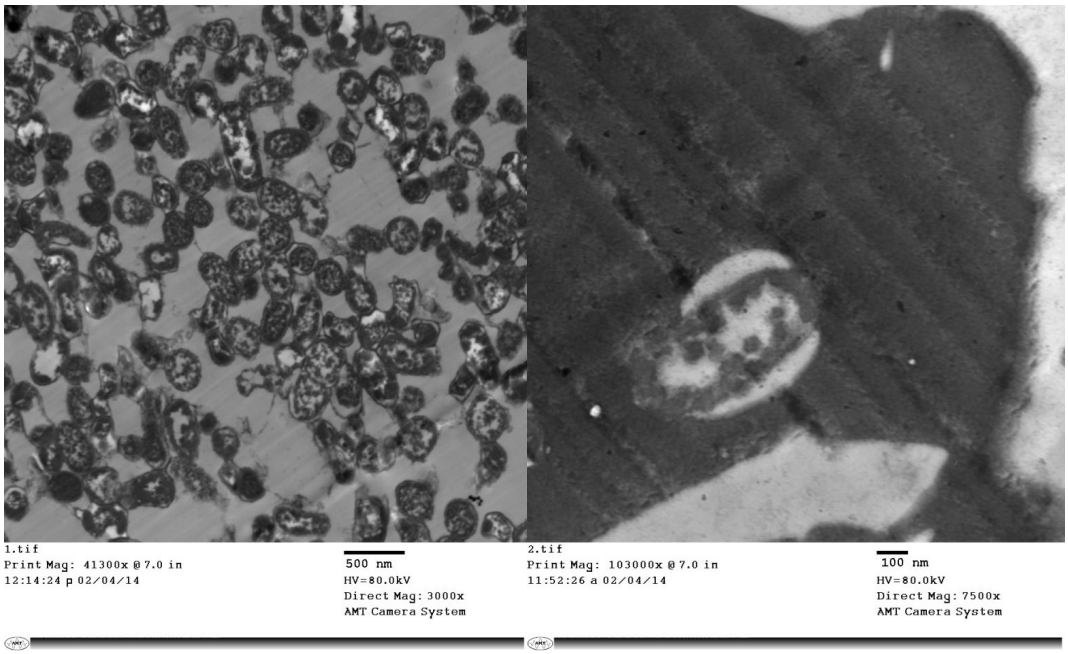
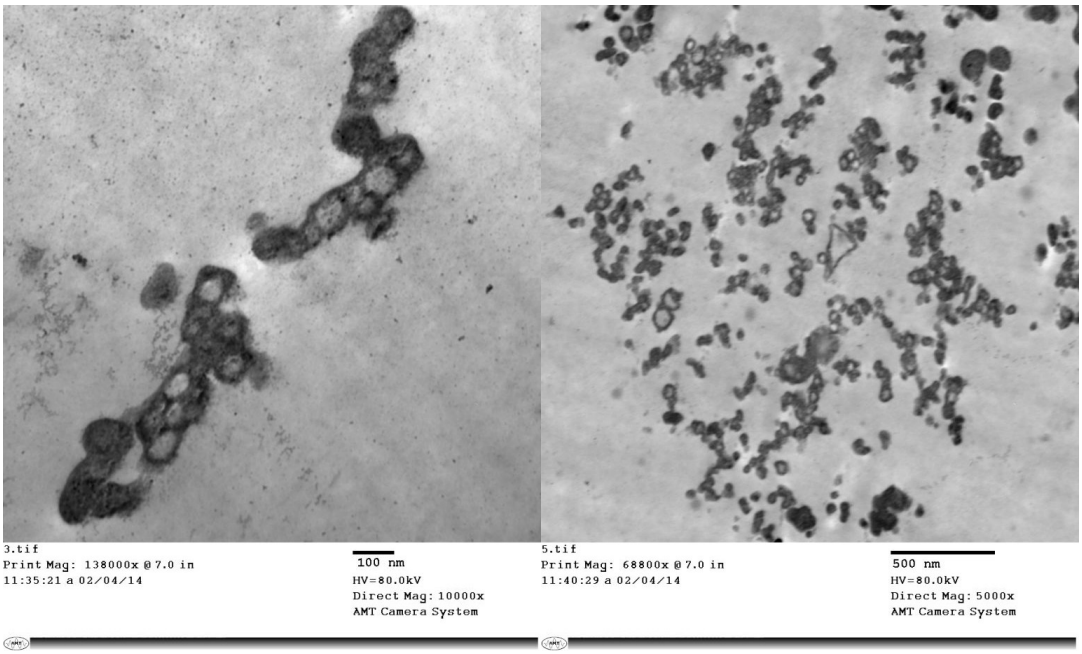


