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Antibacterial potential of indigenous medicinal plants against methicillin-resistant *Staphylococcus aureus* isolated from septic wounds

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ABSTRACT

Aims: This study was aimed to screen indigenous medicinal plants for their antibacterial potential against methicillin-resistant *Staphylococcus aureus* (MRSA).
Methodology and results: Three indigenous plants (*Nigella sativa*, *Zingiber officinale* and *Calotropis procera*) and thymoquinone were screened for antibacterial activity against MRSA, isolated from septic wounds of patients admitted to Mayo Hospital Lahore, Pakistan. Isolated bacteria were screened for methicillin and cefoxitin resistance by the Kirby-Bauer method, followed by *mecA* gene-specific polymerase chain reaction (PCR). Confirmed MRSA was processed for antibacterial activity of plant extracts and thymoquinone followed by cytotoxicity assay of plant extract having least minimum inhibitory concentration (MIC) value. Out of total samples (n=100), *S. aureus* (29%), MRSA (26%) and vancomycin-resistant *S. aureus* (VRSA) (21.7%) isolates were recovered based on morphology, biochemical profile and antibiotic susceptibility testing. *Nigella sativa* showed the highest antibacterial activity (10.06 ± 6.53 mm) against MRSA followed by *Z. officinale* (4.06 ± 3.72 mm) and *C. procera* (3.65 ± 3.33 mm) in comparison to standard thymoquinone (17.93 ± 10.14 mm). The least MIC value recorded was for *Z. officinale* at 36.89 ± 3.75 µg/mL. *Zingiber officinale* was the most effective antibacterial agent, followed by *N. sativa* and *C. procera* and non-toxic for eukaryotic cells at all tested concentrations (1500 µg/mL to 2.92 µg/mL).
Conclusion, significance and impact of study: It was concluded that *Z. officinale* may be used as an effective alternative for treating septic wound infection in local or topical preparations. As pathogenic *S. aureus* is becoming life-threatening among antibiotic-resistant bacteria and traditional plants are in used for centuries to treat septic wound infections.

Keywords: Cytotoxicity, medicinal plants, methicillin-resistant *Staphylococcus aureus*, thymoquinone, *Zingiber officinale*

INTRODUCTION

The developing resistance in infectious organisms to antimicrobials has become a serious predicament to fight infections using traditional antibiotics (Chai *et al*., 2022). Infectious diseases are becoming a major element of morbidity and mortality. About one-third of the total deaths in the world transpire from infectious diseases caused by pathogenic bacteria (Reddy *et al*., 2021). Treatment of MRSA-associated infection is already a challenging trait and rapidly thriving resistance has worsened the situation for public health and hospital clinicians. MRSA had developed resistance to even vancomycin and is increasing worldwide. The situation is constraining the researchers to screen better novel molecules as alternate antibacterial agents to handle a load of pathogens in the future for patients with multiple drug-resistant (MDR) infections (Craft *et al*., 2019).

Herbal remedies are now found as an integral part of the primary health care settings due to their safe and effective physiological effects. World Health Organisation had emphasized the use of medicinal plants in exploiting the treatment aptitudes (anonymous). Screening of novel antibacterial agents of plant origin against MDRs would

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present more treatment preferences to the health care professionals. Medicinal plants could present a useful alternative therapy options due to the presence of beneficial phytochemicals (gums, tannin resins, flavonoids, resins, phenolic components, alkaloids, steroids or fatty acids, etc.) having therapeutic value (Okwu *et al*., 2019). Medicinal plants are now extensively investigated for physiologically active molecules having antibacterial potential (do Nascimento *et al*., 2021).

The present study was planned to evaluate the antibacterial activity of thymoquinone and three medicinal plants (*Z. officinale*, *C. procera* and *N. sativa)* extracts against MRSA isolated from septic wounds from indoor patients in selected tertiary care hospital.

MATERIALS AND METHODS

MRSA was isolated from septic wounds of patients admitted to Mayo Hospital, Lahore and accessed for susceptibility to antibiotics, plant extracts (*Z. officinale*, *C. procera* and *N. sativa*) and thymoquinone.

