

Original Research Article

Comparative Evaluation of Antibacterial Efficacy of Cannabis Sativa, Allium Sativum, Allium Ceba, Thuja Orientalis and Psidium Guajava against Drug Resistance Pathogens

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ABSTRACT

Background: The increase in isolates of *S. aureus* with resistance to methicillin and decreased susceptibility to vancomycin has created an urgent and consideration need for the development of new anti-staphylococcal agents that kill the resistant mutants. So, the identification of an alternative and safer drug for the control of MRSA is necessary to combat this worldwide problem. Nature is the only source to provide a good variety of chemical compounds that can be used for new drug discovery especially from mineral, plant and animal products.

Methods: Clinical strains were isolated from hospitalized patients in Rama Medical College Hospital and Research Centre Mandhana, Kanpur. The ethanolic extracts of plants Cannabis sativa, Allium sativum, Allium cepa, Thuja orientalis and Psidium guajava was taken, of which 1g was prepared with 5% and 10% concentration respectively. The antimicrobial properties of the extracts were determined through disk diffusion method.

Results: The sensitivity of bacteria was detected based on the disk diffusion method, the zone of inhibition was obtained for VRSA (20mm), VISA (19mm), MRcons (32mm), MRSA (20mm), MSSA (22mm) and ATCCS.aureus25923 (23mm) at different concentration of the plant extract. The highest inhibition zone of plant extract was observed in Cannabis sativa followed by Allium sativum then Allium cepa, Psidium guajava and lastly by Thuja orientalis. It was also observed that the zone of diameter increased with the increase of concentration.

Conclusion: The plants included in this study showed mild to moderate anti-microbial activity. This further concluded that these plants can be used as a potent agent for the treatment of MDR infections.

Key Words: Antibiotic resistance, plant extracts, MDR infections

1. INTRODUCTION

Staphylococcus aureus is one of the prominent medically important bacterial pathogen. Its potential to cause wide spectrum of pyogenic lesions involving several organs, hospital outbreaks and community acquired infections are well recognized. *S. aureus* infections are often fatal in nature and are associated resistance to several

beta-lactam antibiotics used in hospitals. ^[1] These strains are known as MRSA (methicillin resistant *S. aureus*). ^[2] They are Gram positive, non spore forming bacteria, spherical in shape, aerobic or facultatively anaerobes. MRSA can take hold in human tissues and eventually become resistant to treatment. Historically, it had drawn special attention since 1970 due to its association

with several nosocomial outbreaks and cross infections. [3] The epidemiology of this organism has changed over years. Life-threatening infections which were limited only in hospitals are now becoming widespread in community. [4]

The initial presentation of MRSA is small red bumps that resemble pimples, spider bites and they may be accompanied by the fever and occasionally. Recently the pathogen has developed resistance towards Methicillin also. High usage of antibiotics in hospitals and selection pressure of these antibiotics has been implicated in development of multidrug resistance (MDR) in hospital acquired MRSA (HA-MRSA) strains. The glycopeptide vancomycin was considered to be the best alternative for the treatment of the multi drug resistant MRSA. [5] However, there are increasing numbers of reports which are indicating the emergence of vancomycin-resistant *S. aureus* (VRSA) strains. Therefore, keeping in view the side effects reported from the use of fourth generation antibiotics scientists are trying to explore the plant products as potential candidates for treating these drug resistant (ESBL) strains of *S. aureus*.