Fig.7 *Klebsiella pneumoniae* green tea treated cells (A) , Cinnamon treated cells(B)



A **B**

Fig.8 ***MRSA*** green tea treated cells (A) , Cinnamon treated cells(B)



A **B**

were cefoxitin (FOX), oxacillin, (OX), ceftriaxone (CRO) and vancomycin (VA) (Abd-El Aal et al., 2007).With respect to *Klebsiella pneumoniae* there was an antagonist effect (fig 5). On the other MRSA there was a highly synergistic increase due to the combination in all antibiotics, compared with inhibition zones of antibiotics alone except for CIP and CRO treated with cinnamon extract against MRSA. (fig 6) Shiota *et al.*(1999) ; Zhao *et al. (2001)* and Stapleton *et al.(2006)* reported that, the galloylated catechins (–)-epicatechin gallate (ECg) and (–)-epigallocatechin gallate (EGCg) found in green tea shared a capacity to reduce PBP2a-mediated oxacillin resistance in *S. aureus*, rendering genotypically resistant strains susceptible to *β*-lactam antibiotic action .The bioactive extracts were evaluated for qualitative determination of major phytoconstituents i.e. flavonoids, glycosides, phenols, saponins, steroids, tanins and terpenoids (Sibi et al.,2013). In this study, phytochemical analysis of cinamon extract revealed the prescence of glycosides, phenol, saponins,tanins and terpenoids and this was consistent with Shiney ramya & Ganesh (2012) findings . Phytochemical analysis of green tea extract explored that prescence of flavanoids, phenol,steroids and tanins as reported by Tariq & Reyaz (2012) findings. Shiney ramya & Ganesh (2012) reported that the presence of phytochemicals in spices has the bacteriostatic and bactericidal activity. Alschuler (1998) and Graham (1992) reported that the major antibacterial phytochemical components in green tea were polyphenols (catechins). Withrespect to cinnamon the major active phtochemical compounds were the terpenoid (eugenol)and the phenol (cinnamaldehyde) (Agaoglu et al., 2007).

Electron microscopy examination of the treated cells showed that the main targets of cinnamon and green tea extracts were cell wall and cell membrane of treated *Klebsiella pneumoniae* and MRSA, plasma membrane seemed to be irregular, dissociated from cell wall, invaginated and associated with the formation of abnormal structures (Fig,7(A&B) and 8(A&B) Cytoplasmic membranes and other membranous structures of organelles, such as nuclei and mitochondria, were also disrupted. Plasmolysis accompanied by an almost complete depletion and disorganization of cytoplasmic structures were found to be the final event which led to cell death.The marked effect of the extract components might have conferred lipophilic properties and the ability to penetrate the plasma membrane (Knobloch et al., 1989). An important characteristic of bioactive plant extracts and their compounds is their hydrophobicity, which enables them to break down the lipids of the bacterial cell membrane and disturbing the structures and rendering them more permeable (Knobloch et al., 1989 and Sikkema et al., 1995).

It was concluded that green tea and cinnamon extracts specifically and bioactive plant extracts in general may be potential sources of new and selective agents for the treatment of MRSA and *Klebsiella pneumoniae*. Further studies will be beneficial in providing data on the possible effects of these plant extracts if it is to be used as a relevant medical agent.

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