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RESEARCH ARTICLE

Antibacterial activity of *Citrus aurantium* leaf essential oil against *S.aureus* and MRSA

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ABSTRACT

Plants based antimicrobials effective in the treatment of infectious diseases and also devoid of side effects which associated with synthetic one. To evaluate antibacterial effect of *Citrus aurantium* leaf essential oil against *S.aureus* and MRSA along with SEM approach. Disc diffusion and Microbroth dilution method were used to determine antibacterial effect of *C. aurantium* leaf essential oil and Linezolid was used as standard. Surface morphology of EO treated *S.aureus* and MRSA was demonstrated by Scanning Electron Microscope. Disc diffusion method showed zone of inhibition were 18 ± 0.2, 23± 0.13 & 26 ± 0.1mm at the concentration of 0.25, 0.5, 0.75 µl/disc for *S.aureus* respectively. Linezolid showed 24±0.2 mm. Zone of inhibition were 14±0.3, 16±0.2, 20±0.1mm at the concentration of 0.25, 0.5, 0.75 µl/disc for MRSA respectively. Linezolid showed 21±0.2mm against MRSA. MIC was determined for VO and Linezolid against *S.aureus* and MRSA were found to be 4, 6 µg/ml and 2, 4 µg/ml respectively by micro broth dilution test. SEM observation showed appearance of blast like bleb structures on the surface, irregular spherical structures and eventual cell ruptures of the bacterial cell wall in other words surface destruction of the MRSA with intervening spaces have been resulted. *Citrus aurantium* leaf essential oil showed antimicrobial activity against *S.aureus* and MRSA.

Key words: *Citrus aurantium*, Essential oil (EO), Methicillin Resistant *Staphyllococcus auerus* (MRSA), Scanning Electron Microscope (SEM), Disc diffusion, Microdilution.

INTRODUCTION:

Growing antibiotic resistance is a global phenomenon in both developed and developing countries. Methicillin-resistant *Staphylococcus aureus* (MRSA) alone infects more than 94,000 people and kills nearly 19,000 in the US every year [1]. Resistance to antibiotic agent has resulted in treatment failures and enhancement in health care costs. Now there is only a little doubt that emerging antibiotic resistance is a serious global problem [2]. The association between increased rates of antimicrobial use along with the resistance has been documented for nosocomial infections and resistant community acquired infections [3, 4].

Only a few new antibacterial agents have received approval by the US Food and Drug Administration in the last 10 years [5]. Plant derived antimicrobials represent a vast untapped source for developing new lead molecules. Continued and further exploration of plant antibiotics needs to occur. Plants based antimicrobials have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [6].

The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment [7].

Citrus aurantium L commonly called as bitter orange. The leaves of *C.aurantium* really do not have any match as a cheap natural and easily available plant. It is traditionally known to be useful for the treatment of wide panel of diseases like stomach ache, vomiting etc [8]. Essential oil of the leaves used as antibacterial and antifungal [9], anxiolytic [10] and antiamoebic [11].

Mode of action of EOs towards several targets in the bacterial cell. The hydrophobicity of EOs enables them to partition in the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents [12]. Depending on type and

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concentration, they exhibit cytotoxic effects on living cells but are usually non-genotoxic [13].

Citrus oils not only lend themselves to use in food but also are generally recognized as safe (GRAS) and have been found to be inhibitory both in direct oil and vapour form against a range of both Gram-positive and Gram-negative bacteria. This group of oils may provide the natural antimicrobials that the food industry requires to fulfill both its requirements and those of the consumer [14, 15].

Antibacterial activity of *Citrus aurantium* leaf [9, 16], peel [17] and seed [18] EO against *S.aureus* were previously reported. Antimicrobial activity of Tunisian variety against both *S.aureus and* MRSA was reported earlier [19]. Chemical composition of Tunisian variety different from Indian variety. The reasons for this variability can be understood if we take into account all the factors influencing the chemical composition of the oils, namely, climatic, seasonal and geographic conditions, harvest period and distillation technique, among others [20].

We previously reported EO composition of Indian variety [21]. No studies available Indian variety leaf essential oil against MRSA. In this present study mainly focused antibacterial activity of *Citrus aurantium* leaf oil against both *S.aureus and* MRSA along with SEM approach.