Isolation and biochemical identification of *S. aureus*

Swab samples (n=100) were collected from septic wounds of the patients admitted to Mayo Hospital, Lahore Punjab. Sample swabs stored at low temperature were transferred to Bacteriology Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore. The swab samples were inoculated on nutrient agar followed by incubation for 24 h at a temperature of 37 °C. Colonies were purified on mannitol salt agar (MSA) plates. Plates were incubated at 37 °C for 24 h.

Bacterial identification was carried out by colony and microscopic morphology. Biochemical testing (catalase test, blood hemolysis and coagulase test) was carried out according to Bergey's manual of determinative bacteriology's protocol (Holt *et al*., 1994). Coagulase-positive isolates were selected and preserved in glycerol (15%) for further reference (Rupert *et al*., 2022).

Screening and molecular confirmation of MRSA

Isolates identified as MRSA were preceded for screening methicillin (oxacillin 1µg) and cefoxitin (30 µg) resistance by disk diffusion method of Kirby-Bauer according to the provided guidelines of Clinical and Laboratory Standards Institute (CLSI) 2020 manual. ATCC-29213 was used as a control for *S. aureus* culture. Isolates that showed methicillin resistance were tested for susceptibility to vancomycin (5 µg) and ampicillin (25 µg). A uniform lawn of standard bacterial inoculum (equal to 0.5 MacFarland) was prepared on Mueller-Hinton agar (MHA). Disks were applied and plates were incubated at 37 °C for 24 h. Plates were observed for resistance based on the Inhibition zone in millimeters.

MRSA was confirmed by PCR targeting *mecA* gene using gene-specific primers i.e., forward (5'-TGGCATTCGTGTCACAATCG-3') and reverse (5'-CTGGAACTTGTTGAGCAGAG-3') (Pu *et al*., 2014). DNA was extracted using a DNA extraction kit (Vivantis, Nucleic acid Extraction Kit GF-1). PCR was carried out using initial denaturation (94 °C for 4 min) followed by 34 cycles of denaturation (92 °C for 1 min), annealing (53 °C for 50 sec), and extension (72 °C for 1 min) and final extension (72 °C for 10 min) and stored at 4 °C. It was visualized under a gel documentation system and observed for *mecA* gene (310 bps) on 1.5% agarose gel.

Antibacterial activity of plant extracts and thymoquinone

Three plants (*N. sativa*, *Z. officinale* and *C. procera*) were collected. Seeds of *N. sativa*, the rhizome of *Z. officinale* and leaves of *C. procera* were crushed down to fine powder. Extraction was carried out by Soxhlet apparatus using ethanol as solvent and ethanolic extracts were prepared (100 mg/mL) (Ertürk, 2006). Extracts were dried in a rotary evaporator and stored in a dark bottle. The percentage yield of the extracts was calculated and compared statistically.

The stock solution of each plant extract (0.1 g/mL) and thymoquinone (10 µg/mL) was prepared in dimethyl sulphoxide (DMSO). Antibacterial activity of the plant extracts and thymoquinone was determined by the well diffusion method (Magaldi *et al*., 2004). MHA plates were prepared and inoculum from fresh culture was swabbed over test plates to make a bacterial lawn. Wells were formed by well borer and sealed with molten agar. About the volume of 100 µL of plant extracts (100 mg/mL) and thymoquinone (10 µg/mL) were poured into wells. Plates were incubated for 24 h at 37 °C. Zone of Inhibition was recorded using the measuring scale in millimeters.

MIC of the plant extracts and thymoquinone was determined by the micro broth dilution method using 96 well micro-titration plates (Fon *et al*., 2018). Bacterial inoculum was prepared and standardized. Two-fold serial dilution was made having a constant volume of Mueller Hinton Broth with different concentrations (5000 to 9.765 µg/mL) of test agents (plant extracts and thymoquinone). Standard bacterial inoculum (100 µL) was added to each well. After 24 h of incubation at 37°C, the optical density at wavelength 620 nm was determined using an enzyme-linked immunosorbent assay (ELISA) reader.

