

## IN VITRO – ANTIMICROBIAL ACTIVITY OF CASHEW (*ANACARDIUM OCCIDENTALE*, L.) NUTS SHELL LIQUID AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) CLINICAL ISOLATES

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### ABSTRACT

Acetone, 70% acetone and methanol extracts of cashew nut shell liquid (CNSL) of *Anacardium occidentale*, L. were investigated for their ability to inhibit 15 clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) from patients of different corporate hospitals of Coastal Andhra Pradesh, South India. The antibacterial activity of three organic solvents of cashew nut shell crude extract against MRSA isolates was tested by well diffusion with zone of inhibition 19 – 25mm. MIC and MBC for 15 clinical isolates of MRSA were 0.00024 µg/ml – 0.00375 µg/ml. Similarly the antibacterial activity of three organic solvents of cashew nut shell crude extract against MRSA (ATCC 33591) and for MSSA (ATCC 25923) by the well diffusion showed zone of inhibition 19 and 23 mm respectively. MIC and MBC values for MRSA are 0.00024 µg/ml and for MSSA are 0.00375 µg/ml respectively. Thin layer chromatography with acetone and 70% acetone extracts of CNSL revealed only one fraction. The separated compound with R<sub>f</sub> value of 0.6 in acetone and 0.8 in 70% acetone extract revealed inhibition zone on the MRSA culture medium in Agar overlay bioautography. The strong *in vitro* antibacterial activity of the separated compound against MRSA suggests the wide pharmaceutical applications.

**Keywords:** MRSA, Cashew nut shell liquid, Well-diffusion, MIC, TLC-Bioautography

### INTRODUCTION

Multiple drug resistance in human and animal pathogenic microorganisms have been commonly reported in recent years from all over the world, particularly in developing countries, due to indiscriminate use of commercial antibiotics in the treatment of infectious diseases<sup>1</sup>. Though, the resistance development by microbes cannot be stopped, appropriate action will reduce the mortality and health care costs by using antibiotic resistant inhibitors of plant origin<sup>2</sup>. Moreover, traditional remedies utilizing plants still occupy a central place among rural communities of developing countries for curing various diseases in the absence of an efficient primary health care system<sup>3</sup>. Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs<sup>4</sup>.

*Anacardium occidentale*, L. belonging to anacardiaceae member have great economic and medicinal value. The commercial importance of cashew is due to its richness in nutrient that constitutes of 47% fat, 21% protein and 22% carbohydrates, vitamins and all essential amino acids especially thiamin<sup>5</sup>. Cashew nut shell liquid (CNSL) a byproduct in cashew-processing factories, is among the sources of renewable alkenyl phenols, whose structural properties permit wide range of applications including the synthesis of highly cross-linked polymers<sup>6,7</sup> and bioactive compounds<sup>8</sup>. It is well-known that anacardic acid (AnAc) is the main component of natural cashew nut shell liquid (CNSL) constituting more than 80% of the total solvent extracted CNSL<sup>9</sup>. The liquid obtained from the shell of the nut, CNSL have wide commercial application<sup>10-12</sup>, biological and medicinal properties. The biological properties of CNSL such as larvicidal<sup>13</sup>, molluscicidal<sup>14,15</sup>, antifungal and antimicrobial<sup>16,17</sup> were also reported. The medicinal properties of phytochemicals present in CNSL reported are cytotoxic activity against several tumor cell lines<sup>18</sup>, anti-diabetic<sup>19</sup>, anti-inflammatory and analgesic effects<sup>20,21</sup>.

*Staphylococcus aureus* has been reported as a major cause of community and hospital acquired infections<sup>22</sup>. The organism has a differential ability to spread and cause outbreaks in hospitals<sup>23</sup>. Infections caused by *S. aureus* used to respond to β-lactam and related group of antibiotics. However, due to development of methicillin resistance amongst *S. aureus* (MRSA) isolates, treatment

of these infections has become problematic. Indiscriminate use of multiple antibiotics, prolonged hospital stay, intravenous drug abuse, and carriage of MRSA in nose are few important risk factors for MRSA acquisition<sup>24</sup>. In recent years, there have been several reports of community-associated MRSA (CA-MRSA) infections throughout the world strains of methicillin-resistant *S. aureus* (MRSA) are known to be resistant to many antibiotics and currently represent a serious problem to hospitalized patients as well as their caretakers<sup>25</sup>.

