Evaluation of *Conocarpus erectus* against multidrug resistant
*Staphylococcus aureus*: Cell to animal study

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**Abstract**: Antibiotic resistant infections by *Staphylococcus aureus* (*S. aureus*) in high risk patients is critical challenge for all clinicians across globe. In an effort to achieve robust bactericidal effect, therapeutic approach based on antimicrobial plant extract of *Conocarpus erectus* (*C. erectus*) been assessed *in-vitro* and *in-vivo* against *S. aureus* resistant clinical strains isolated from burn patients and antibiotic susceptibility was conducted using Kirby-baur disc diffusion technique. *C. erectus* plant extract obtained and characterized for phytochemical constituents, its hemocompatibility and for antioxidant potential. Minimum inhibitory concentration studied for *C. erectus* extract against multidrug resistance (MDR) *S. aureus* clinical isolates *in-vitro* and in rat’s sepsis model. Therapeutic activity along acute toxicity was evaluated in rat’s model. *C. erectus* extract showed marked antioxidant activity attributed to its phenolic components predominately along others. Hemocompatibility results were significantly different (*p*<0.05) compared to vancomycin (positive control). Statistically significant reduction in bacterial colony count (*p*<0.05) observed in rat’s sepsis model with *C. erectus* treated group vs. controls. *C. erectus* extract offered higher bactericidal effect both *in-vitro* and *in-vivo* along no acute toxicity at therapeutic dose. We infer that it can serve as alternative promising treatment option against antibiotic resistant against MDR *S. aureus* strains.

**Keywords**: *S. aureus*, *C. erectus* extract, multidrug resistant, acute toxicity.

**INTRODUCTION**

The critical healthcare challenge faced across globe are bacterial infections resistant to antibiotics (Andersson and Hughes, 2010), (Aslam et al., 2018). Multidrug resistant (MDR) bacteria worldwide are rapidly increasing and becoming major threat of 21st century. It has drawn tremendous attention of media and global surveillance authorities (Nii-Trebi, 2017). Worldwide over 13 million deaths occurring every year are ascribed to new infectious diseases emergence or re-emergence of resistance strains of prevailing pathogens (Nii-Trebi, 2017). Burn wounds are more prone to infections leading up to 75% deaths in burn wards comparing to surgical wounds, osmotic shock and hypovolemia (Agnihotri et al., 2004), (Zhang et al., 2021). From burn injuries commonly isolated microorganisms include *S. aureus*, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus pyogenes* (*S. pyogenes*) (Halstead et al., 2018). *S. aureus* was isolated more frequently comparing others from burn patients. The susceptibility of *S. aureus* differs in countries where antibiotics irrational use is common causing reduce efficacy of antimicrobial agents (Bilal et al., 2021), (Bukhari et al., 2004). Over the time *S. aureus* has acquired significance and appeared as super bug from an innocuous organism. It exhibited both intrinsic and acquired resistance towards available treatment options (Harding et al., 2018). Infectious Diseases Society of America has ranked *S. aureus* in top six hit list of pathogenic microbes against which treatment options are not available or very limited (Cho et al., 2017). Methicillin resistant *S. aureus* (MRSA) due to multidrug resistance has become a major public health threat causing both hospital and community acquired infections. MRSA is leading cause of skin and soft tissue infections, bacteraemia, endocarditis, bone and joint infections posing challenging clinical threat with persistent inclined rate of morbidity and mortality (Kshetry et al., 2016). MRSA showed resistance to even last resort antibiotics including vancomycin (Ahmed et al., 2016). Successful therapeutic approaches are still challenging and demanding assessment of novel antimicrobials agents, adjunct care parameters along source control (Turner et al., 2019).

Medicinal plants on account of safety, multi targeting potential and inexpensive comparing to synthetic agents offers promising approach for exploring more drugs (Anand et al., 2019). *C. erectus* extract and its bioactive constituents has been explored via biological assays by scientists tried to justify its biological actions (Rehman et al., 2019). In this study, *in-vitro/in-vivo* antimicrobial efficacy of *C. erectus* leaves extracts against MDR *S. aureus* infections isolated from burn patients was evaluated. Current study findings may serve as translational link for clinical trials for assessment of

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efficacy and safety of *C. erectus* extracts after substantial testing and guidance for further studies leading to clinical evaluation to determine safety margins to make it a future drug delivery system.