Cytotoxicity assay

Plants extracts showing MIC were selected. Cytotoxicity was determined by MTT assay for plant (*Z. officinale*) using a confluent layer of Vero cell line grown in 96 well flat bottom micro-titration plates. Stock solution (0.3g/2mL DMSO) of *Z. officinale* was prepared. Required concentration was made by reconstituting the 0.1 mL of stock solution in 9.9 mL of cell culture media. From this 100 µL of the extract was taken and added into 1st well followed by two-fold serial dilution i.e., concentration ranging from 1500 to 2.92 µg/mL. Each dilution (100 µL) was added to plates having a confluent layer of Vero cell

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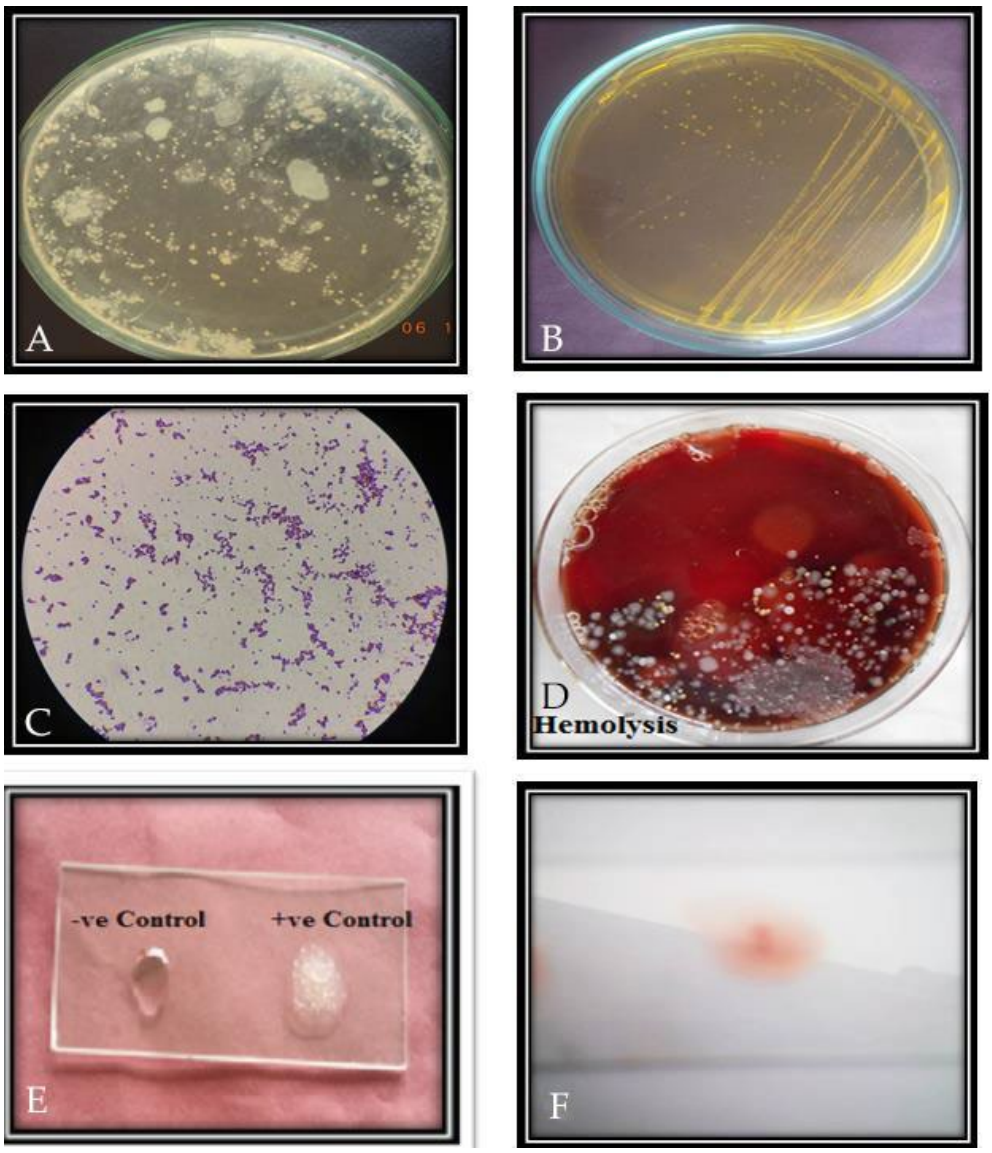