Phytochemicals have great potential as antimicrobial compounds and have been proven to have great therapeutic potential. [6] The control of major diseases by synthetic products is decreasing there is increased interest in the revival of herbal medicines leading to the current widespread belief that the “green medicine” is safe, more accessible and also more affordable than the costly synthetic drugs, many of which have the adverse side effects. [7]

2. MATERIALS AND METHODS

Identification:

a) Study area, sampling, and sample processing

It is a cross sectional case control study. The study was carried out in the Microbiology Laboratory of the Department of Microbiology, Rama Medical College Hospital and Research

Centre, Mandhana, Kanpur during June to November 2016. Clinical specimens [urine, pus, blood, sputum and throat swab etc] was collected aseptically with a sterile cotton swab. The samples was inoculated on the Mannitol salt agar (MSA) (HiMedia, India) plates immediately after sample collection for isolation of *S. aureus* and transported to the laboratory as early as possible.

b) Isolation and identification of *Staphylococcus aureus*

All MSA plates were incubated for 24 hours at 37°C. After incubation, isolated colonies suspected to be *Staphylococcus* were allowed to grow on nutrient agar plates (HiMedia, India) and then identified microscopically, biochemically according to the standard guidelines. [8,9]

For microscopic observation, a pure colony was selected and subjected to Gram staining. Then the shape, arrangement, and Gram reactions of the isolates was observed under a light microscope. [10] Required confirmatory biochemical tests including catalase and triple sugar iron agar tests was also performed to identify suspected *S. aureus* following standard protocols. [10]

Biochemical Identification of *S. aureus*

a) By Gram Staining: 1.) Crystal violet-1min, 2.) Grams iodine-1min, 3.) acetone/alcohol-30sec, 4.) Saffarine-30sec

b) Catalase Test: using 3 % H₂O₂

c) Coagulase test: Can be done by using two methods:

a) **Tube Coagulase Test:** About 0.1ml of a young broth culture or agar suspension of the isolate is added to 0.5 ml of human or rabbit plasma in a narrow test tube. EDTA, oxylate or heparin may be used as the anticoagulant for preparing plasma. Positive and Negative controls are also set up. The tubes are incubated in water bath at 37°C for 3-6 hours. If positive, the plasma clots and does not flow when the tube is tilted.

- b) **Slide Test:** The isolate is emulsified in a drop of saline on a slide. After checking for absence of auto agglutination, a drop of human or rabbit plasma is added to the emulsion and mixed. Prompt clumping of the cocci indicated a positive test. Positive and Negative controls are also set up.
- c) **Hemolytic activity:** The hemolytic activity of *S. aureus* isolates will be tested using blood agar plates containing 5% defibrinated sheep blood. An isolated colony from a nutrient agar (NA) plate will be inoculated on blood agar and incubated at 37°C for 24 hours. The hemolytic zones will be characterized as alpha (partial hemolysis), beta (complete hemolysis), and gamma (no hemolysis) depending on the extent of each colony. [10]

Assay of antibacterial susceptibility

A standard agar-disc diffusion (Kirby-Bauer) assay using Mueller-Hinton agar (MHA) (HiMedia, India) plates will be conducted to determine the susceptibility of the isolated *S. aureus* to different antibiotics. [11-13] A suspension of the test organism will be prepared by adjusting the turbidity of the broth in a phosphate buffer saline by comparing it with that of the McFarland standard solution of 0.5. [12,13] By means of a sterile cotton swab, a uniform lawn of bacterial growth will be prepared on the MHA plates. Antibiotic disc including Ciprofloxacin (CIP) (30µg), Tobramycin (TOB) (10µg), Cotrimoxazole (COT) (25µg), Penicillin (P) (10µg), Imipenem (IPM) (10µg), Cefoxitin (CX) (30µg), Amikacin (AK) (30µg), Netilmicin sulphate (NET) (30 µg), Oxacillin (OX) (1µg), Erythromycin (E) (15µg), Clindamycin (CD) (2µg), Azithromycin (AZM) (15µg), Gentamicin (GEN) (10µg), Teicoplanin (TEI) (30µg), Linezolid (LZ) (30µg), Tetracycline (TE) (30µg), Vancomycin (VA)(30µg), Norfloxacin (NX) (10µg), Nitrofurantoin (NIT) (300µg), Ofloxacin (OF) (5µg), Chloramphenicol (C) (30µg) will be applied aseptically on the surface of

the inoculated plates in an appropriate spatial arrangement using a sterile needle.