**MATERIALS AND METHODS**

**Clinical isolates of multidrug resistant *S. aureus***

Clinical isolates of MDR *S. aureus* used in this study were taken from burn wounds of patients admitted at Allied Hospital Faisalabad, Pakistan after taking consent from Ethical Review Committee (ERC) Government College University Faisalabad (Ref No: GCUF 767/2017). Antimicrobial susceptibility was conducted for erthromycin, clindamycin, methicillin, vancomycin, levofloxacin and moxifloxacin by standard disc diffusion method (Organization, 2003). Interpretation of results were recorded as sensitive and resistant according to clinical and laboratory standard institute guidelines (Wayne, 2015).

**Preparation of plant extract**

Plant sample was collected from botanical garden of Government College University Faisalabad and identified, confirmed by taxonomist Associate Professor Dr. Uzma Hanif at Department of Botany, GCU Lahore and specimen has been kept in GC University herbarium museum, Lahore under voucher number via GC-Herb-Bot. 1938. Plant leaves were washed, dried in shade, powdered and used for extraction. Fifty grams of air-dried and coarsely powdered plant leaf material was extracted successively with 200 ml each of hexane, ethyl acetate, methanol and water in increasing order of their polarity using buchner funnel and whatman No. 1 filter paper for 12 h and stored at 25°C (Dahiya and Purkayastha, 2012).

**Characterization of plant extract**

**Phytoconstituents screening of extracts**

Thin Layer Chromatography (TLC) (Fluka, silica gel F 254) was utilized for extract fractional separation analysis using 10µl ethyl acetate *C. erectus* extracts according to reported method (D’Sousa’Costa et al., 2015). Eluted plates were visualized at 360 nm in ultraviolet light and sprayed with freshly prepared vanillin spray reagent to check separated compounds. Phytoconstituents present in extract was evaluated to detect phenols, flavonoids, alkaloids, tannins, glycosides (Yasin and Al-Azawi, 2019).

**In-vitro bactericidal activity**

MDR clinical isolates of *S. aureus* with their antibiotic resistance profiles performed in above section were used. Standard strains *S. aureus* ATCC 25923 was used for quality control. All test strains were kept on nutrient agar slants (Hi-Media Laboratories Pvt. Limited, Mumbai, India) at 4°C and sub-cultured 24h prior to their testing. For antibacterial activity assay these bacteria served as test pathogens. Ethyl acetate *C. erectus* extracts antibacterial potential was evaluated by agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) (Wayne, 2015). Standard antibiotic discs and distilled water served as positive and negative controls respectively. After incubation zone of inhibition diameter was measured in mm. Experiment was conducted in triplicate.

**Minimum inhibitory concentration**

Based on preliminary screening ethyl acetate *C. erectus* extracts that exhibited antimicrobial potential was further evaluated for minimum inhibitory concentration (MIC). Vancomycin was kept as control. Different dilutions of ethyl acetate *C. erectus* extract was prepared in range of (0.01-60µg/ml). Microplate Reader (Biotek Model FL×800, USA) was used for recording of results after incubation period of 24 h at 37°C.

**LC-MS/MS Analysis**

The ethyl acetate *C. erectus* extracts fractions demonstrated antimicrobial activity were analyzed for bioactive compounds identification by LC MS/MS analysis (LTQ XL, Thermo Electron Corporation, USA via Electrospray ionization (ESI) interface). The ionization source temperature was set at 250°C, voltage of source and lens set at 3 kV and 70V respectively. All the data was analyzed by Xcalibur software version 2.0.7 (Thermo Fisher Scientific USA). The spectrums of observed compounds in ethyl acetate *C. erectus* extracts was examined and compared with Sci Finder, Metlin, Mass bank and NIST library.

**Free Radical scavenging activity by DPPH**

Ethyl acetate *C. erectus* extracts DPPH radical scavenging capacity was studied as reported earlier (Shirwaikar et al., 2006). Samples were diluted in ethanol ranging from 0.1-1.0µg/ml. DPPH (1.0 ml) plus ethanol (2.5 ml) kept as negative control and ascorbic acid as positive control. Absorbance of each dilution was recorded at 518 nm after 30 min of reaction in dark and at 25°C. DPPH scavenging ability was expressed as IC50. The experiment was run in triplicate and antioxidant capacity was calculated as:

\[
\%\text{Inhibition} = \frac{100 - (\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100
\]

where Abs sample is absorbance of sample; Abs control is absorbance of control.