Figure 1: Primary culture, microscopic view and biochemical profile of *Staphylococcus aureus*. (A) Primary culture, (B) Yellow colonies of *S. aureus* on mannitol salt agar, (C) Blue color gram-positive cocci in clusters, (D) Blood hemolysis, (E) Catalase test and (F) Coagulase test.

line. Negative (20% DMSO) and positive (Vero cell line) controls were run along with the test. Plates were incubated at 37 °C for 24 h. The percentage of cells survival was calculated using the formula:

CSP = [(Mean OD of test – Mean OD of negative control)/Mean OD of positive control] × 100

Statistical analysis

Results obtained were analyzed by one-way analysis of variance (ANOVA) using Duncan's multiple range test (DMRT) by statistical package for social sciences (SPSS, 20.0).

RESULTS

Isolation and identification

All of the 100 isolates revealed bacterial growth in primary culture on nutrient agar. Out of 100 cultured bacteria, 81 isolates were able to grow on MSA. Out of 81 isolates, only 33 were positive for mannitol fermentation. Among 33 isolates, 29 isolates have microscopic morphology (Gram-positive cocci as clusters of blue colored grapes) of *S. aureus*. *S. aureus* isolates were further identified based upon biochemical profile i.e., blood hemolysis, catalase test and coagulase test. Out of 29 isolates, 26 were declared as pathogenic *S. aureus* (Figure 1).

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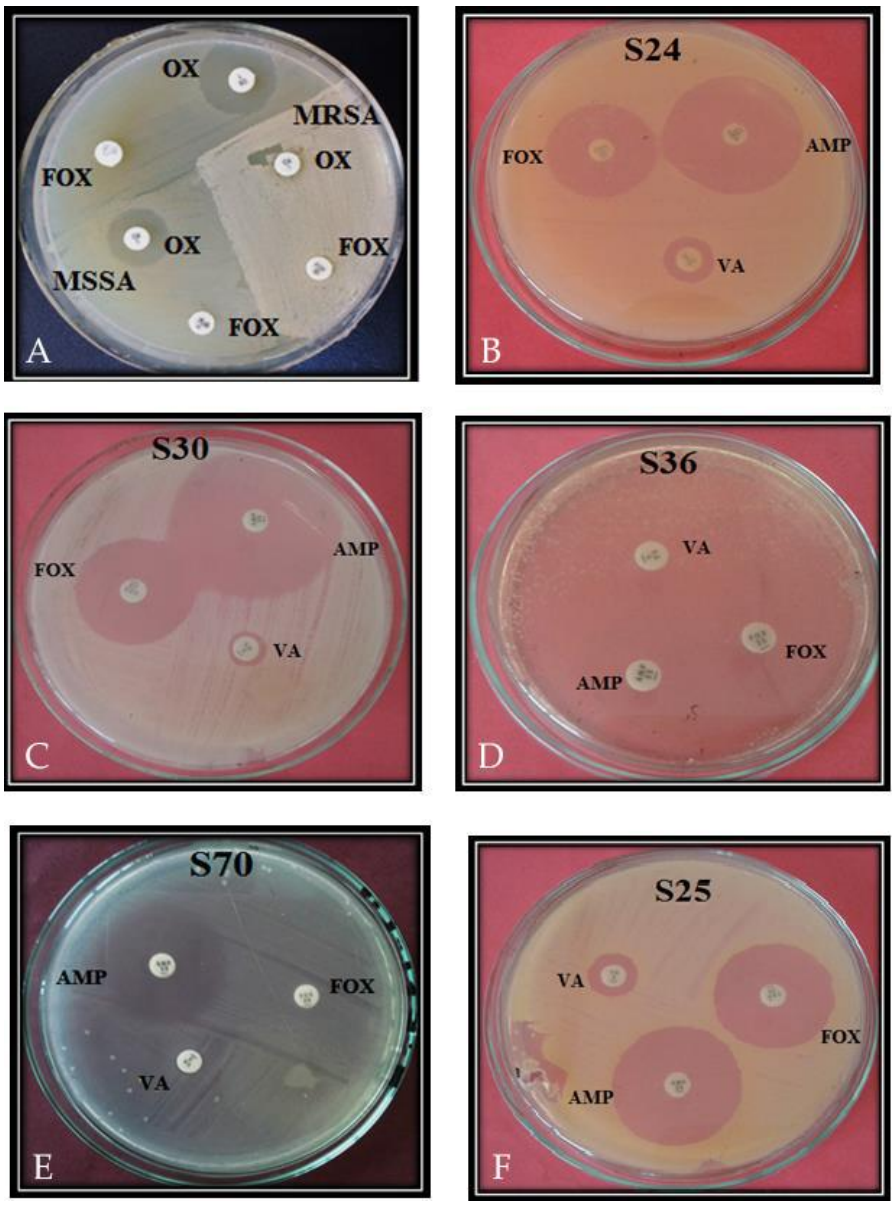