Hence the present investigation was carried out to evaluate the effect of anacardic acid present in the CNSL against MRSA isolated from the patients of different corporate hospitals of Coastal Andhra Pradesh, South India and compared with type cultures of MRSA and MSSA.

### MATERIALS AND METHODS

The cashew nuts of *Anacardium occidentale* were collected from cashew plantation of East Godavari district of Andhra Pradesh, South India. The seed coat of cashew nuts were separated and washed with sterile distilled water and dried at room temperature under shade, ground into fine powder using a blender and stored in air tight container till further analysis.

#### Extraction procedure

Three separate samples of 10g each were extracted with 100ml of 100% acetone, 70% acetone (30 ml of water + 70 ml of acetone) and 100% methanol, respectively. The mixture is kept for twenty four hours on rotary shaker at 190-220 rpm. The mixtures were filtered using Whatman No.1 filter papers. The precipitates were discarded and the filtrate was collected. Each extract was concentrated using rotary evaporator.

#### Preparation of stock solution for each extract of cashew nut seed coat powder

Stock solution of each extract was prepared by weighing 10 mg of each dried solvent extract dissolved in 1 ml of dimethyl sulphoxide (DMSO) giving a final concentration of 10,000 µg/ml. The stock solution was kept in screw capped bottles for subsequent use.

### Source of clinical isolates and identification of MRSA

MRSA isolates were consecutively isolated from diabetic care centres and intensive care units of various corporate hospitals in East Godavari, West Godavari and Krishna districts of Coastal Andhra Pradesh, South India. Samples comprised of blood, urine, pus, ear swabs, eye swabs and anterior nasal swabs. The swabs and body fluids of patient's samples were inoculated onto blood agar plates, each plate inoculated with a sample of single patient. The inoculated plates were incubated at 37°C for 18-24 h. After inoculation on blood agar, the swabs were placed in brain heart infusion (BHI) with 7.5% sodium chloride, which were also incubated at 37°C for 18-24 h. Inoculated BHI broth was sub cultured onto blood agar plates. From these blood agar plates, the colonies which were opaque, circular, pigmented with  $\beta$  hemolytic were identified as *S. aureus* by the Grams staining, catalase and coagulase (slide and tube) test<sup>26</sup>. Adequate controls were put up at every stage. A total of 153 coagulase positive *S. aureus* strains were isolated and identified from 478 clinical samples.

Antibiotic susceptibility testing was performed for the antibiotics; oxacillin (1 $\mu$ g) gentamycin (10  $\mu$ g), erythromycin (15  $\mu$ g), cotrimoxazole (25  $\mu$ g), vancomycin (30  $\mu$ g) (Hi-media) by Kirby-bauer disc diffusion technique with quality control strain of *S. aureus* ATCC 25923 as per National Committee for Clinical Laboratory Standards (NCCLS). Bacterial suspension matching 0.5 McFarland turbidity standards were inoculated on Muller-Hinton agar containing 4% NaCl and 6  $\mu$ g/ml oxacillin. Isolates showing visible growth after 24h incubation at 33-35°C were identified as MRSA. Oxford strains of *S. aureus* (ATCC 25923) sensitive to methicillin and *S. aureus* (ATCC 33591) resistant to methicillin were used as control organisms. Final identification was made on detection of *mecA* gene by PCR. Finally 82 MRSA were identified. Out of 82 MRSA, 15 isolates were screened for inhibitory action of anacardic acid.

