International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2022.1219.1228

Research Article
Carthamus tinctorius L., as an Anti-virulence Intervention Against Methicillin Resistance *Staphylococcus aureus*

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Abstract

Background and Objective: Methicillin-Resistant *Staphylococcus aureus* (MRSA), is a common antibiotic-resistant bacterium leading to severe community-acquired infections with limited treatment options. The present study was designed to analyze the antibacterial activity of hydroalcoholic extract of *Carthamus tinctorius* L., against Methicillin-Resistant *Staphylococcus aureus* (MRSA). **Materials and methods:** The evaluation of *Carthamus tinctorius* L. was done by agar well diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Further, the production of virulence factors like adhesion, biofilm formation, hemolysis, staphyloxanthin production and related genes were also investigated to confirm antibiofilm activity. **Results:** In the agar well diffusion method, the zone of inhibition was observed at low dosage (1.6 mg mL^{-1}) and increased with higher dosage. In, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), 50% inhibition of Methicillin-Resistant *Staphylococcus aureus* (MRSA) were observed in 25 mg mL^{-1} and 100% was observed in 50 mg mL^{-1} which showed decreased biofilm formation, hemolytic activity, staphyloxanthin production and its Hydrogen Peroxide resistance. Modulation of mecA and spA genes was observed on treatment with extract by decreasing its gene expression. **Conclusion:** Since, hydroalcoholic extract act against the virulence factor involved in biofilm formation, it can be used as an alternative substitute for antibiotic treatment.

Key words: MRSA, *Carthamus tinctorius*, hydrophobicity, anti-biofilm, anti-virulence, antibacterial, gene expression

Citation: Abdulhaq, A.A., 2022. *Carthamus tinctorius* L., as an anti-virulence Intervention against methicillin resistance *Staphylococcus aureus*. Int. J. Pharmacol., 18: 1219-1228.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Antimicrobial resistance has created a global threat due to the increased usage of antibiotics, which resulted in the inefficacy of antimicrobial agents[1]. Transmission of bacterial resistance has increased morbidity and mortality[2] which raised great demand for alternative treatments for infectious diseases. Methicillin-Resistant *Staphylococcus aureus* (MRSA) is common antibiotic-resistant bacteria, predominant all over the world leading to many nosocomial and community-related infections[3]. Although vancomycin is considered a first-line antibiotic to treat MRSA, it should not exceed 2 gm[4]. So, it is very crucial to explore herbal medicine which can provide antimicrobial action without allowing the emergence of a new resistant strain.

Carthamus tinctorius L., is safflower or false saffron and belongs to the compositae or asteraceae family. It is used as an analgesic and antipyretic is useful in patients with poisoning[5]. But traditionally used for cardiovascular, cerebrovascular and the gynaecological complications. Furthermore, used as an anticoagulant, antioxidative and anticancer agent[6]. It possesses several phytochemical components like phenolics, flavonoids, alkaloids, lignans, carboxylic acids, steroids, polysaccharides, quinochalcone C-glycosides and quinone-containing chalcones[7].

Though antimicrobial screening of safflower has been carried out previously, its mechanism of action remains to be studied against resistant bacteria, especially MRSA. The current study was carried out to evaluate the effect of safflower on MRSA to determine its bacteriostatic and bactericidal activity. Further, experiments were performed to assess the effect of bacterial hemolysis and the formation of biofilm and virulence factor-related gene expression.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Medical Laboratory Technology in Jazan University, Jazan-Kingdom of Saudi Arabia from February-April, 2022.

Plant extract preparation: *Carthamus tinctorius* L. flower was dried and immersed in 70% ethanol (200 g L^{-1}) for 48 hrs with periodic stirring. After 48 hrs, the hydroalcoholic extract was filtered using a whatman filter No.1 (Sigma Aldrich, US) and the ethanolic solution was evaporated at 40°C. The dried extract was stored at 4°C until use.

