Efficacy of *Azadirachta indica* organic extracts against clinical methicillin resistant *Staphylococcus aureus* isolates

Jaweria Anwar[1], Muhammad Asif Zahoor[1*], Muhammad Kashif Zahoor[2], Abu Baker Siddique[1], Zeeshan Nawaz[1], Muhammad Hidayat Rasool[1], Muhammad Usman Qamar[1], Muhammad Waseem[1], Syed Zajif Hussain[3] and Aysha Yasmin[4]

[1]Department of Microbiology, Government College University, Faisalabad, Pakistan
[2]Department of Zoology, Government College University, Faisalabad, Pakistan
[3]Department of Chemistry, Syed Babar Ali School of Science and Engineering, Lahore University of Management Sciences, Pakistan
[4]Department of Biochemistry, Government College University, Faisalabad, Pakistan

Abstract: In current study we investigated the efficacy of organic extracts of *Azadirachta indica* leaves against Methicillin Resistant *Staphylococcus aureus* (MRSA) clinical isolates. For this purpose fresh leaves were used to prepare ethanol, methanol and chloroform extract. Secondly, a cross sectional study was conducted to isolate MRSA in clinical samples from patients having surgical/ non-surgical wounds from Allied Hospital and District Head Quarter Hospital, Faisalabad. The *S. aureus* isolates were initially identified by biochemical characterization, followed by identification of MRSA using cefoxitin disc diffusion test that was finally confirmed by genomic amplification of *mecA* gene, responsible for resistance. All MRSA isolates were tested to find vancomycin resistant *S. aureus* (VRSA) using E-strips (M.I.C. Evaluator™, Oxide, UK). The data showed an overall 37% prevalence of *S. aureus* including 56.75% clinical MRSA isolates while none of the isolated *S. aureus* showed resistance to vancomycin. The antimicrobial activity was measured as mean zone of inhibition for each extract against all MRSA isolates and it was found as 15.38±2.26, 16.09±3.09 and 17.42±2.48 for methanol, ethanol and chloroform extracts respectively. Chloroform extract showed significantly high antimicrobial activity against MRSA isolates. Altogether, the current study exposed the high prevalence of MRSA isolates from tertiary care hospitals. However, all MRSA isolates were found susceptible to organic extracts of *A. indica* leaves.

Keywords: MRSA, VRSA, antimicrobial, wound infection.

INTRODUCTION

Staphylococcus aureus is the most widely isolated pathogen that causes variety of infections including cutaneous abscesses, wound infections, fatal necrotic fasciitis and life threatening necrotic pneumonia (Miller *et al*., 2005). More than 50% infections are considered to be caused by methicillin resistant *S. aureus* (MRSA) as described previously (Drago *et al*., 2007). Therefore, MRSA infections are considered as foremost cause of increased morbidity rates throughout the world (Applebaum *et al*., 2006). During the last two decades, the world-wide spread of MRSA resulted in major therapeutic problems in several hospitals around the world. A multi centric study from India reported prevalence of MRSA as 41% in 2008-2009 from tertiary care hospitals (Joshi *et al*., 2013). Similarly, in Pakistan, the incidence of nosocomial infections caused by MRSA has increased continuously and ranged from 5-61% (Qureshi *et al*., 2000; Hafiz *et al*., 2002; Hussain *et al*., 2005; Akhter *et al*., 2009). Overall MRSA showed resistance to various antibiotics including aminoglycosides, macrolides, fluoroquinolones, clindamycin, chloramphenicol and β-lactamases (Mutto *et al*., 2003).

**Corresponding author:* e-mail: drasifzahoor@gcuf.edu.pk

World Health Organization (WHO) estimated that about 80% of the world population is using herbal medicines (Ansari *et al*., 2011). Traditionally, herbs and herbal products have been used for thousands of years for treatment of various problems particularly wound infections. *Azadirachta indica* plant is present in tropical and sub-tropical regions of the world including South East Asian countries (Reigo *et al*., 2001). *A. indica* leaves are effective in treatment of chronic wounds, diabetic foot and gangrenous conditions. Similarly, a lot of people throughout the world rely on medicinal plants for treatment of minor and even in some cases major diseases (Zabta, 2010). Therefore, keeping in view the importance of MRSA and herbal treatments, the present study has focused to find out the prevalence based on molecular identification of MRSA clinical isolates in tertiary care hospitals and to measure the antibacterial activity of organic extracts of *A. indica.*

MATERIALS AND METHODS

This cross sectional study was conducted between January and August 2015. The study was approved from the Ethical Review Committee of the Governement College University, Faisalabad (GCUF) along with consent of the patients.