### Agar well diffusion method

The antibacterial activity of acetone, 70% acetone and methanol of CNSL against MRSA clinical isolates from hospitalized patients were evaluated by using agar well diffusion method according to the National Committee for Clinical Laboratory Standard (NCCLS). Briefly, 3-5 morphologically identical colonies from each agar plate were lifted with a sterile wire loop and transferred into a tube containing 5 ml of tryptic soya broth (TSB). The turbidity of each bacterial suspension was adjusted to reach an optical comparison to that of 0.5 McFarland standards, resulting in suspension containing approximately  $1$  to  $2 \times 10^8$  CFU/ml. Muller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plates approximately 60° each time to ensure even distribution of the inoculum. As a final step the rim of the agar plate was also swabbed. After allowing the inoculum to dry at room temperature, 6mm diameter wells were bored with sterile metal borer. Each extract was checked for antibacterial activity by introducing 10 $\mu$ l of 100 mg/ml concentration into duplicate wells by using sterile micro pipette. The plates were allowed to stand at room temperature for 1 h to allow diffusion of extract into the agar. Then, plates were incubated at 37°C for 18 h in upright position. The presence of zone of inhibition was regarded as the indicator of antimicrobial action and the antimicrobial activity of extract was expressed in terms of average diameter of zone inhibition measured in millimeters. Each test was carried out in triplicate.

### Determination of minimal inhibitory concentration and Minimal bactericidal concentration

Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) were determined for the extracts by broth dilution method as described by Ayafor et al. (1994). The concentration at which there was no visually detectable bacterial growth was taken as the MIC and the concentration at which there was no bacterial growth after inoculation in Mueller Hinton agar was taken as MBC.

### Thin layer chromatography (TLC)

TLC was performed on a silica gel aluminum plate (Silica gel 60 F<sub>254</sub>, Merck) to fractionate active compound of 5mg/ml CNSL extracts. The TLC plates were cut into 6 cm x 6 cm and dried in oven at 90°C for 10 minutes. This is to activate the TLC plates by absorbing the moisture content from the plates. Then the 5  $\mu$ l of extract was spotted on the bottom of the plates. Each sample spot was located about 2 cm apart and away from the bottom of TLC plate. Sample spots were performed with a micropipette, thus the spot diameter was about 4 mm. The plates were placed in ascending direction in a jar with benzene: ethyl acetate: methanol (35:35:30) solvent as mobile phase. The plates were observed under UV light. The separated spots were marked and the R<sub>f</sub> value was calculated.

### Bioautography technique

Bioautography method is another technique that is used in screening antibacterial activity. Bioautography method is basically to localize the antibacterial compound from crude extract into chromatogram. This is a supportive and quick search method for antibacterial activity compound. The TLC plates that were used to separate active compounds in CNSL extract were directly mounted on to seed culture medium. The culture plate was kept for 5 h at room temperature to make sure separated compound diffuse into the medium. Then the TLC plates were taken out and incubated at 37°C for 24 hours<sup>27</sup>. Plates were sprayed with an aqueous solution of *p*-iodonitrotetrazolium violet (INT) 0.2 mg/ml. After incubating for about 1 h at 37°C; clear zones on chromatograms indicates inhibition of growth.

### RESULTS

Result obtained in the present study revealed that the tested cashew nut shell solvent extracts possess potential antimicrobial activity against 15 clinical isolates of MRSA from hospitalized diabetic and intensive care unit (ICU) patients by using standard microbiological technique. All the isolates showed multiple antibiotic resistances in the study area, which may be due to large portion of the bacterial isolate being previously exposed to several antibiotics

In the present study, the antibacterial activity of the acetone, 70% acetone and methanol extracts of CNSL against MSSA (ATCC 25923), MRSA (ATCC 33591) and 15 clinical isolates of MRSA from hospitalized diabetic and intensive care unit (ICU) patients were examined, both qualitatively and quantitatively by the presence or absence of MRSA growth and zone inhibition. Results from the antimicrobial well diffusion are summarized in Table 1 and Fig.1. The one of inhibition for MSSA (ATCC 25923) was 23 mm and for MRSA (ATCC 33591) was 19 mm. The control dimethyl sulphoxide (DMSO) did not inhibit any of the MRSA isolates.