Agar well diffusion: Agar well diffusion method was performed by the previous authors[8] with slight modification. For the well diffusion method, the MHA plates were cultured with MRSA from the overnight broth by swab culture method and then, the wells were prepared with the help of a cork-borer. About 100 µL of the samples of different concentrations (1.6, 3.125, 6.25, 12.5, 25, 50, 100 and 200 mg mL^{-1}) was added to the well. The plates were incubated overnight at 37°C. The zone of inhibition was observed and the diameter of the zone was measured. The results were compared with the standard antibiotic chloramphenicol (30 mcg/disc).

MIC: The antibacterial potential was determined by the standard broth dilution method (CLSI M07-A9)[9] through visualization of bacterial growth in the broth. The extract was serially two-fold diluted in different concentrations in the range of 0.1-200 mg mL^{-1} and control with MRSA alone was incubated at 37°C for 24 hrs. The lowest concentration of extract that shows the absence of microbial growth is the minimum inhibitory concentration.

MBC: From MIC well with no turbidity, 50 µL of aliquots was seeded in MHA agar plates and incubated at 37°C for 24 hrs. The lowest concentration which inhibits 99.9% of bacterial growth was determined as the minimum bactericidal concentration.

Time kill assay: Tubes with Muller Hinton Broth with MRSA inoculum of 5×10^6-1×10^7 CFU mL^{-1} with different concentration of extract (12.5, 25 and 50 mg mL^{-1}) was analysed for bacterial survival at 0, 4, 8, 12 and 24 hrs. Surviving bacteria were counted at 0, 4, 8, 12 and 24 hrs by culturing 50 µL serial dilutions (10^{-1}, 10^{-2} and 10^{-4}) of samples taken in normal saline and plated in Mueller-Hinton agar at 1/2 MIC, MIC and 2x MIC of extract.

Biofilm assay: Biofilm assay was performed by Kimyon *et al.*[10] with slight modification. The overnight culture was normalized to OD600 = 0.4 and treated with different concentrations (12.5, 25 and 50 mg mL^{-1}) of extract in microtiter plates and incubated for 24 hrs at 37°C with MRSA culture alone as a positive control. Each well was washed with PBS and fixed with 200 µL of 99% methanol for 15 min. After removing methanol, bacterial biofilm was stained with 0.1% crystal violet for 15 min. The stain was removed by washing with distilled water and dried completely. The 99% ethanol was added to dilute crystal violet bound biofilm and read at 570 nm in a microplate reader. (BioTek Instruments, Inc.US).

Hemolysis assay: The hemolysis was quantified by the potential of extract to lyse human red blood cells. Various concentration of extract (12.5, 25 and 50 mg mL^{-1}) was added along with MRSA culture to 3% RBC suspension and incubated

for 1hr at 37°C and shaking at 100 rpm. The complete mixture was centrifuged at 3000xg for 10 min and read at 543 nm using a UV spectrophotometer (Thermo fisher scientific, US).

Microbial surface hydrophobicity index: The adhesion of microbes to hydrocarbon was carried out to determine Bacterial surface hydrophobicity[11]. MRSA was treated with the extract (12.5, 25 and 50 mg mL^{-1}) and incubated at 37°C for 24 hrs and centrifuged to harvest and washed with phosphate-buffered aline and adjusted to 0.3 OD at a 600 nm spectrophotometer. The adjusted bacterial suspension was mixed with Toluene (200 µL) and absorbance was recorded after phase separation from the aqueous phase (Af). The hydrophobicity index (HPBI) was calculated by:

$$\frac{(Ai\text{-}Af)}{Ai}\times 100\%$$

Quantification of staphyloxanthin: Staphyloxanthin quantitative analysis was carried out by Silva *et al.*[12] with slight modification. For the extraction of carotenoid pigments the culture pellet was suspended in 0.2 mL of methanol with vortexing and heated at 55°C for 30 min. The cell debris was separated by centrifugation at 16,600 g for 10 min for pigment extraction and repeated thrice to maximize staphyloxanthin yield. The extraction was measured at 465 nm using UV-spectrophotometer.

Evaluation of staphyloxanthin hydrogen peroxide resistance: MRSA was cultured with extract for 24 hrs and harvested by centrifugation and washed with sterile saline. The bacterial suspension was adjusted to OD600 of 0.150 with Phosphate buffered saline solution and incubated with 1.5% of H_2O_2 for 60 min at 37°C under 150 rpm shaking. The cell survival percentage was calculated by several colony-forming units (CFU mL^{-1}).