The plates will be incubated at 37°C for 24 hours and examined for zones of inhibition (mm). [11,14]

Optimum temperature required for the Antibiotic Cefoxitin is at 37°C and Oxacillin at 35°C. If large doses of vancomycin might still be able to kill the *S. aureus*, it is called Vancomycin-intermediate *S. aureus* (VISA). If no amount of vancomycin will kill the *S. aureus*, it is called Resistance to Vancomycin (VRSA).

Cefoxitin and Oxacillin if not resistance: MSSA

Cefoxitin and Oxacillin if resistance: MRSA

Vancomycin if resistance: VRSA

Vancomycin large doses if able to kill: VISA

Identification of MRSA

For the detection of MRSA, oxacillin (1µg) and cefoxitin (30µg) will be introduced on the MHA plates against the growth of *S. aureus*. For this purpose, a bacterial suspension will be prepared in sterile saline by selecting colonies produced by the overnight incubation on Nutrient agar plates. After 5-7 hours of incubation, the cell turbidity will be adjusted to 0.5 McFarland standards. [12,13] Subsequently, the suspensions will be inoculated onto MHA plates and the antibiotic discs will then be placed onto the plates. [9,15] All plates will be incubated for 24 hours at 35°C and 37°C to observe for oxacillin and cefoxitin resistant *S. aureus*.

Identification of VRSA through disc diffusion methods

MHA plates will be inoculated with the bacterial suspension which was previously adjusted to 0.5 McFarland standards. Afterward, a 30 µg vancomycin disc and a blank disc as a control will be aseptically placed over the surface of the MHA plates at a distance of 5mm to observe the range of the zone diameter for the detection of strains of VRSA. [9,15]

Determination of vancomycin resistance by minimum inhibitory concentration test

The minimum inhibitory concentration (MIC) of vancomycin will be determined by the tube dilution method. [16-19] Muller-Hinton Broth will be prepared with 2-128 µg/mL of vancomycin. By using a direct colony suspension method, 0.5 McFarland equivalent bacterial inoculums will be prepared in normal saline after culturing for 24 hours on an agar plate. The suspension was further diluted to achieve the desired inoculum concentration. If it is ≤ 2 µg/mL then it is Sensitive, if it is ≥ 4 -8 µg/mL then it is Intermediate and if it is ≥ 16 µg/mL then it is Resistance, according to the CLSI guidelines 2016. All strains will be spotted onto Muller-Hinton plates containing different concentrations of vancomycin. The plates will be incubated for 24 hours at 37°C and checked for any visible growth. [11]

Plant material

The plant leaves of Cannabis sativa followed by Allium sativum, Allium cepa, Psidium guajava and Thuja orientalis were collected from Botanical garden of Rama Medical College Hospital and Research Centre, Mandhana, Kanpur.

Preparation of the plant extract

Extraction procedure

The plant leaves of Cannabis sativa followed by Allium sativum bulbs, Allium cepa, Psidium guajava and Thuja orientalis was washed thoroughly and then shade dried in room temperature for 4 days, finely powdered and stored in a sterile airtight container for further use. Now 1 g of

powder was dissolved in 10 ml of ethanol in a conical flask. The flask was Plugged with cotton and kept on a rotary shaker at 250 rpm for three days.

After three days filtered with the help of Whatman No.1 filter paper and the solvent was evaporated carefully at low temperature until reach its maximum concentration (Harbone et al., 1973) [20] supposed final concentration as 10% and it diluted in respected solution to form concentration as 5% to test their antimicrobial properties.

Screening for antimicrobial activity

The antimicrobial activities were screened on MHA media by using disc diffusion method (Bauer et al., 1996, Andrews's et al., 2001). [21] Taken loopful bacterial culture from peptone and Swabbing on MHA agar plate with the help of sterile swab stick. Marked the swabbed plate with marker as 10% and then 5%. Himedia discs (6 mm in diameter) were impregnated with respective 25µl of the respected percentage solution of the plants extract and then placed on the swabbed MHA plates. Meanwhile, 25µl of the Ethanol was also pipetted onto the Himedia discs a control.