**Table 1: Well diffusion method for CNSL extracts of *Anacardium occidentale*, L. against methicillin sensitive *S. aureus* ATCC, methicillin resistant *S. aureus* ATCC and clinical isolates of MRSA (n=15)**

Extract	Volume of extract	Diameter of zone inhibition (mm) methicillin sensitive <i>Staphylococcus aureus</i> (ATCC 25923)	Diameter of zone inhibition (mm) MRSA (ATCC 33591)	Diameter of zone inhibition (mm) MRSA clinical isolates (n=15)
Acetone	10 $\mu$ l (100 $\mu$ g)	22	21	21-22
70% Acetone	10 $\mu$ l (100 $\mu$ g)	25	24	23-24
Methanol	10 $\mu$ l (100 $\mu$ g)	21	20	19-21
Oxacillin	Disc 9(1 $\mu$ g) Hi-media	13	-	-
DMSO	10 $\mu$ l	-	-	-

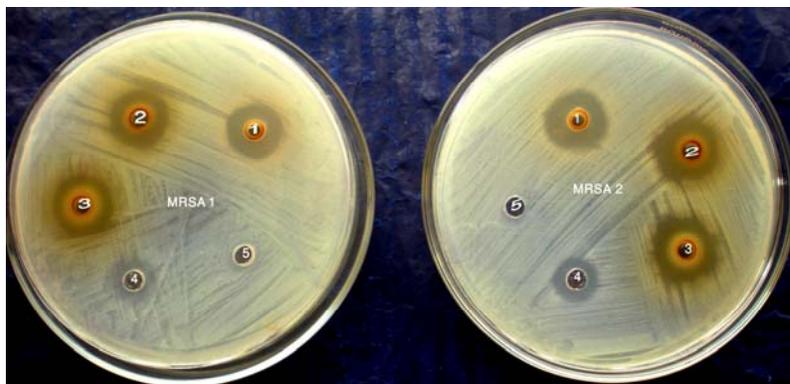


Fig. 1: Antibacterial activity of different solvent CNSL extracts against MRSA (1 and 2 ) clinical isolates. 1. Acetone extract, 2. 70% Acetone extract, 3. Methanol extract, 4. Oxacillin and 5. DMSO.

The antimicrobial activity of the CNSL extracts of *Anacardium occidentale, L.* and their potency were quantitatively assessed by determining the MIC and MBC, respectively, as given in Table 2 and

Fig.2. The MIC and MBC values were same for three extracts ranging between 0.00024  $\mu\text{g/ml}$  – 0.00375  $\mu\text{g/ml}$ , for all clinical isolates of MRSA, standard strain of MRSA and MSSA.

Table 2: Antibacterial activity (MIC & MBC in  $\mu\text{g/ml}$ ) of the acetone, 70% acetone and methanol extracts of CNSL of *Anacardium occidentale, L.* against MRSA

Extract	Methicillin sensitive <i>S. aureus</i> (ATCC 25923)	MRSA (ATCC 33591)	MRSA Clinical isolates
Acetone	0.00024 $\mu\text{g/ml}$	0.00188 $\mu\text{g/ml}$	0.00188 $\mu\text{g/ml}$
70% acetone	0.00024 $\mu\text{g/ml}$	0.00188 $\mu\text{g/ml}$	0.00188 $\mu\text{g/ml}$
Methanol	0.00188 $\mu\text{g/ml}$	0.00375 $\mu\text{g/ml}$	0.00375 $\mu\text{g/ml}$
Oxacillin (1mg/ml)	125 $\mu\text{g/ml}$	500 $\mu\text{g/ml}$	500 $\mu\text{g/ml}$