PCR reaction to mecA and spA: The PCR reaction was carried out on a final volume of 25 µL containing 2.5 µL of DNA, 1 µL of primer, 8 µL of distilled water and 12.5 uL PCR Master Mix (Takara). The temperature of the PCR reaction includes the initial denaturation at 95°C for 2 min and the 35 cycles with the denaturation at 95°C for 30 sec, the annealing at 52.2°C for 1 min (mecA) and 54.1°C for 1 min (spA) and the extension at 72°C for 1 min and the final extension at 72°C for 1 min. The amplified PCR products were run on 1% agarose gel electrophoresis with ethidium bromide staining. mecA primers (Forward-5'CAGGTACTGCTATCCACCCTC3') and (Reverse-5' TGAGTTCTGCAGTACCGGAT 3') gene were designed. spA primers (Forward-5'GAAGACGGCAACGGAGTACA 3') and (Reverse-5'GCGACGACGTCCAGCTAATA 3') gene were designed.

Statistical analysis: Triplicates were performed for all the assays and the results obtained were subjected to statical analysis using GraphPad Prism version 5.1. The data was explained in Mean ± Standard deviation and analysed by One-way Analysis of Variance (ANOVA) with Tukey's multiple t-tests.

RESULTS

Agar well diffusion: The hydroalcoholic extract of *Carthamus tinctorius* L. showed good antibacterial activity against MRSA compared with Chloramphenicol. The diameter of the zone of inhibition shown by extract in increased dosage is represented in Table 1 and Fig. 1a-c.

Determination of MIC and MBC: The MIC of the extract against MRSA was determined by the microdilution method. The MIC value was observed at 25 mg mL^{-1} showing 50% inhibition of bacterial growth depicted in Fig. 2. In the case of the MBC study, complete inhibition of bacterial growth was observed at 50 mg mL^{-1} using the spread plate method shown in Fig. 3a-c.

Time kill assay: Figure 4 showing an inhibitory effect in 12.5, 25 and 50 mg mL^{-1} of extract. The culture treated with extract showed a decrease in CFU count with an increased concentration of extract compared to control. The ideal growth behaviour of MRSA was observed at 24 hrs of incubation in the control group. The CFU count reduced with an increase in concentration at 4 hrs which ended up with significant growth inhibition followed by 8, 12 and 24 hrs of incubation shown in Table 2.

Table 1: Zone of inhibition of MRSA with extract

Dose (mg mL^{-1})	Zone of Inhibition (in mm)
Water	-
DMSO	-
Chloramphenicol	10
1.6	06
3.125	07
6.25	10
12.5	13
25	13
50	14
100	15
200	18

Zone of inhibition of MRSA with *C. tinctorius* L. extracts at different concentrations in mg mL^{-1}, chloramphenicol disc 30 mcg/disc used as standard

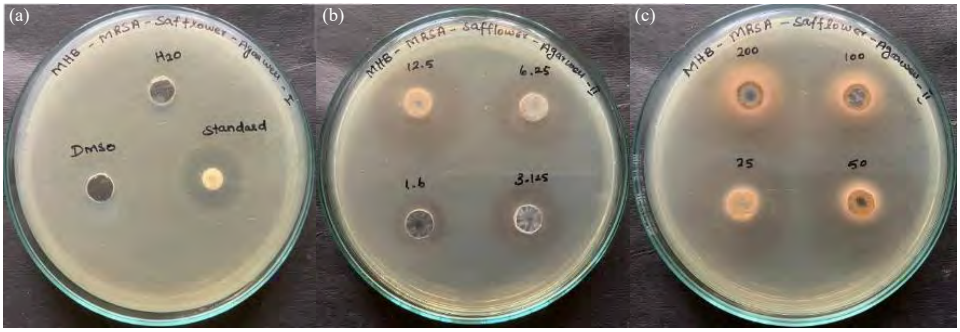

Fig. 1(a-c): Figure showed the zone of inhibition, (a) Water, DMSO and chloramphenicol standard (30 mcg/disc), (b) *C. tinctorius* L. hydroalcoholic extract at 1.6, 3.125, 6.25 and 12.5 mg mL^{-1} and (c) 25, 50,100 and 200 mg mL^{-1}

