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Acetone extract of Cashew (*Anacardium occidentale, L.*) nuts shelliquid against Methicillin resistant *Staphylococcus aureus* (MRSA) by minimum inhibitory concentration (MIC)

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ABSTRACT

Acetone extracts of cashew nut shell of Anacardium occidenatle, L. were investigated for their ability to inhibit 15 clinical isolates of methicillin resistant Staphylococcus aureus (MRSA) from patients of diabetic care centers. Antibacterial activity was determined for cashew nut shell liquid (CNSL) extract by finding minimum inhibitory concentration (MIC) using 96 well microtitre plates. A twofold serial dilution of compounds (100 µl) in sterile normal saline was prepared in 96-well microtitre plate and 50 µl overnight fresh bacterial cultures of one McFarland unit were added to each well. The plates were incubated overnight at 37^{0}C and bacterial growth was detected by adding and incubating for 30 min with 20 µl of p-iodonitrotetrazolium violet (INT) to each well. The well that remained color less when there was no bacterial growth and that concentration was taken as MIC. A negative control was also maintained in which all wells with bacterial growth in red color were found. MIC values(µg/mL) obtained for CNSL against 15 clinical isolates of MRSA were 0.00024 – 0.00375, Similarly the acetone extract of CNSL against MRSA (ATCC 33591) and for methicillin sensitive Staphylococcus aureus (MSSA) (ATCC 25923) MIC values for MRSA are 0.00024 µg/ml and for MSSA are 0.00375 µg/ml respectively. The strong in vitro antibacterial activity of the separated compound against MRSA suggests the wide pharmaceutical applications.

Keywords: MRSA, Cashew nut shell, MIC, Antibacterial.

INTRODUCTION

In recent years, drug resistance to human pathogenic bacteria is being commonly reported from all over the world [1]. 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 [2]. 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 [3]. 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 [4].

Ancardium occidentale 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 aminoacids especially thaiamin [5]. 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 [6,7] and bioactive compounds [8]. 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 [9]. The liquid obtained from the shell of the nut, cashew nut shell liquid (CNSL) have wide commercial application [10-12], biological and medicinal properties. The biological properties of CNSL such as larvicidal [13], molluscicidal [14,15]; antifungal and antimicrobial [16,17] were also reported. The medicinal properties of phytochemicals present in CNSL reported are cytotoxic activity against several tumor cell lines [18], anti-diabetic [19], anti-inflammatory and analgesic effects [20,21].

Staphylococcus aureus has been reported as a major cause of community and hospital acquired infections [22]. The organism has a differential ability to spread and cause outbreaks in hospitals [23]. 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 [24]. 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 [25].

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. And they were identified and authenticated by A.Ramakrishna, Principal and Head, Department of botany, VKR College, Budhavaram, Andhra

Pradesh, India. The seed coat of cashew nuts were separated and washed with sterile distilled water and dried using laminar air flow, ground into fine powder using a blender and stored in air tight container till further analysis.

Extraction procedure

Ten grams of cashew nut shell fine powder weighed into a 250 ml conical flask and 100 ml of acetone was added then on a rotary shaker at 190 – 220 rpm for 24 hours [27]. This was filtered with whatman No1. Filter paper, the residue discarded, and the filter were evaporated to dryness in a water bath temperature at 80^{o} C.

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

Stock solution 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 Kirshna 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 (BHA) 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 β hemolytic were identified as *S. aureus* by the Grams staining, catalase and coagulase (Slide and tube) test [26]. 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 (1µg) Gentamycin (10 µg), Erythromycin (15 µg), Co-trimoxazole (25 µg), vancomycin (30 µg) (hi-media0 by Kirby-bauer disc diffusion technique with quality control strain of *S. aureus* ATCC 25923 as per national Committee for Clinical laboratory standards [39]. Bacterial suspension matching 0.5 McFarland turbidity standards were inoculated on Muller-Hinton agar containing 4% NaCl and 6 µg/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.

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 er 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.

RESULTS

Result obtained in the present study revealed that the tested cashew nut shell acetone solvent extracts posses potential antimicrobial activity against 15 clinical isolates of methicillin resistant *Staphylococcus aureus* from hospitalized diabetic patients by the using MIC. 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.

Table 1A. Antibacterial activity (MIC in µg/ml) of the acetone extracts of CNSL of *Anacardium occidentale, L.* against clinical isolates of MRSA, ATCC strains of MRSA and MSSA

S.No.	MRSA clinical isolates	Acetone (10mg/ml) extract	Oxacillin(1mg/ml)
01	MRSA1	0.00024 µg/ml	500 µg/ml
02	MRSA2	0.00024 µg/ml	500 µg/ml
03	MRSA3	0.00188 µg/ml	500 µg/ml
04	MRSA4	0.00024 µg/ml	500 µg/ml
05	MRSA5	0.00024 µg/ml	500 µg/ml
06	MRSA6	0.00375 µg/ml	500 µg/ml
07	MRSA7	0.00188 µg/ml	500 µg/ml
08	MRSA8	0.00375 µg/ml	500 µg/ml
09	MRSA9	0.00375 µg/ml	500 µg/ml
10	MRSA10	0.00024 µg/ml	500 µg/ml
11	MRSA11	0.00024 µg/ml	500 µg/ml
12	MRSA12	0.00188 µg/ml	500 µg/ml
13	MRSA13	0.00375 µg/ml	500 µg/ml
14	MRSA14	0.00024 µg/ml	500 µg/ml
15	MRSA15	0.00375 µg/ml	500 µg/ml

Table 1B. Antibacterial activity (MIC in µg/ml) of the acetone extracts of CNSL of *Anacardium occidentale, L.* against ATCC strains of MRSA and MSSA.