The plates were kept at 4°C for 1 hr for diffusion of extract, thereafter were incubated at 37°C for 24 hrs. Antibacterial activities were observed by zone of inhibition in millimeter.

3. RESULTS

The results were presented using tables and figures.

TABLE 1(Refer FIG 1) Details of the Test Isolates:

S.NO.	PATIENT'S NAME	ID NUMBER	SEX	AGE	CLINICAL SAMPLE	TYPE ISOLATED
1.	A1	712810	F	68yrs	Pus	VISA
2.	A2	821900	M	18yrs	Pus	VISA
3.	A3	766811	F	16yrs	Pus	MRCns
4.	A4	755190	F	18yrs	Pus	VRSA
5.	A5	713258	M	34yrs	Blood	MRSA
6.	A6	708984	F	36yrs	ET tube	MRSA
7.	A7	613242	F	1yr	Blood	MRSA
8.	A8	608109	M	20yrs	Pus	MSSA
9.	A9	614630	M	55yrs	Pus	MSSA
10.	A10	609180	F	25yrs	Pus	MSSA
11.	ATCC	25923				S.aureus

TABLE 2(Refer FIG 6(A) and Refer FIG 6(B)) Preliminary Screening of Ethanolic plant extracts with Disc Diffusion Assay using 5% and 10% Concentration:

S.NO	PATIENT'S NAME	TYPE ISOLATED	CLINICAL SAMPLE	PLANT EXTRACT	ZONE OF INHIBITION (IN mm) (For 5% Plant Extract Concentration)	ZONE OF INHIBITION (IN mm) (For 10% Plant Extract Concentration)
1.	A1	VISA	Pus	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	7mm 6mm 12mm 6mm 6mm	10mm 7mm 14mm 12mm 7mm
2.	A2	VISA	Pus	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	7mm 11mm 18mm 10.5mm 10mm	13mm 12mm 19mm 15mm 10mm
3.	A3	MRCons	Pus	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	7.5mm 6mm 17mm 11mm 8mm	13mm 8mm 32mm 18mm 12mm
4.	A4	VRSA	Pus	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	7mm 6mm 12.5mm 7mm 6mm	10mm 10mm 20mm 10mm 7mm
5.	A5	MRSA	Blood	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	6.5mm 6mm 14mm 8mm 6.5mm	10mm 7mm 14mm 10mm 7mm
6.	A6	MRSA	ET tube	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	8mm 6mm 11mm 7mm 7mm	10mm 7mm 20mm 14mm 7mm
7.	A7	MRSA	Blood	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	7mm 7mm 13mm 8mm 7mm	7mm 7mm 19mm 10mm 7mm
8.	A8	MSSA	Pus	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	7.5mm 7mm 20mm 8mm 7mm	14mm 8mm 21mm 13mm 7mm
9.	A9	MSSA	Pus	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	8mm 7mm 12mm 8mm 6mm	10mm 7mm 21mm 14mm 7mm
10.	A10	MSSA	Pus	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	8mm 7mm 22mm 8mm 7mm	10mm 9mm 22mm 12mm 7mm
11.	ATCC	S.aureus	25923	<i>Psidium guajava</i> <i>Thuja orientalis</i> <i>Cannabis sativa</i> <i>Allium sativum</i> <i>Allium cepa</i>	7mm 6mm 13mm 8mm 7.5mm	14mm 8mm 23mm 13mm 11mm
12.	A8	MSSA	Pus	Control (Ethanol)	No zone of inhibition	No zone of inhibition
13.	A6	MRSA	Et-tube	Control (Ethanol)	No zone of inhibition	No zone of inhibition



FIG 1: Suspension of the test organism was prepared by adjusting the turbidity of the broth in a phosphate buffer saline by comparing it with that of the McFarland standard solution of 0.5(Refer table 1)