Figure 2: Antibiotic susceptibility testing of methicillin-resistant *Staphylococcus aureus* isolates. (A) Oxacillin/Cefoxitin disk plate, (B) Isolate S24, (C) Isolate S30, (D) Isolate S36, (E) Isolate S70 and (F) Isolate S25.

Antibiotic susceptibility testing

Isolates were marked as resistant, intermediates and sensitive according to the CLSI-2020 breakpoint. Isolates that were showing ZOI less than the standard ZOI or no ZOI were recorded as resistant (Figure 2). Out of 26 coagulase-positive *S. aureus* isolates, 23 (88.4%) showed resistance to oxacillin and cefoxitin.

MRSA isolates (n=23) were tested for vancomycin (VA) and ampicillin (AMP) susceptibility by well diffusion method according to the CLSI-2020 recommended standards. Out of 23 isolates, 20 isolates were found sensitive to ampicillin. Whereas out of a total of 23 isolates, 8 (34.7%) were sensitive, 11 (47.8%) were intermediate and 5 (21.7%) were resistant to vancomycin. The isolation rate of the vancomycin-resistant MRSA in the present research study recorded was 21.73% (Figure 2).

Molecular confirmation of MRSA

MRSA isolates showing resistance to VRSA were selected for *mecA* gene amplicons by PCR. Amplicons of 310 bp were considered positive for MRSA (Figure 3). All of the tested isolates were positive for *mecA* gene amplification by PCR.

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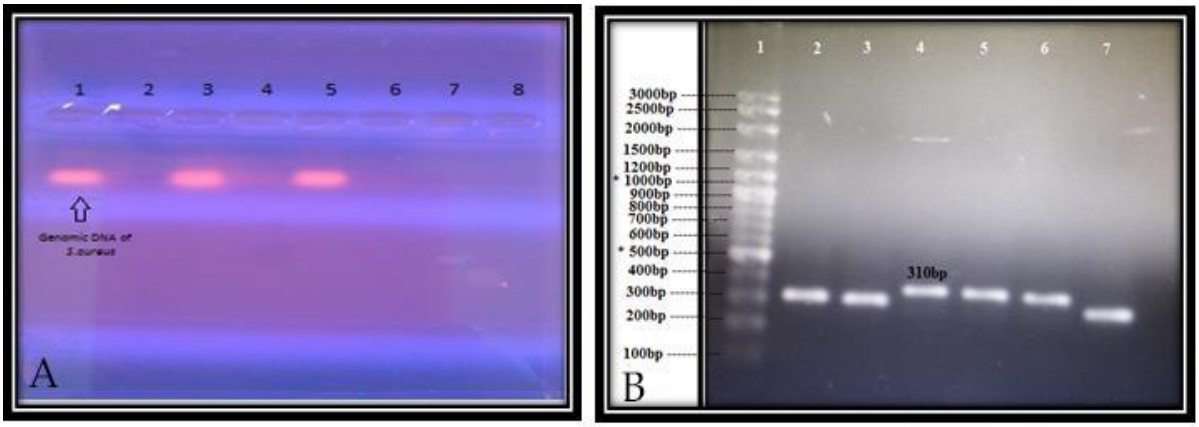