A total of 100 clinical samples were collected aseptically from patients having infected surgical/ non-surgical wounds from Allied Hospital and Distric Head Quarter Hospital, Faisalabad. Samples were cultured on nutrient agar, blood agar and mannitol salt agar (Oxide, UK) and incubated at 37^{o}C for 18-20 hours aerobically.

Initial identification of the clinical isolates was carried out on the basis of colony morphology, a cultural characteristic and is further confirmed by biochemical tests including catalase and coagulase (Wayne, 2010). *S. aureus* clinical isolates were initially tested for methicillin resistance by modified Kirby-Bauer disk diffusion method using cefoxitin (30µg) disc according to CLSI guidelines (Wayne, 2010).

Molecular confirmation of MRSA was carried out by amplification of *mecA* gene. For this purpose genomic DNA was extracted using DNA extraction kit (Bio Basic Inc, Ontario, Canada) and subjected to polymerase chain reaction using *mecA* specific primers targeting 310bp DNA fragment (Ahmed *et al*., 2014). Briefly, PCR was performed in 20µl final volume containing master mix (Bio Basic Inc, Ontario, Canada) and 1µl of *mecA* forward and *mecA* reverse primers. The conditions were optimized as initial denaturation at 95^{o}C for 1 minutes followed by first denaturation at 95^{o}C, annealing of primers at 56^{o}C, extension at 72^{o}C and final extension for 10 minutes at 72^{o}C. The analysis of PCR product was conducted using 1.2% agarose gel electrophoresis.

Fresh *A. indica* leaves were collected which are identified and confirmed by Botanical Society of GCUF. Leaves were washed with sterilized water, dehydrated at room temperature and grinded into fine powder by Pestle and Mortal. A 50g of leave powder were soaked in 100mL of absolute ethanol, absolute methanol and absolute chloroform in each Erlenmeyer flasks and left for 48 hours at 20^{o}C and later on filtered through Whatman filter paper (Sigma-Aldrich, UK) as described (Dahiya *et al*., 2012; Siddiqui *et al*., 2003). Each filtrate was allowed to dry to obtain crude extract and dissolved in dimethyl sulfoxide (DMSO) to prepare different working solutions.

Finally, all extracts were screened against MRSA isolates by agar well diffusion assay. The subcultured isolates were adjusted to 0.5 McFarland standards and spread on Mueller Hinton (MH) agar. Each extract was tested for antibacterial activity. Bacteria showed mean zone of inhibition (>12 mm) were considered to be inhibited (Dahiya *et al*., 2012).

The data was analyzed statically applying ANOVA and Tukey (HSD) test using software XLSTAT Premium.

RESULTS

Out of 100 clinical samples 80 were positive for bacterial growth. On the basis of cultural and biochemical characteristics 37 isolates were identified as *S. aureus*. Cefoxitin disk susceptibility (fig. 1A) or amplification of *mecA* gene revealed 21 (56.75%) MRSA isolates. While none of the isolate was found resistant to vancomycin (fig. 1B).

The antimicrobial data of each extract was analyzed statistically. Overall the chloroform extract showed significant mean zone of inhibition (17.42±2.48) as compared to ethanol and methanol extracts. Mean zone of inhibition was observed as 16.09±3.09 and 15.38±2.26 for ethanol and methanol extract respectively. The statistical data is shown in table 1.

DISCUSSION

In the present study, a total of 80 clinical samples were found positive for bacterial growth including 37 *S. aureus* isolates out of which 21 (56.75%) were identified as MRSA on the basis of cefoxitin disk susceptibility or amplification of *mecA* gene which indicated a high prevalence or emergence of drug resistance among clinical isolates from tertiary care hospitals. These findings are in accordance with the previous studies that also described *S. aureus* as major pathogen isolated from infected wound of skin along with their antimicrobial profiles (Zafar *et al*., 2008; Wong *et al*., 2013; Yasidi *et al*., 2015).

The current study showed significant antibacterial activity of *A. indica* extracts (mean ± SD) against MRSA. Historically, lot of literature is available which describe the effectiveness of *A. indica* leaves and extracts only for the treatment of chronic wounds, diabetic foot and other gangrenous conditions of skin which does not describe its effectiveness against multi drug resistant or MRSA clinical isolates (Zafar *et al*., 2008; Wong *et al*., 2013; Yasidi *et al*., 2015). However in the current study we have characterized the MRSA clinical isolates by amplification of *mecA* gene and showed antibacterial activity of organic extracts of *A. indica*, whereas only two studies published recently have described the effects of *A. indica* against drug resistance bacteria and biofilm formation (Dahiya *et al*., 2012; Quelemes *et al*., 2015).