MATERIALS AND METHODS:

Chemicals: Cartridges containing paper discs (HiMedia), Mueller-Hinton Agar Medium, Barium sulphate, Sulphuric acid, 0.5McFarland standard and Luria-Bertani10 (LB10) medium were used in this study.

S.aureus (MTCC strain no.260) and MRSA (ATCC 33591) were obtained from Hi-media Pvt Ltd, Mumbai.

Instrument: UV spectroscopy (UV1800 Shimadzu, Japan), GC-2010 Shimadzu capillary gas chromatography directly coupled to the mass spectrometer system (GC-MS, model QP 2010) and Hitachi Scanning electron Microscope 3000H model were used in this experiment.

Plant collection and authentication:

The leaves of *Citrus aurantium* L. selected for our study was collected from Chinthamani, Villupuram District, Tamil Nadu, India during the month of January and was authenticated by Dr. P. Jayaraman, Director of Plant Anatomy Research Institute, Tambaram, Chennai, Tamil Nadu, India.

Extraction of essential oil:

The fresh leaves of *C.aurantium* were hydro distilled using Clevenger apparatus for 3 hours with an average yield of 0.4% (w/v). The oil obtained was dried over anhydrous Sodium sulphate and stored in closed glass vials at 4°C until analysis.

Gas chromatography – Mass spectrometry analysis (GC-MS) and evaluation of isolated Volatile oil:

Procedure for GC-MS analysis and organoleptic evaluation of *C.aurantium* leaf oil were previously reported [21].

Preparation of MH Agar:

It includes the following steps:

1. It was prepared from commercially available (HiMedia) dehydrated base according to the manufacturer's instructions.
2. It was allowed to cool in a 45-50°C water bath. The freshly prepared cooled medium was poured into glass flat bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30ml for plates with a diameter of 100mm.
3. This medium was allowed to cool to room temperature and if it was not used in the same day, stored in a refrigerator (2°C to 8°C) and used within 7days and wrapping in plastic was undertaken to minimize drying of the agar.
4. Sample of each batch of plates was examined for sterility by incubating at 30-35° C for 24hrs or longer.

We used formulations that have been tested according to and that meet the acceptance limits described in NCCLS document M62-A7- protocols for evaluating Dehydrated Muller-Hinton Agar.

Turbidity standard for inoculum preparation:

To standardize the inoculum density for the susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland Standard was prepared.

1. 0.5ml aliquot of 0.048ml/L $BaCl_2$ (1.175%w/v $BaCl_2.2H_2O$) was added to 99.5ml of 0.18 ml/L $H2SO_4$ (1%v/v) with constant stirring to maintain a suspension.
2. The correct density of the turbidity standard was verified by using a spectrophotometer (Shimadzu UV1800). The absorbance at 625nm was adjusted to be 0.008 to 0.01 for the 0.5McFarland standard.
3. Barium sulphate suspension was transferred into 4 to 6 ml aliquots into screw cap tubes of the same size as those used in growing or diluting bacterial inoculum tightly sealed and stored in the dark at room temperature.
4. It is vigorously agitated on mechanical vortex mixer before each use and inspected for a uniformly turbid appearance.

Disc diffusion method:

The Kirby-Bauer and Stokes' methods are usually used for AST. Kirby-Bauer method recommended by NCCLS is usually recommended. The accuracy and reproducibility

of this test are dependent on maintaining a standard set of procedure described here. NCCLS is approved by FDA-USA and recommended by WHO.

PROCEDURE:

Inoculum preparation:

Growth method: At least 3 to 5 isolated colonies of MTCC No.260 strain of *S.aureus* were selected from an agar plate culture. The top of each colony is touched with a loop and the growth is transferred in to a tube containing 4 to 5ml of broth medium. Broth culture was incubated at 35°C until it achieves turbidity of the 0.5 McFarland standards (usually 2 to 6hrs). The turbidity of the actively growing culture was adjusted with sterile saline or broth to obtain a turbidity optically compared to that of the 0.5McFarland standard. This results in a suspension containing approximately 1 to 2 × 108 CFU/ml for *E.Coli* ATCC 25922. To perform this step properly, visually adequate light is needed to compare the inoculum tube and the 0.5 McFarland standards against a card with a white background and contrasting black lines.