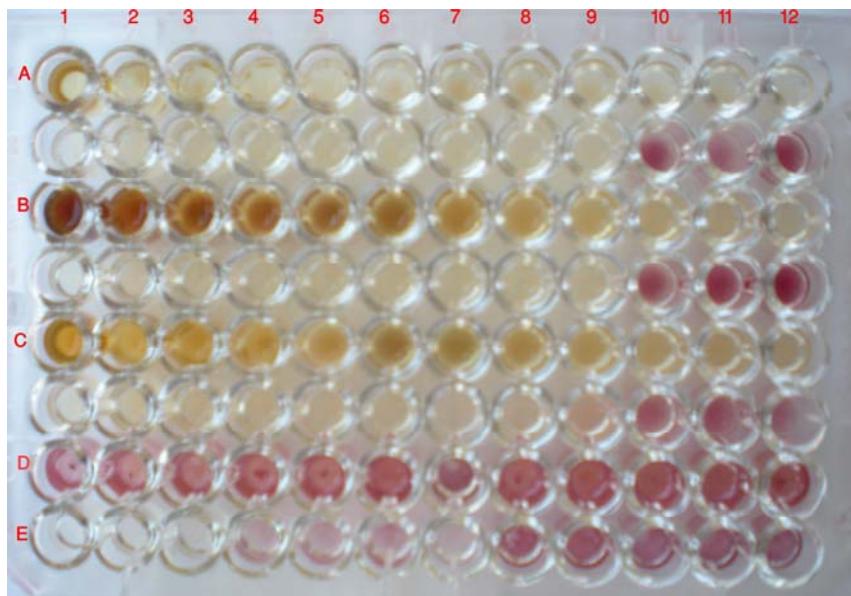


Fig. 2: Minimum inhibitory concentration (MIC) of different extracts of CNSL of *anacardium occidentale, L.* against MRSA ( pink colour indicates growth).

Rows A and B: Acetone extract (compound in serial dilution + broth culture + saline + indicator)

Rows C and D: 70% acetone extract (compound in serial dilution + broth culture + saline + indicator)

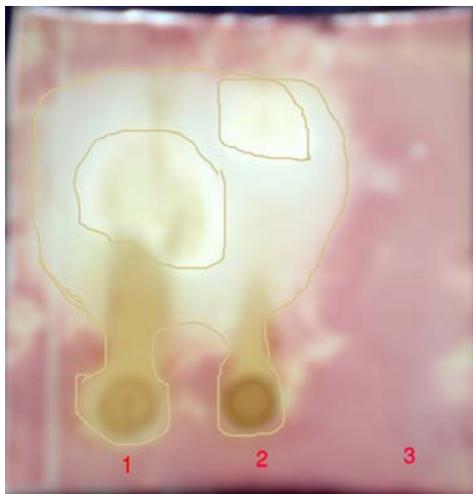
Rows E and F: Methanol extract (compound in serial dilution + broth culture + saline + indicator)

Row G: Normal saline+ broth culture + Indicator

Row H: Oxacillin in serial dilution + broth culture +saline + indicator)

In TLC, acetone and 70% acetone extracts of CNSL of *Anacardium occidentale, L.* separated fragments were visualized under ultraviolet (UV) light at 254 nm and the  $R_f$  value was calculated. The TLC plate of both extracts had only one separated fragment (Fig.3) Agar overlay bioautography method was used to localize antibacterial activity of extracts of CNSL of *Anacardium occidentale*. The separated

compound to  $R_f$  value of 0.6 in acetone 0.8 in 70% acetone extract revealed inhibition zone on the MRSA culture medium (Fig. 3). Therefore, it can be presumed that only one compound may be present in the CNSL extract and it may have antibacterial activity against MRSA.