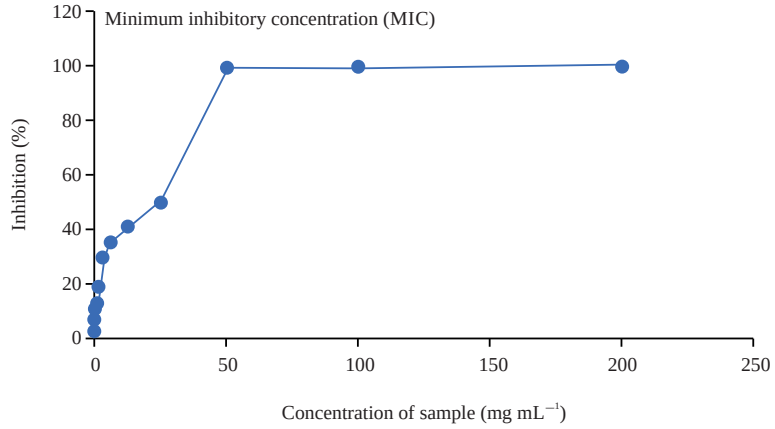

Fig. 2: MIC of MRSA by *C. tinctorius* extract at a concentration range between 0 - 200 mg mL^{-1}
Graph showed the 49.51% inhibition at 25 mg mL^{-1} of extract

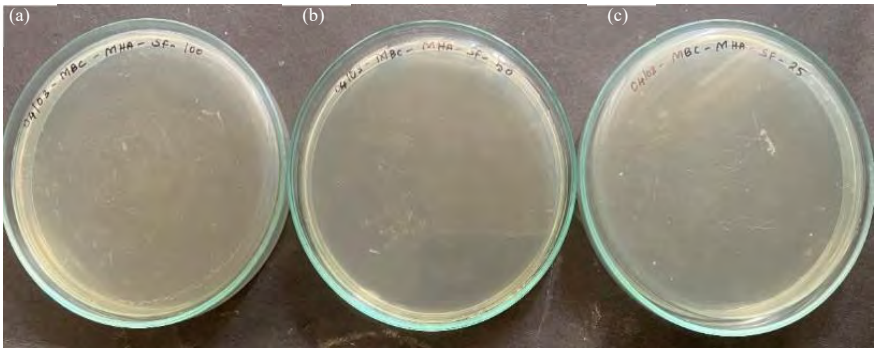

Fig. 3(a-c): Figure showed the MBC of MRSA, (a, b) Showed nil colonies at 100 and 50 mg mL^{-1}, respectively considered as MBC value for the extract and (c) Showed <10 colonies

Table 2: Colony-forming unit in time-kill assay

	0 hr	4 hrs	8 hrs	12 hrs	24 hrs
Control (mg mL^{-1})	4.8×10^{5}	TNTC	TNTC	TNTC	TNTC
12.5	3.5×10^{5}	1.4×10^{4}	1.2×10^{4}	1.0×10^{4}	7×10^{2}
25	3.6×10^{5}	1×10^{3}	6×10^{2}	3×10^{2}	0.3×10^{1}
50	4.3×10^{5}	4×10^{2}	0.4×10^{2}	0.01×10^{2}	-

Table showed the number of colonies of MRSA treated with extract of various concentrations (12.5, 25 and 50 mg mL^{-1}) at different periods such as 0, 4, 8, 12 and 24 hrs and the result showed the decrease in colony count as prolonging incubation time in a dose-dependent manner