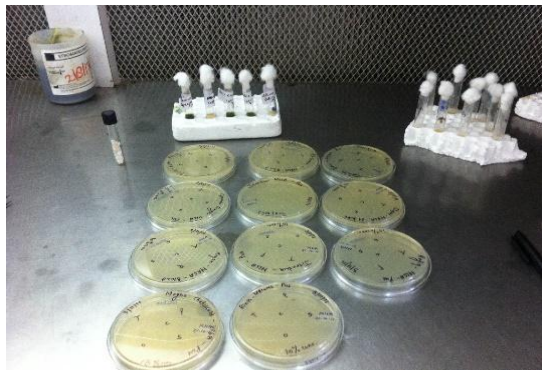


FIG 2: By means of a sterile cotton swab, a uniform lawn of bacterial growth was prepared on the MHA plates

- a.) T: Thuja orientalis
- b.) P: Psidium guajava
- c.) C: Cannabis sativa
- d.) O: Allium cepa
- e.) S: Allium sativum

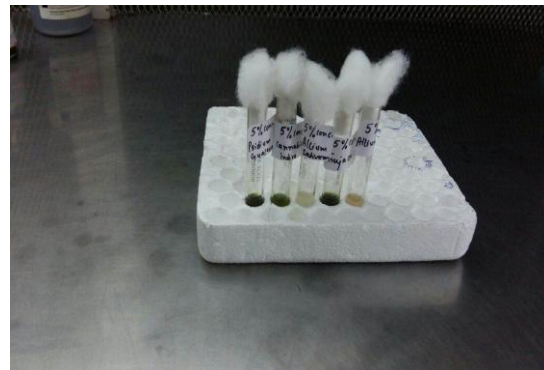


FIG 4: Plant extract concentration



FIG 3: Plant extract concentration at 10%.

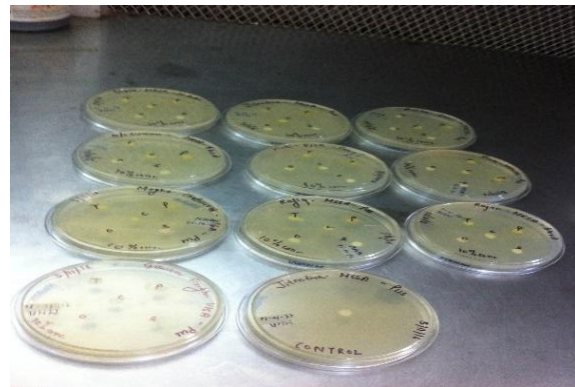
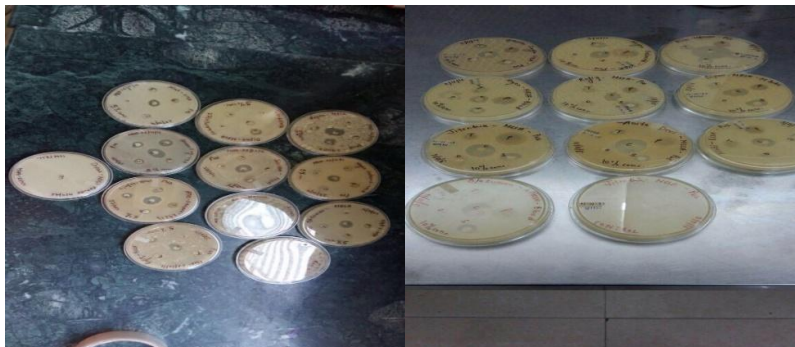


FIG 5: Himedia disc was impregnated with 25µl of the respective percentage solution of the plants and then placed the swabbed MHA plates. *Meanwhile, 25µl of the Ethanol was also pipette onto the Himedia paper discs a control



6A.) At 5% Plant extract concentration 6B.)At 10% Plant extract concentration FIG 6:The plates was incubated at 37 °C for 24 hours and then aruler scale was used to measure the inhibition zones in millimetres(mm) of the plant extract.(Refer table 2 for FIG 6(A.) and FIG 6 (B.))