**In-vitro biocompatibility assay**

Ethyl acetate *C. erectus* extracts biocompatibility was conducted using RBCs of rat. Blood sample was withdrawn for rat RBCs isolation, centrifuged at 1500 rpm for 5 min and supernatant was discarded and RBCs were washed thrice using sterile Dulbecco phosphate buffer saline (PBS). RBC pellet was purified and diluted 9 times (v/v) using PBS. 100µl RBC suspension was added in each well of 96-well plate (Nadhman et al., 2015) and...
treated with varying concentrations of *C. erectus* as test sample vs vancomycin taken as positive control. Samples were allowed to incubate at 37°C for 2 h followed by centrifugation. Supernatant was collected and absorbance was measured at 485 nm using Microplate Reader (Biotek Model FL×800, USA). Percent hemolysis was calculated.

**Animals**
Rats of age 7-9 weeks and weight 180-200 g taken from Department of Pharmacy Government University College of Faisalabad, Pakistan for this study. Prior to study an ethical approval was obtained from Animal Ethics Committee, Government College University of Faisalabad, Pakistan (Protocol No AEC/GUCF-675/2017). All the experiments in animals in this work were undertaken in accordance with International Conference on Harmonization ICH guidelines (ICH, 2018).

**Multidrug Resistant Sepsis Model in Mice and therapy Induction of sepsis in rats**
To determine therapeutic efficacy of ethyl acetate *C. erectus* extracts in rats initially sepsis model was developed. *S. aureus* isolated from burn patients that was resistant to all tested antibiotics was selected for sepsis induction in rats. For animal inoculation freshly prepared bacterial suspension was used and stored at 2-4°C. Four groups (n=6 per group) of rats were taken Group I (untreated) given normal saline was kept as negative control, while animals in Group II and IV were given single dose with 40μl of inoculum at different concentrations for sepsis induction, Group II (3×10⁷ CFU/ml) Group III (5×10⁷ CFU/ml) and Group IV (6×10⁷ CFU/ml). Animals were monitored for morbidity and mortality for duration of 7 days. At 8th day rats were anesthetized and blood sample was collected through intra-cardiac puncture and stored at 4°C in EDTA vials. The experiments were conducted in triplicate.

**Therapeutic monitoring of sepsis model**
To determine therapeutic potential of ethyl acetate *C. erectus* extracts group that has received 5×10⁷ CFU/ml inoculum in sepsis model study was selected for subsequent experiments. Three groups; n=6 were taken (Group I) untreated animals as negative control, (Group II) dosed with vancomycin as positive control, (Group III) given *C. erectus* extract at dose of 200mg/kg in accordance to reported earlier at clinical significant dose for period of 5 days on daily basis (Arshad et al., 2017). At the end period rats were anesthetized and blood was drawn by cardiac puncture, stored at 4°C in EDTA vials from control and test groups.

**Acute oral toxicity**
Acute oral toxicity of plant extract was evaluated in rats for 14 days to establish LD50 (median lethal dose) according to Acute Toxic Class Method (OECD 2001) for acute toxicity test of single dose (Guideline 423). Healthy male rats 188-200 g and 8-10-week age, were used. Rats were divided into 3 groups n=6 and were kept under standard conditions of food and water under controlled environment. Group 1 administered with normal saline served as negative control, Group II received therapeutic dose of 200mg/kg *C. erectus* studied in above section. Group III received vancomycin at dose of 10mg/kg (Bruniera et al., 2014). The rats were kept under observation for 24 h for any change in body weight and visual observations for mortality, behavior pattern, physical appearance changes and signs of illness were conducted daily throughout the week.

**Organ to body ratio**
Organ weight change was measured for toxicity evaluation after exposure for specific time. The weights of organs from treated groups were compared with control group and body mass index was calculated using formula (Sohail et al., 2017).

$$\text{organ} – \text{body weight index} (\%) = \frac{\text{Wet organ weight}}{\text{Body weight} \times \text{10}^6}$$

**STATISTICAL ANALYSIS**
All experiments were conducted in triplicates and data are presented as mean ± SD. Graph Pad Prism 7.0 was employed to compare all parameters using two-way ANOVA with post-hoc Tukeys test. Significance was considered at p value (<0.05).