Figure 3: Molecular confirmation of methicillin-resistant *Staphylococcus aureus*. (A) Detection of extracted DNA using agarose under 260 nm wavelength and (B) The amplicons of *mecA* gene along with DNA ladder (VC 100 bp, 0.5 µg/uL) on 2% agarose gel in gel documentation system at 260 nm wavelength.

Table 1: Comparison of ZOI and MIC of *Nigella sativa*, *Zingiber officinale* and *Calotropis procera* with thymoquinone against *S. aureus* (n=5).

Sr. no	Plant extract	ZOI	MIC
		Mean ± SD	Mean ± SD
1	*Nigella sativa*	10.06 ± 6.53[b]	172.52 ± 169.89[a]
2	*Zingiber officinale*	4.06 ± 3.72[a]	36.89 ± 3.75[a]
3	*Calotropis procera*	3.65 ± 3.33[a]	694.44 ± 524.29[b]
4	Thymoquinone (Positive control)	17.93 ± 10.14[b]	4.37 ± 1.25[a]

Values having the same superscripts (a, b) differ non-significantly and with different superscripts differ significantly. Each value represents the means of three replicates.

Anti-MRSA activity of plant extracts and thymoquinone

N. sativa, *Z. officinale* and *C. procera* plants were got identified by the Department of Botany, Government College University (GCU), Lahore, Pakistan. The reference number allotted to *N. sativa* was GC. Herb. Bot. 2991, for *Z. officinale* was GC. Herb. Bot. 2993 and for *C. procera* was GC. Herb. Bot. 2992 (Figure 4). Commercially available thymoquinone having reported anti-MRSA activity was imported from America and its stock solution (10 µL) was prepared. The mean percent yield of *Nigella sative*, *Z. officinale* and *C. procera* was 1.2, 2.08 and 1.58, respectively. Statistically, a significant difference was observed in the mean yield of plant extracts.

Zones of inhibition were recorded for three tested plant extracts against five vancomycin-resistant MRSA isolates (Figure 5). Thymoquinone was used as a standard drug. All three plant extracts showed antibacterial activity against some tested MRSA isolates. Thymoquinone and *N. sativa* show no significant difference with each while differ significantly from *C. procera* and *Z. officinale.* However, *Z. officinale* and *C. procera* differ non-significantly from each other (Table 1). Statistically, *Z. officinale* had exhibited maximum efficacy in the present research study against *S. aureus* after the standard drug thymoquinone.

MIC values of plant extracts were calculated vancomycin-resistant MRSA isolates which showed sensitivity in the well diffusion method. Overall Mean MIC values of *N. sativa*, *Z. officinale*, *C. procera* and thymoquinone were compared statistically (Table 1). The lowest effective concentration observed was for *Z. officinale* and the highest for *C. procera.* All three plant extracts had shown antibacterial activity against tested MRSA isolate*. Z. officinale* showed more comparable results with thymoquinone, thus found most effective against MRSA among other tested extracts.

Cytotoxicity assay

Cell survival percentage (CSP) using mean OD ± SD was calculated (Table 2). The CSP for concentration ranging from 1500 to 2.92 µg/mL recorded were 56.91, 64.77, 67.29, 69.96, 79.44, 65.72, 64.46, 65.72, 66.03 and 65.72%, respectively. It was concluded based upon present findings that ethanolic extract of *Z. officinale* had higher *in vitro* efficacy against vancomycin-resistant MRSA isolates comparable with thymoquinone and was non-toxic for eukaryotic cells.