Inoculation of Test Plates:

1. Optimally, within 15mts after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculums from the swab.
2. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60°C each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

Application of Discs to inoculated Agar plates:

The predetermined battery of antimicrobial discs was dispensed onto the surface of the inoculated plates distributed evenly so that they are no closer than 24mm from centre to centre. Each disc was pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set to 35^{o} C within 15mts after the discs were applied.

Reading Plates and Interpreting Results:

After 24 hours (16 to 18 for some organisms) of incubation, each plate was examined for resulting zones of inhibition uniformly circular and confluent lawn of growth. The diameters of the zones of complete inhibition as judged by unaided eye were measured, including the diameter of the disc, using ruler held on the back of the inverted Petri plate, to the nearest whole millimeters. Transmitted light (plate held up to light) was used to examine the zones for light growth of methicillin resistant colonies, within apparent zones of inhibition.

Dilution Method:

Dilution susceptibility testing methods are used to determine the MIC (Minimum inhibitory concentration-The minimal concentration of antimicrobial to inhibit or kill the microorganism) this can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials were tested in log_2 serial dilutions (two fold).

Procedure:

Sterile graduated pipettes of 10, 5, 2 and 1ml sterile capped 7.5 x 1.3 cm tubes /small screw capped bottles, Pasteur pipettes overnight broth culture of *S.aureus*, antibiotic powder form and a suitable rack to hold tubes, DMSO sterile tubes were arranged in rack in two rows. 2ml of broth containing the specified quantity of VO was transferred using a pipette to the first tube in each row. Using a fresh pipette 4ml of broth was added and mixed well and transfer 2ml to the second tube in each row and dilution was continued. 2ml Control broth containing vehicle without test drug or standard drug was placed in the last tube in each row. Inoculation was done with one drop of an overnight broth culture of the test organism (10^{6}organism/ml) and then incubated for 24 hr at 37^{o}C. A tube containing 2ml broth with the organism was kept at 4^{o}C in a refrigerator overnight to be used as standard for the determination of complete inhibition.

Micro-broth dilution test:

MTCC strain No. 260 of *S.aureus* was cultured in Luria-Bertani10 (LB10) medium (10g bactotryptone, 5g yeast extract, 0g NaCl per litre) at 37^{o}C for 24 hr. The full growth culture was diluted to 1/1000 in Mueller-Hinton (MH) medium and then a 100µl aliquot was added to each well 96 well plates. VO of the leaf of *C.aurantium* was serially diluted 2 fold and 100µl of each dilution was added to the bacteria solution and cultured at 37 ^{o} C for 24hr. Bacterial growth was visually determined. The lowest concentration showing inhibition of growth was considered the MIC. Same test was carried out for the standard Linezolid.

Reading of the result:

MIC is expressed as the highest dilution which inhibited growth judged by the lack of turbidity in the tube. As very faint turbidity may be given by the inoculum itself, the inoculated plate kept in the refrigerator overnight was used as the standard for the determination of complete inhibition. Control run with the test was used as the control to check the reagents and conditions.

Surface morphological changes of VO treated *S.aureus* using SEM:

Scanning electron microscopy is a complementary technique and importance in analyzing surface character of the sample [22].

SEM sample preparation:

Sample for SEM analysis were mounted on the specimen stub using carbon adhesive sheet. Small sample were mounted with I sq. cm glass slide And kept in carbon adhesive sheet. Samples were coated with gold to a thickness of 100AO using Hitachi vacuum evaporator. Coated sample were analysed in a Hitachi Scanning electron Microscope 3000 H model.

RESULTS AND DISCUSSION:

The yield of essential oil from these leaves was 0.4% w/v through hydro distillation. The oil was greenish yellow with aromatic odour and has pungent taste with refractive index of 1.4570. The yield of the oil depends upon the type, situation of the place in the stem, time of collection, method of isolation, nature of the material distilled, fresh or dried, bleached or unbleached etc.

GC-MS analysis of isolated Volatile oil:

The isolated VO was subjected to GC-MS analysis showed 35 peaks. The important known major constituents were α-Pinene, β-Pinene, Sabinene, β-Myrcene, O-Cymene, D-Limonene, Eucalyptol (1, 8 Cineole), β Linalool, 4 terpineol, α Terpineol etc [21].

Some variations were observed in the composition and percentage of constituents in the essential oil when compared to previous studies. This is may be due to climate change, soil, altitude and other conditions.