**RESULTS**
Antibiotics susceptibility of bacterial isolates performed on erythromycin, clindamycin, methicillin, vancomycin, levofloxacin, moxifloxacin by disc diffusion method. Antibiogram of studied isolates revealed that *S. aureus* was resistant to all antibiotic applied. While the isolates were sensitive and resistant to both vancomycin used in this study.

Ethyl acetate *C. erectus* extracts fractional separation analysis by Thin Layer Chromatography (TLC) visualization at 360 nm revealed presence of various compounds (fig.1). Selected extract phytochemical analysis revealed existence of phenols, alkaloids, flavonoids and tannin as well.

Antibacterial activity of ethyl acetate *C. erectus* extract of leaves determined by agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) revealed comparable results to vancomycin served as positive control. Ethyl acetate *C. erectus* extract showed comparable zone of inhibition at all concentrations against MRSA isolates to vancomycin (fig.2).
Ethyl acetate C. erectus extract mass spectrum showed characteristic peaks at 169.08 (Gallic acid), 301.08 (Quercetin), 285.08 (Luteolin), 329.08 (5-hydroxy-3′4′7 trimethoxy-flavanone), 343 (Caffeoyl-O-hexoside), 423 (Equisetumpyrone) in (fig.3).

Radical scavenging activity of different concentrations of ethyl acetate extracts of C. erectus showed different activity with IC50 values. The highest showed antiradical activity toward DPPH was at IC 0.5µg/ml (fig. 4).

Percent hemolysis of ethyl acetate C. erectus extract and vancomycin at all concentrations presented in fig. 6. C. erectus extracts showed 0.35% hemolysis at its highest concentration, 1.0µg/ml showing biocompatibility with RBCs. Vancomycin demonstrated significantly higher (<0.01) hemotoxicity.

In-vivo sepsis model was optimized in rats using inoculums of varying concentrations (3×10^8 CFU/ml, 5×10^8 CFU/ml, 6×10^8 CFU/ml S. aureus). Animals treated with 3×10^8 CFU/ml showed no significant change in weight and no mortality was observed in this group. The rats inoculated with 5×10^8 CFU/ml presented lethargic condition, increase body temperature and loss in body weight. While animals inoculated with higher dose of 6×10^8 CFU/ml, all rats showed adverse clinical signs of raised body temperature, body weight loss, and 50% animals died by 4th day. The remaining rats were sacrificed at 8th day. All doses effected hematological parameters (Table 1). After 24 h of incubation at 37°C, colony forming unit per ml (CFU/ml) was calculated and represented as log_{10} value.
extract treated group was 0%. Hematological parameters effect shown in Table 2.

**DISCUSSION**

The treatment of drug resistant *S. aureus* infections is a challenge for clinicians all over the world. Bacteria acquire resistance against conventional antibiotics through various mechanisms (Sultan et al., 2018). *S. aureus* resistance mechanism against vancomycin may be due to cell wall thickening. In addition; prior exposure to vancomycin increases chances of isolation of strains of *S. aureus* with reduced susceptibility and reason due to haphazard use of antibiotic (Amatya et al., 2014). At present when infections due to MRSA have become serious public health concern; development and rapid spread of resistance of *S. aureus* to reserve drug (vancomycin) is very fearsome and immediate actions should to be taken to halt it (Amatya et al., 2014). To prevent situation of drug resistance from worsening; use of herbal origin treatment for patients should be preferred. These rapidly emerging drug resistant pathogens impede development process of novel antibiotics, as these learn to resist effect of antibiotics in no time. To counter pan-drug resistant bacteria, plant extracts offer promising direction (Cruz et al., 2014); (Ruddaraju et al., 2020). In present study, ethyl acetate *C. erectus* extract was evaluated which effectively targeted MDR *S. aureus* clinical isolates, both in-vitro and in-vivo.