DISCUSSION

A disquieting upturn of the antibiotic resistance in superbugs (pathogenic strains) has lowered the treatment

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Figure 4: Medicinal plants with their identification reference number. (A) *Nigella sativa* seeds, (B) *Nigella sativa* reference number, (C) *Calotropis procera* leaves, (D) *Calotropis procera* reference number, (E) *Zingiber officinale* rhizome and (F) *Zingiber officinale* reference number.

Table 2: MTT assay for cell survival percentage of ethanolic extract of *Z. officinale* at various concentrations.

Sr. no	Extract concentrations (µg/mL)	Mean ± SD	Cell survival percentage (%)
1	I500	0.269 ± 0.033	56.91
2	750	0.294 ± 0.007	64.77
3	375	0.302 ± 0.022	67.29
4	187.5	0.312 ± 0.016	69.96
5	93.75	0.296 ± 0.044	79.44
6	46.85	0.297 ± 0.025	65.72
7	23.43	0.293 ± 0.018	64.46
8	11.71	0.297 ± 0.005	65.72
9	5.85	0.298 ± 0.022	66.03
10	2.92	0.297 ± 0.014	65.72
Negative control	20% DMSO	0.088 ± 0.024	0.00
Positive control	Cell culture media	0.318 ± 0.022	72.32

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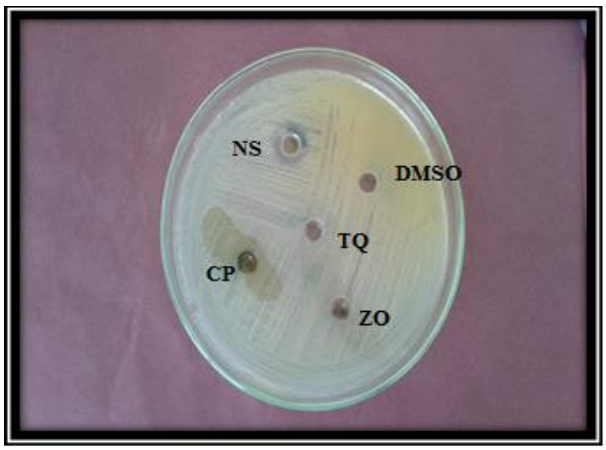

Figure 5: Antibacterial activity of plant extracts against methicillin-resistant *Staphylococcus aureus* isolates. NS, *Nigella sativa*; ZO, *Zingiber officinale*; TQ, Thymoquinone; CP, *Calotropis procera*; DMSO, Dimethyl sulfoxide.

options and medicinal plants would present more treatment preferences to the health care professionals. In the present study, 29% of *S. aureus* isolates were recovered on MSA based upon morphology and biochemical characteristics from septic wounds of patients admitted to the tertiary care (Mayo) hospital and is in agreement with Al-Zoubi *et al.* (2015) as well Ayub *et al.* (2015) studies i.e., 31.6 and 48.89% *S. aureus* isolates respectively. While, a comparatively low percentage (23.94%) was reported in research (Hassan *et al*., 2019). MRSA (26%) recovered in present findings were in agreement with Kahsay *et al.* (2014) i.e. 49.7% MRSA from surgical wounds and Rai *et al.* (2017) findings i.e., 35.5% MRSA. Lowered percentage (6.57%) of the MRSA isolated from wounds in a study by Lena *et al.* (2021) was due to the improved hygienic status in hospitals in the UK. Mechanism of resistance includes altered membrane permeability, production of PBP's (penicillin-binding proteins) and modification in the drug. Antibiotic sensitivity of *S. aureus* isolates was tested against oxacillin (1 µg), cefoxitin (30 µg), ampicillin (25 µg) and vancomycin (5 µg) antibiotics. The maximum number (88.46%) of the *S. aureus* isolates had shown resistance to oxacillin and cefoxitin. Similar findings were reported by researchers (Farahani *et al*., 2013; Dibah *et al*., 2014). While, oxacillin identified 80% of MRSA while sensitivity to cefoxitin gave 100% MRSA (Pourmand *et al*., 2014). The emergence of the resistance compelled health care providers to emphasize the use of 2nd line antibiotics use but it would increase the economics and decrease the treatment options (Sharma and Vishwanath, 2012). A pathogenic strain has developed to vancomycin for the treatment of MRSA strains lowering the treatment options. In the present study, 21.7% of isolates were found resistant to vancomycin and these findings are in agreement with the study of Hasan *et al.* (2016) i.e. 62% vancomycin resistance MRSA strains from burn wounds, whereas, only 0.4% vancomycin-resistant strains were reported in an investigation due to steady increase in MIC range with time (Naghavi-Behzad *et al*., 2014). Furthermore, these five isolates were processed for molecular confirmation by PCR and 100% of the isolates were found positive for *mecA* gene similar findings were reported by researchers (Roghmann, 2000).