Altogether, there is no doubt that anti-bacterial, anti-fungal, anti-parasitic, anti-viral properties and insecticidal properties of *A. indica* plant have been reported previously (Reigo *et al*., 2001). In the present findings the antibacterial activity of *A. indica* extracts against MRSA was estimated and good results were obtained.

CONCLUSION

High prevalence of MRSA among tertiary care hospitals was observed. However, all of MRSA were found susceptible to organic extracts of *A. indica* leaves.

Table 1: Comparison of antibacterial activity of organic extracts of *Azadirachta indica* (mean zone of inhibition) using Tukey test (HSD) with a confidence interval of 95%

Comparison of Extracts	Difference	Standardized Difference	Critical value	P value	Significant
Methanol vs. Chloroform	2.048	2.514	2.403	0.038	Yes
Chloroform vs. Ethanol	1.333	1.637	2.403	0.238	No
Ethanol vs. Methanol	0.714	0.877	2.403	0.657	No

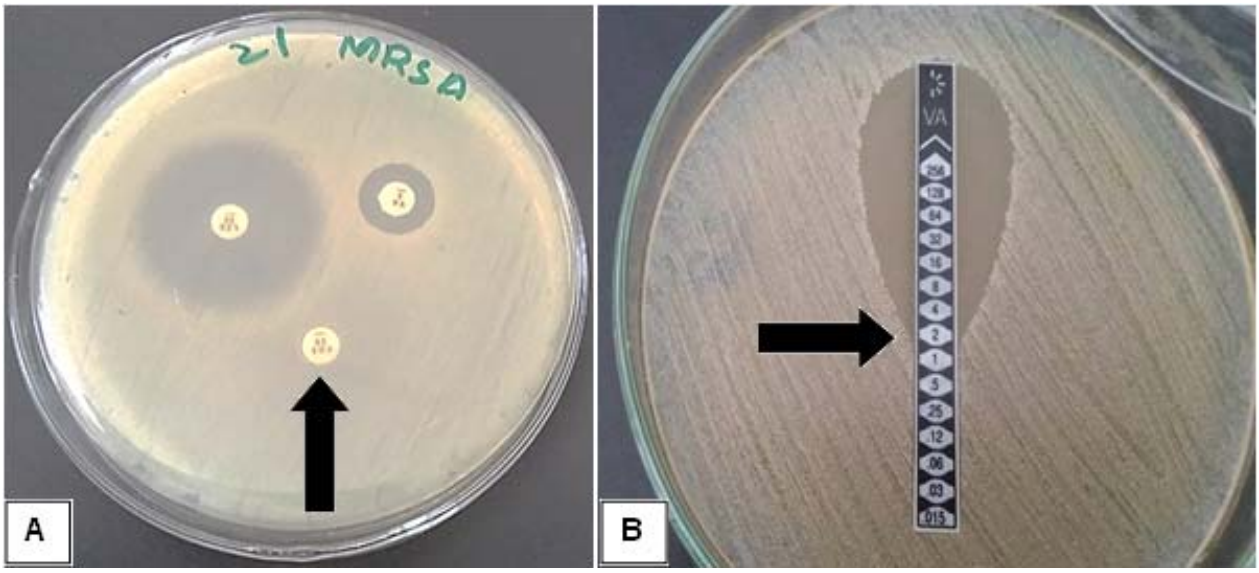

Fig. 1: Clinical *Staphylococcus aureus* isolates showed A). Methicillin resistant *S. aureus* (MRSA) on Mueller-Hinton agar plate, arrow indicates the antimicrobial resistance of the MRSA. B). Screening of MRSA to find vancomycin resistant *S. aureus* (VRSA) using E-Strips (M.I.C. Evaluator™, Oxide, UK), arrow indicates susceptibility to vancomycin.

Further, the antimicrobial activity could not be attributed to crude extracts, instead there is need to purify and assess potential activity of active ingredients.

REFERENCES

Ahmed EF, Gad GF, Abdalla AM, Hasaneen AM and Abdelwahab SF (2014). Prevalence of Methicillin Resistant *Staphylococcus aureus*among Egyptian Patients after Surgical Interventions. *Surg. Infect.,* **15**: 404-411.

Akhter R, Khan KMA and Hassan F (2009). Isolation and antimicrobial susceptibility pattern of methicillin resistant and sensitive *Staphylococcus aureus*. *J. Surgery Pak.,* **14**: 161-165.