**Fig. 3: Bioautography of fraction and known antibiotic sprayed with active growing MRSA. White areas indicate zone of growth inhibition 1. Acetone extract of CNSL ( $R_f$  Value 0.6), 2. 70%acetone extract of CNSL ( $R_f$  value 0.8) and 3. Oxacillin drug**

## DISCUSSION

Methicillin resistant *Staphylococcus aureus* (MRSA) as a hospital pathogen presented many clinical problems in India. In Indian hospitals, MRSA is one of the common cause of hospital-acquired infections and different hospitals have reported about 30% to 80% methicillin resistance based on antibiotic sensitivity tests<sup>28</sup>. The identification of hospital isolates bacterial strains is very important to confirm the presence of MRSA infection in patients. During identification coagulase test was carried out. This is an important test to differentiate *S. aureus* from other species especially from *S. epidermidis* and screening to oxacillin disc test was conducted. The choice of drugs to be used against MRSA is shrinking day by day as susceptibility of MRSA to drugs is decreasing by target site alteration, enzyme modification and permeability changes<sup>28</sup>. Although strategies have been proposed in an attempt to control the spread, the searches for new ways to treat MRSA infections stimulate the investigation of natural compounds as an alternative treatment of these infections.

In the present study the growth inhibition activity of MRSA by well diffusion method showed, that the 70% acetone extract of CNSL gave maximum inhibitory activity i.e. 19-23mm for MRSA (ATCC 33591), clinical isolates of MRSA and MSSA (ATCC 25923 ) when compared to pure acetone and methanol extracts of CNSL. The relatively wide spectrum of activity of the 70% acetone extract of CNSL over acetone and methanol extract is difficult to explain since all the extracts contained same types of phytochemicals. Perhaps, the active principles were more soluble in 70% acetone extract than the pure acetone and methanol solvents. The excellent inhibitory activity against MRSA was explained due to the presence of 80 % anacardic acid<sup>9</sup> in cashew nut shell liquid extract prepared in acetone, 70% acetone and methanol organic solvents. The checkerboard assay is probably the most convenient way of assessing the antimicrobial potential of plant extracts. In this method, the text extracts are able to diffuse more easily into the media. Advantage over the agar disc diffusion method includes increased sensitivity in small quantities of extract, ability to distinguish between bacteriostatic and bactericidal effects and quantitative determination of minimum inhibitory concentration (MIC). The MIC and MBC values were same for acetone, 70% acetone and methanol extracts of CNSL.

The bioautography technique has been used to identify the bioactive constituents present in acetone and 70% acetone extracts of CNSL. Inhibition zones of antibacterial components were observed as spots on a purple background. White areas indicate the presence of antibacterial components that inhibited the growth of bacteria that did not support the reduction of INT to the colored formazan<sup>29</sup>.

The antibacterial activity of CNSL can be attributed to amphipathic anacardic acid which enters into the membrane lipid bilayers where various enzymes, especially components of energy converting systems such as electron transport chains (ETCs) and ATPases, are embedded. The amphipathic anacardic acids entered into the lipid bilayers may disrupt the ETC and/or ATPases as surfactants. Anacardic acids were also reported to inhibit lipid synthesis of bacterial cells by inhibiting glycerol-3-phosphate dehydrogenase<sup>30</sup>. Chelation might also play a role in the antimicrobial activity of anacardic acids as it show high selectivity toward  $Fe^{2+}$  and  $Cu^{2+}$  and there by reducing their bioavailability for bacteria<sup>31</sup>. It was also reported that Anacardic acid exerted  $\beta$ -lactamase inhibitory activity<sup>32</sup>.

## CONCLUSION

The use of plants to heal diseases including infectious ones has been extensively applied by people. Data from literature as well as the present study results revealed great potential of cashew nut shell extracts/fractions for therapeutic treatment and the importance of cashew nut shell extracts /fractions, when associated with antibiotics to control multidrug resistant pathogenic bacteria, a major threat to human health.

In the present study, we concluded that cashew nut shell extract of *Anacardium occidentale, L.* was active against Methicillin resistant *Staphylococcus aureus* at a very low concentration in both well diffusion and MIC. The crude extracts of cashew nut shell exhibits powerful *in vitro* antimicrobial activity against control and clinical isolates of methicillin resistant *Staphylococcus aureus*. These antimicrobial characteristics of cashew nut shell of *Anacardium occidentale* potentially valuable for the future as a bioenhancer in antimicrobial drug resistance reversal therapy for *Staphylococcus aureus* infections.

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