Herbal medicines provide an effective alternative for the treatment of ailments for centuries due to phytochemicals having therapeutic value (Valle *et al*., 2015). Three local plants (*Z. officinale*, *N. sativa* and *C. procera)* and thymoquinone were investigated for antibacterial activity against VRSA. Thymoquinone was imported from America as it is not yet been manufactured in Pakistan. Percentage yield obtained for *Z. officinale*, *N. sativa* and *C. procera* was 1.2, 2.08 and 1.58%, respectively were in agreement with researchers i.e., *Z. officinale* (4.7%), *N. sativa* (3%) and *C. procera* (9.13%), respectively (Ogudo *et al*., 2014). Plant contents vary with cuts, age of the plant and the environment with soil fertility and composition etc. About 60% of the isolates were susceptible to *Z. officinale* and *C. procera* to similar results reported by Salem and his co-workers, whereas 80% of isolates showed antibacterial activity with *N. sativa.* All three plant extracts had showed antibacterial activity against some MRSA isolates compared with standard thymoquinone for anti-MRSA activity (Salem *et al*., 2014).

MIC was determined for the isolates showing ZOI against plant extracts. The lowest effective concentration of 36.89 ± 3.75 µg/mL observed was for *Z. officinale* after thymoquinone (standard) and the highest was 694.44 ± 524.29 µg/mL for *C. procera.* Statistically, thymoquinone, *Z. officinale* and *N. sativa* differ non-significantly from each other, while *C. procera* differ significantly. MIC values reported in the literature for *Z. officinale* were 78.125 µg/mL, for *C. procera* was 14.5 µg/mL and for *N. sativa* was 409.6 µg/mL (Liaqat *et al*., 2015). The degree of antibacterial activity of these three tested plant extracts against vancomycin-resistant MRSA can be graded in the following order: *Z. officinale* > *N. sativa* > *C. procera*.

The safety profile of *Z. offIcinale* was determined by MTT assay on Vero cell line. It was found non-toxic for eukaryotic cells at all tested concentrations (1500 µg/mL to 2.92 µg/mL) even at the highest dose of 5000 mg/kg of the bodyweight no significant toxicity is observed (Plengsuriyakarn *et al*., 2012). So, *Z. officinale* may effectively be used as an alternate for treating septic wound infections caused by vancomycin-resistant MRSA. The irrational use of antibiotics, lack of newer antibiotics and emergence of the resistance have limited the treatment options. Traditional plants are in used to treat ailments for years, but not much is known about their pharmacologically active principles and *in vivo* safety profile; thus, needs further support from the animal and human studies to find out therapeutic efficacy, therapeutic window, optimum dosage regimen and *in vivo* clinical trials to confirm safety profile before considering traditional plants for routine use.

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CONCLUSION

It was concluded that ethanolic extract of *Z. officinale* has higher *in vitro* efficacy against vancomycin-resistant MRSA isolates comparable with thymoquinone and is non-toxic for eukaryotic cells at all tested concentrations in *in vitro* cytotoxicity testing. So, *Z. officinale* extract can be the alternative therapeutic option to treat septic wound infections caused by vancomycin-resistant MRSA.

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