FIG 7: Zone of inhibition by plants extracts Cannabis sativa followed by Allium sativum, Allium cepa, Psidium guajava and Thuja orientalis.

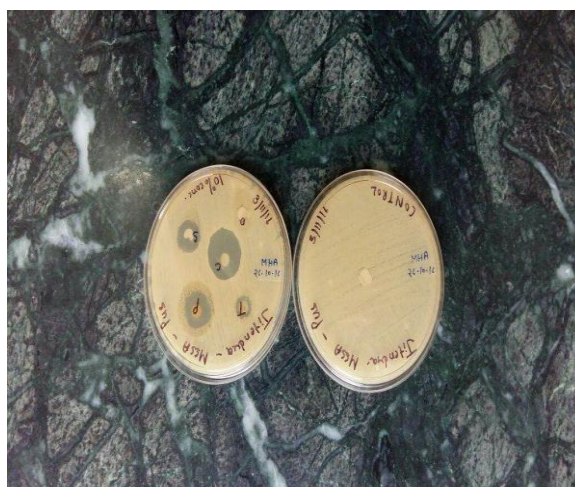


FIG 8: Zone of inhibition by plant extract; No zone of inhibition by ethanolic extract.



FIG 9: Ethanol as a control for both 5% and 10% plant extract concentration.

4. DISCUSSION

Antimicrobial drug resistance is an important global public health concern. Recent epidemiological reports from India states that there is an alarming increase in antimicrobial resistance. *Staphylococcus aureus* continues to be a persistent and dangerous pathogen for both community-acquired and hospital associated infections. The control of MRSA is important to curtail

the introduction and spread of infection. [22]

In order to control these infections, there is a need for the development of alternative therapeutic compounds from cheaper natural resources with marked antibacterial activity and less toxicity.

Plant derived products have been used for the medicinal purposes for centuries. Currently, it is estimated that about 80% of the world's population uses the herbal

preparations to meet their health needs. Herbs and spices have been generally considered safe and proved to be effective against certain ailments. [23]

Because of the side effects and resistance of the bacteria against the antibiotics, the scientist developed new drugs from the natural sources such as plants, which have been extensively used as an alternative treatment for disease [24,25] as antibacterial, [26-29] antifungal, antioxidants [30,31] and anticancer [32] due to that most of these plants contain many of active compounds such as flavonoids, tannis, saponins, alkaloids, terpenes, heavy metals. [33]

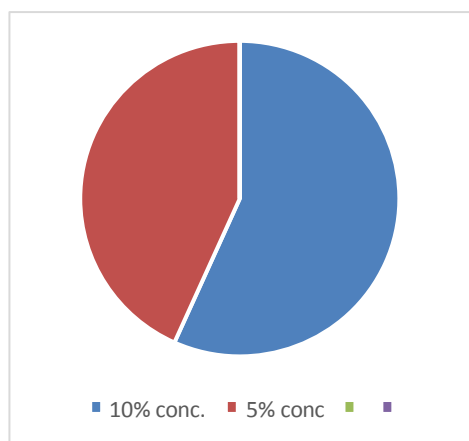


FIG 10: The 10% concentration of the plant extract possess more antimicrobial activity when compared to 5% extract

The antimicrobial and other medical benefits of Garlic and Cannabis have been widely recognised. Allicin is considered to be the most potent antibacterial agent in crushed garlic extracts. The highest inhibition zone of plant extract was observed in Cannabis sativa followed by Allium sativum then Allium cepa, Psidium guajava and lastly by Thuja orientalis. It was also observed that the zone of diameter increased with the increase of concentration.

5. CONCLUSION

It was concluded from this study that Cannabis sativa extract and Allium sativum extract possess the most antimicrobial activity among all the five plant extracts including Thuja orientalis, Psidium guajava and Allium cepa against multiple drug

resistance gram positive pathogens and it is expected that using natural products as therapeutic agents will probably not elicit resistance in microorganisms. Further studies are essential that this research should continue to isolate and purify the active components of this natural herb and use in experimental animals.

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