Ansari M, Larijani K and Tehrani MS (2011). Antibacterial activity of *Lippacitriodora* herb essence against MRSA *Staphylococcus aureus*. *Afr. J. Microbiol. Res.,* **6**: 16-19.

Applebaum PC (2006). MRSA-the tip of the iceberg. *Clin. Microbiol. Infect.,* **12**(Suppl 2): 3-10.

Dahiya P and Purkayastha S (2012). Phytochemical screening and antimicrobial activity of some medicinal plants against multi-drug resistant bacteria from clinical isolates. *Indian J. Pharm. Sci.,* **74**: 443-450.

Drago L, Veechi EL, Nicola L and Gismondo MR (2007). In vitro evaluation of antibiotics' combinations for empirical therapy of suspected methicillin resistant *Staphylococcus aureus* severe respiratory infections. *BMC Infect. Dis.,* **7**: 2334-2337.

Hafiz S, Hafiz AN, Ali L, Chughtai AS, Memon Band Ahmed A (2002). Methicillin resistant *Staphylococcus aureus*: a multicenter study. *J. Pak. Med. Assoc.,* **52**: 312-315.

Hussain S, Shams R, Ahmad K, Parveen R and Riaz B (2005). Prevalence of methicillin resistant *Staphylococcusaureus* in surgical site infections. *Int. J. Pathol.,* **3**: 81-85.

Joshi S, Ray P, Manchanda V, Bajaj J, Chitnis DS, Gautam V, Goswami P, Gupta V, Harish BN, Kagal A, Kapil A, Rao R, Rodrigues C, Sardana R, Devi KS, Sharma A and Balaji V (2013). Methicillin resistant Staphylococcus aureus (MRSA) in India: Prevalence & susceptibility pattern. *Indian J. Med. Res.,* **137**: 363-369.

Miller LG, Remington FP, Rieg G, Mehdi S, Perlroth J, Bayer AS. Tang AW, Phung TO and Spellberg B (2005). Necrotizing fasciitis caused by community-associated methacilline-resistant *Staphylococcusaureus* in Los Angeles. *N. Engl. J. Med.,* **352**: 1445-1453.

Mutto CA, Jenigan JA, Ostrowsky BE and Ricket HM (2003). SHEA guideline for preventing nosocomial transmission of multidrug-resistant strains of

Staphylococcus aureus and enterococcus. *Infect. Cont. Hosp. Epi.,* **24**: 362-368.

Quelemes PV, Perfeito ML, Guimarães MA, Santos RC, Lima DF, Nascimento C, Silva MP, Soares MJ, Ropke CD, Eaton P, Moraes J and Leite JR (2015). Effect of neem (*Azadirachta indica A. Juss*) leaf extract on resistant Staphylococcus aureus biofilm formation and Schistosoma mansoni worms. *J. Ethnopharmacol.,* **175**: 287-94.

Qureshi AH, Qamar MA, Marri MH, Ali N and Ijaz A (2000). Prevalence of methicillin resistant Staphylococus aureus (MRSA). *Pak. Armed Forces Med. J.,* **50**: 91-93.

Reigo JA and Untalan CM (2001). *In vitro* analysis of the antibacterial properties of *Basellarubra*linn, (alugbati) and *Plumeraacuminata* (kalachuchi) against most common bacterial wound isolates among pediatric patients. *PIDSP J.,* **5**: 14-19.

Siddiqui SB, Afshan F, Gulzar T and Sultan R (2003). Tetracyclic triterpenoids from the leaves of Azadirachta indica and their insecticidal activities. *Chem. Pharm. Bull.,* **51**: 415-417.

Wayne PA (2010). Clinical and Laboratory Standards Institute (CLSI) performance standards for antimicrobial disk diffusion susceptibility tests. 20th. Ed., 30.

Wong S, Manikam R and Muniandy S (2013). Analysis of bacterial diversity in healing and non-healing wounds among Malaysian subjects by phenotypic identification and 16S rDNA sequencing. *Biomed. Res.,* **24**: 389-395.

Yasidi BM, Akawu DB, Oihoma OJ, Bara JY, Mohammed UH and Mohammed GN (2015). Retrospective analysis of bacterial pathogens isolated from wound infections at a Tertiary Hospital in Nguru, Yobe State Nigeria. *Am. J. Biomed. Life Sci.,* **3**: 1-6.

Zabta K (2010). Medicinal plants research in Pakistan. *J. Med. Plants Res.,* **4**: 161-176.

Zafar A, Anwar N and Ejaz H (2008). Bacteriology of infected wounds- A Study conducted at children's Hospital Lahore. *Biomedica.,* **24**: 71-74.