Antimicrobial susceptibility test:

Agar disc diffusion technique is frequently used to screen plant extracts and volatile oils for antibacterial activity [23]. This method is limited to the generation of preliminary, qualitative data only, as the hydrophobic nature of most essential oils and plant extracts prevents the uniform diffusion of these substances through the agar medium [24]. Agar and broth dilution methods are also commonly used [25].

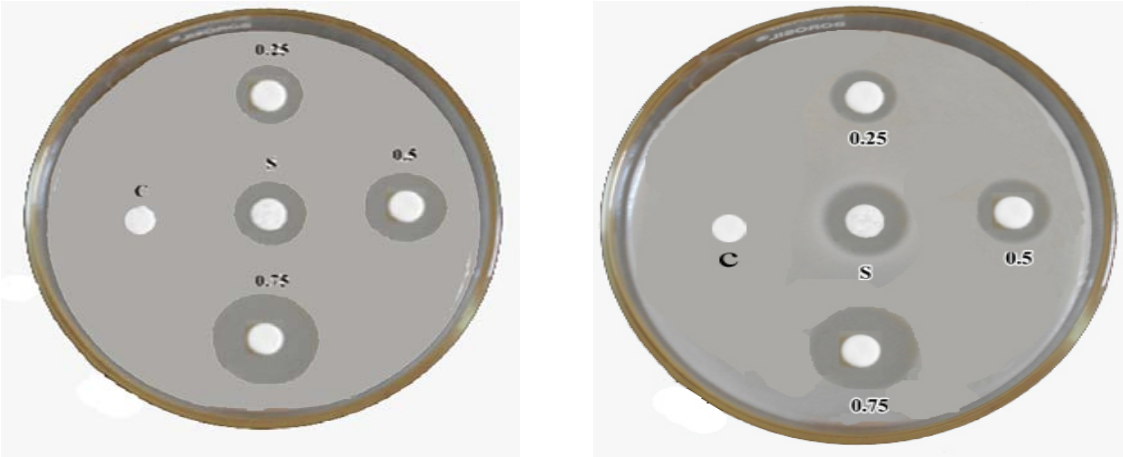
Disc diffusion method:

Screening of volatile oil of the leaves of *C.aurantium* was carried out to determine the antibacterial activity against the pathogenic organism of both *S.aureus* and MRSA by Disc diffusion method (Table 1 & Fig. 1). Zone of inhibition were 18 ± 0.2, 23± 0.13 & 26 ± 0.1mm at the concentration of 0.25, 0.5 & 0.75 µl/disc for *S.aureus* respectively. Linezolid was used as standard, showed 24±0.2mm. Zone of inhibition were 14±0.3, 16±0.2 & 20±0.1mm at the concentration of 0.25, 0.5 & 0.75 µl/disc for MRSA respectively. Linezolid showed 21±0.2mm against MRSA.

For both *S.aureus* and MRSA, 0.5 µl/disc volatile oil showed similar zone of inhibition as compared to linezolid. The results were encouraging that it is highly effective than that of the standard drug linezolid which is effective than vancomycin in MRSA infection both orally and parentally.

Table 1: Antibacterial activity of VO of leaf of *C.aurantium* against *S.aureus* and MRSA

Organism	Conc	Zone of inhibition
S.aureus	0.25	18 ± 0.2
	0.5	23± 0.13
	0.75	26 ± 0.1
	STD	24±0.2
MRSA	0.25	14±0.3
	0.5	16±0.2
	0.75	20±0.1
	STD	21±0.2