S.No.	MRSA clinical isolates	Acetone (10mg/ml) extract	Oxacillin(1mg/ml)
01	MRSA (ATCC)	0.00024 µg/ml	500 µg/ml
02	MSSA (ATCC)	0.00375 µg/ml	500 µg/ml

In the present study, the antibacterial activity of the aceonte extracts of CNSL extracts of *Anacardium occidentale, L.* against MSSA (ATCC 25923), MRSA (ATCC 33591) and 15 clinical isolates of MRSA from hospitalized diabetic patients were examined, and their potency were quantitatively assessed by determining the MIC values as given in Table1A & 1B and Fig.1. The MIC values same for both clinical isolates of MRSA and standard strain of MRSA and MSSA

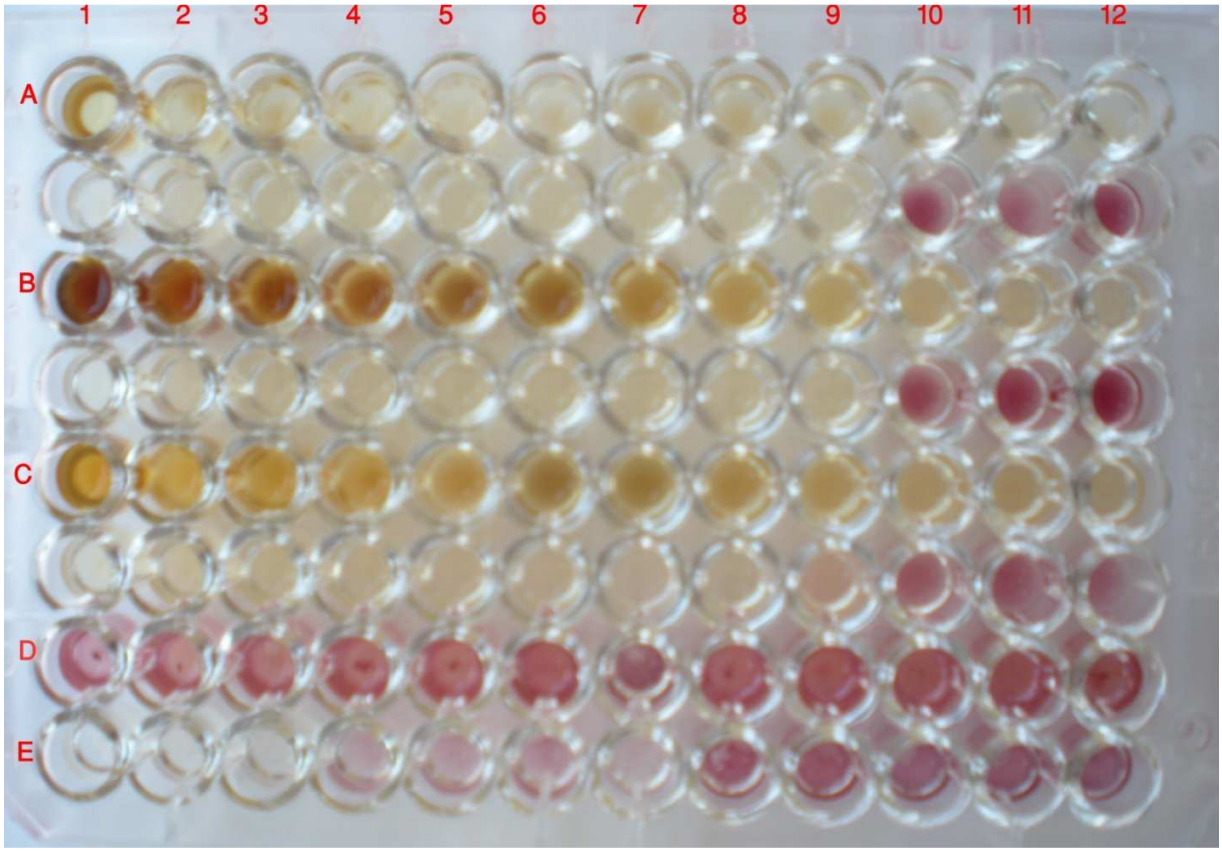

Fig. 1. Minimum inhibitory concentration (MIC) of Acetone extracts of CNSL of *anacardium occidentale, L.* against clinical isolates of MRSA, ATCC MRSA and ATCC MSSA (pink colour indicates growth).

Rows A and B: Acetone extract (compound in serial dilution + clinical isolates of MRSA broth culture + saline + INT indicator)
Rows C and D: acetone extract (compound in serial dilution + ATCC MRSA broth culture + saline + INT indicator)
Rows E and F: Acetone extract (compound in serial dilution + ATCC MSSA broth culture + saline + INT indicator)
Row G: Normal saline+ ATCC MRSA broth culture + INT Indicator
Row H: Oxacillin in serial dilution + broth culture + saline + INT indicator)

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[28].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 [28].

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 The MIC method 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 values were same for some of the clinical isolates but different in ATCC strains of MRSA and MSSA. The relatively wide spectrum of activity of the acetone extract of CNSL because the active principles were more soluble in acetone than the other solvents. The excellent inhibitory activity against MRSA was explained due to the presence of 80 % anacardic acid [9] in CNSL extract prepared in acetone.

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 [29]. Chelation might also play a role in the antimicrobial activity of anacardic acids as it show high selectivity toward Fe2+ and Cu2+ and there by reducing their bioavailability for bacteria [30]. It was also reported that Anacardic acid exerted *β*-lactamase inhibitory activity [31].

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

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