Antibiogram of clinical isolates from burn patients revealed *S. aureus* was resistant to all antibiotic applied and was little bit sensitive to vancomycin. Resistance of local bacterial isolates may be due to production of beta-lactamase enzymes such as ESBL enzymes which degrade penicillin’s and cephalosporin’s (Salman and Ghaima, 2018). The issue regarding increased sensitivity of subjected drug molecules is higher dosage requirement, which further create drug resistance toxicity. The drug toxicity outcomes represent existing research gap and need to explore natural substances, to replace toxic and resistant drugs for treatment of *S. aureus* resistant strains (Othman et al., 2019).

Phytochemical findings agreed with previous who reported presence of flavonoids, phenols, tannins, glycoside and saponin as active compounds in *C. erectus* aqueous extracts (Nascimento et al., 2016). Current isolation and chemical purification methods used include solvent extraction processes that utilize solvent polarity as major separation technique. These methods frequently include use of ethyl acetate, phenol/chloroform, aqueous, and several other approaches (Tohidi et al., 2017). Ethyl acetate extract of *C. erectus* exhibited positive therapeutic produced zones of inhibitions comparable to vancomycin. Vancomycin MIC against MRSA isolates demonstrated susceptibility to vancomycin MIC ranging from 0.01-1.0 μg/ml consistent with earlier reported results (Kshetry et al., 2016). However, ethyl acetate *C. erectus* extract showed comparable zone of inhibition to positive control. LC-MS has been found to enable accurate identification of compounds in complex extracts with co-eluting peaks and compounds were characterized by comparing obtained molecular (precursor) ions and fragmentation patterns (i.e., product ions) from our LC-MS/MS data with data from NIST database libraries for standard compounds. Ethyl acetate extracts of *C. erectus* leaf showed highest antiradical activity toward DPPH at IC 0.5µg/ml. These results elucidate presence of polyphenols in *C. erectus* extract attributing to antioxidant capacity consistent with earlier results.

Hemolytic activity of vancomycin was increasing as concentration was increased fig. 6. Safety and toxicity of plant extracts must be ensured using scientific validations methods before their recommendation for human use. For injectable formulations erythrocyte % hemolysis served as toxicity indicator (Greco et al., 2020).
Hemolysis resulted from rupturing of lipid bilayer membrane leading to red blood cells destruction. *C. erectus* extracts revealed 0.35% hemolytic activity at its highest concentration showing biocompatibility with RBCs. While vancomycin represented hemolytic activity at all concentrations consistent with previously reported result (Cafiso *et al.*, 2012).

Sepsis model was created and optimized using MDR *S. aureus* strains in rats to evaluate efficacy of plant extract (Harris *et al.*, 2017). Blood samples were prone to hematological assessment to observe changes in blood parameters of *S. aureus* infected rat model organisms treated with vancomycin and *C. erectus* respectively. Significant impact of *C. erectus* treatment over blood cells count was observed declaring validity of *C. erectus* extract to treat drug resistant infections. While vancomycin treated animals showed insignificant change in hematological parameters compared to control. It can be attributed to its hemolytic activity as mentioned in this study in also consistent with previously reported results. *C. erectus* being biocompatible *in-vivo* showing compatibility with RBCs as compared to vancomycin make it favorable therapeutic choice in future against MDR infections. Results obtained in rat sepsis models may not manifest all pathological mechanisms occurring in human body. Despite intrinsic limitation of models, these studies provide basic understanding for this critical human pathogen and helps to establish mechanism of action of novel extracts. Further work is necessary to evaluate pharmacokinetics behavior of suggested plant extract.

**CONCLUSION**

The study enables to explore therapeutic potential of *C. erectus* extract both *in-vivo* and *in-vitro* from cellular level to animal study. *C. erectus* extract demonstrated biocompatibility with RBC, compared to vancomycin. Organ weights remained unaffected with *C. erectus* extract. The ability of extract to treat sepsis model against *S. aureus* may make it efficient future drug delivery system for clinical applications. However, detailed biodistribution studies are required to substantiate the above.

**Table 1**: Effect of inoculum treatments on hematological parameters of rats. Results are represented as mean ± S.D.

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<td>RBC (10⁶/L)</td>
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<td>Hb (g/dL)</td>
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<td>WBC (/mm³)</td>
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<td>Lymphocyte (%)</td>
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**Table 2**: Effect of treatments on hematological parameters of rats. Results are presented as mean ± S.D.

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