S.aureus MRSA

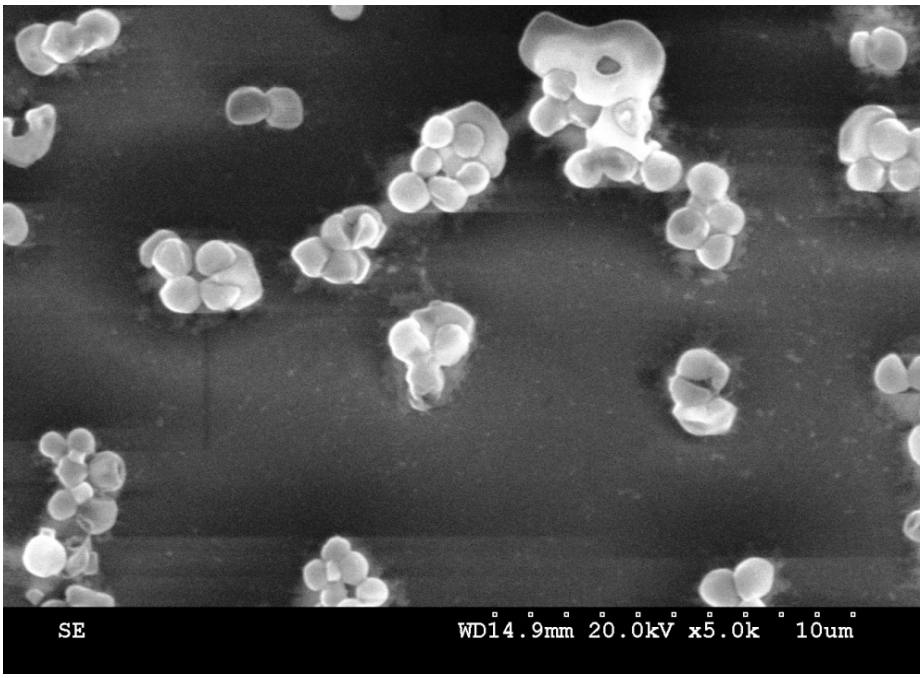
Figure 1: Antibacterial activity of volatile oil of leaf of *Citrus aurantium* on *S.aureus* and MRSA

Minimum inhibitory concentration by Microdilution method:

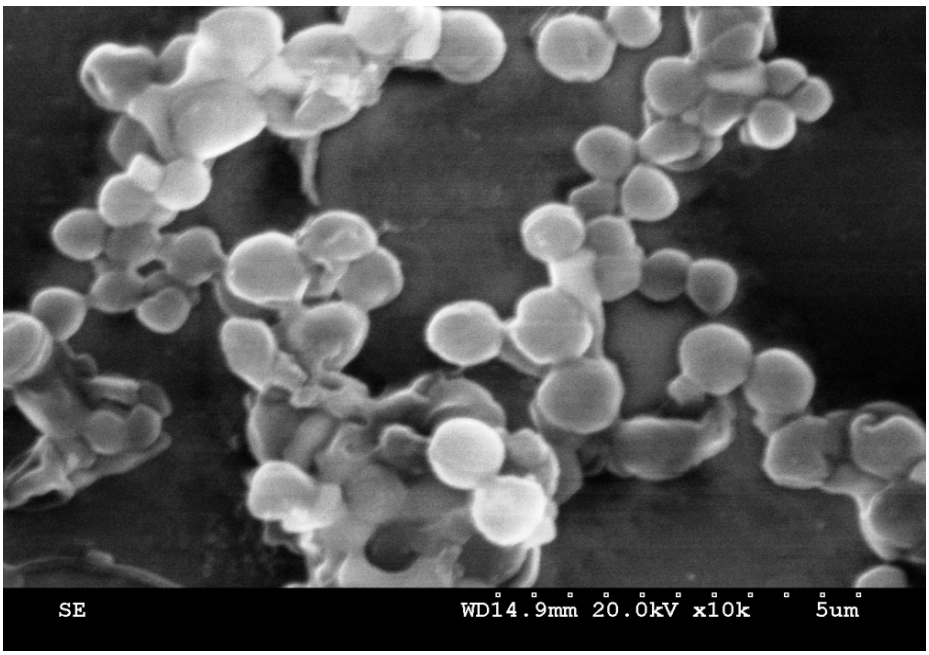
- MIC was determined for VO against *S.aureus* and MRSA.
- It was 4µg/ml and 6µg/ml respectively.
- MIC for the standard drug Linezolid was 2, 4µg/ml respectively.

Observation VO induced alterations on the surface morphology of *S.aureus* and MRSA under SEM:

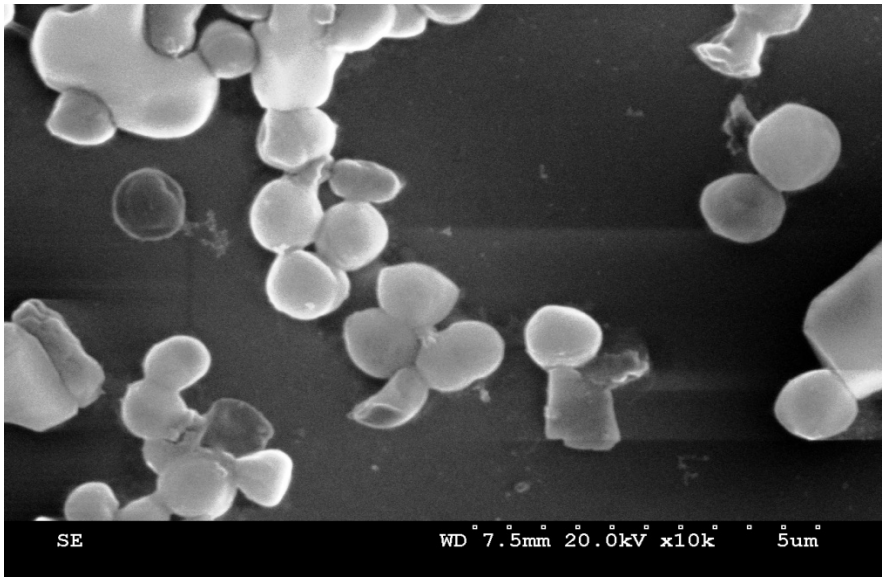
SEM mainly used for observe effect of the VO on the surface morphology of *S.aureus* and MRSA (Fig. 2). The VO has different levels of activity against bacteria as measured by the minimum inhibitory concentration. Although the mode of action of VO is unclear, the SEM examination showed that it had some activity on the cell surface that resulted in morphologic abnormalities. The result suggests biochemical activities occurring in several layers of the cell wall. Appearance of blast like bleb structures on the surface, irregular spherical structures and eventual cell ruptures of the bacterial cell wall in other words surface destruction of the MRSA with intervening spaces have been resulted.



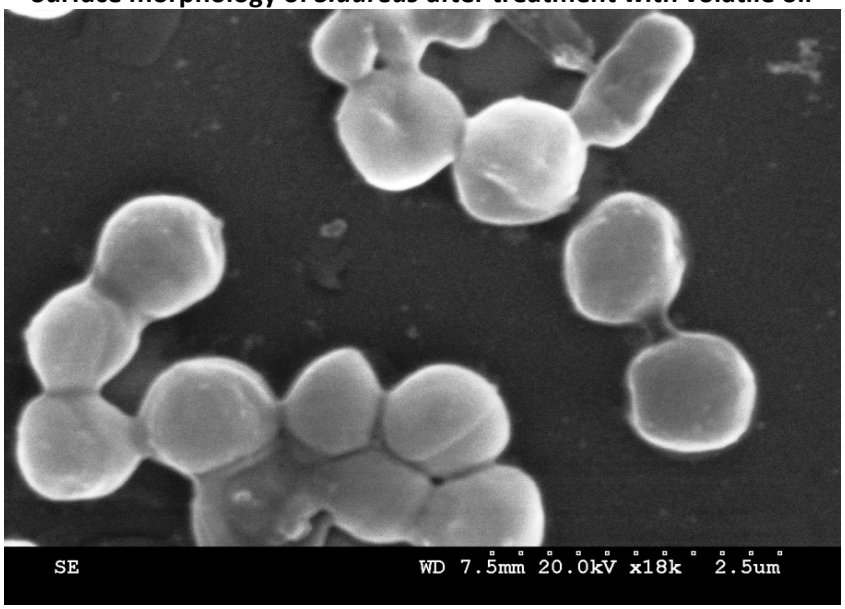
Untreated *S.aureus*



Untreated MRSA



Surface morphology of *S.aureus* after treatment with volatile oil



Surface morphology of MRSA after treatment with volatile oil

Figure 2: Surface morphology of *S.aureus* and MRSA (untreated & treated)

CONCLUSION:

The leaf essential oil of *C.aurantium* proved to be effective against both *S.aureus* and MRSA with resistance modifying property and stimulation of innate immunity. Its medicinal application are still to be explored well so as to minimize the menacing wastage and to maximize the revenue generated by this crop to boost up our national economy as well as the proper exploitation of the plant for the therapeutic purposes. By investigating its bioactivity of its essential oil we can meet the situation of unsettling facts of modern pharmaceutical industry which facing lately it pipeline of new drug discovery seems to be almost empty. Further *in vivo* screening recommended confirming antibacterial activity.

Conflict of interest statement: We declare that we have no conflict of interest.

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