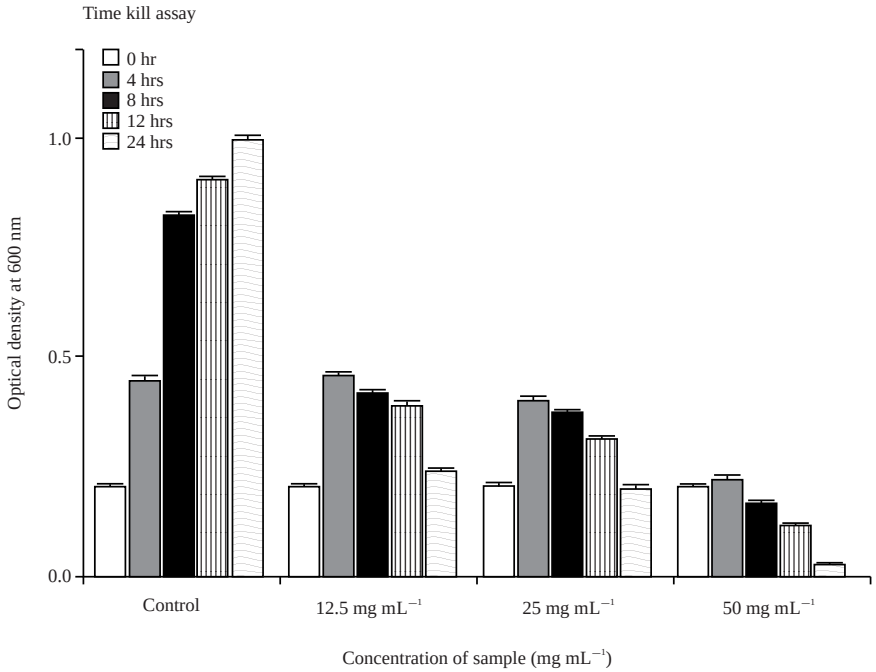

Fig. 4: Time-kill assay in absorbance (OD at 600 nm) value of growth of MRSA at different periods treated with various concentrations (12.5, 25 and 50 mg mL^{-1}) of *C. tinctorius* L. extract

Data are expressed as Mean±SD and treated groups showed high significance compared to the induced group ($p<0.0001$)

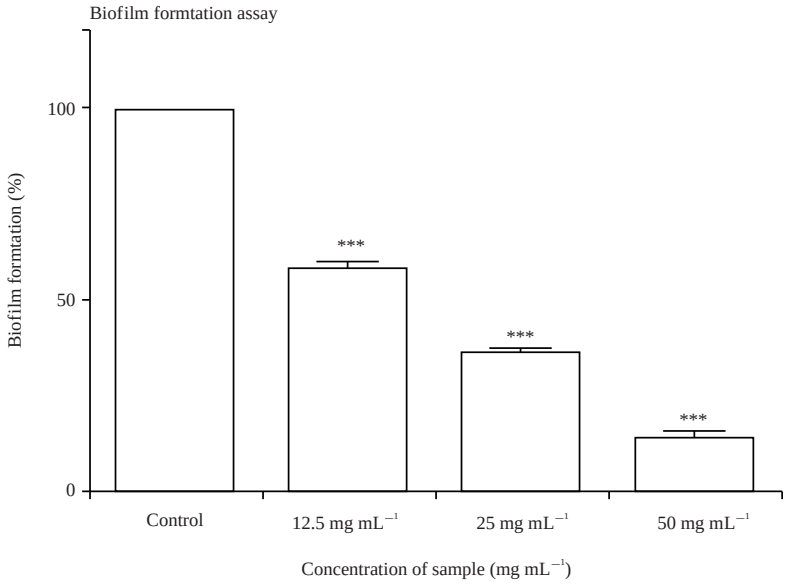

Fig. 5: Biofilm formation assay with the crystal violet staining method

Formation of biofilm decreased as extract concentration increased compared to control, data are expressed as Mean±SD, treated groups showed high significance compared to the induced group and ***($p<0.0001$) as compared to the control group

Biofilm inhibition assay: Antibiofilm activity of hydroalcoholic extract on biofilm formation of MRSA was represented by the percentage of inhibition as shown in Fig. 5. About 0-100% of inhibition of biofilm was observed with an increase in the concentration of extract (12.5, 25 and 50 mg mL^{-1}). Even in low dosage, 50% inhibition was observed which indicates the potential efficacy of extract against MRSA.

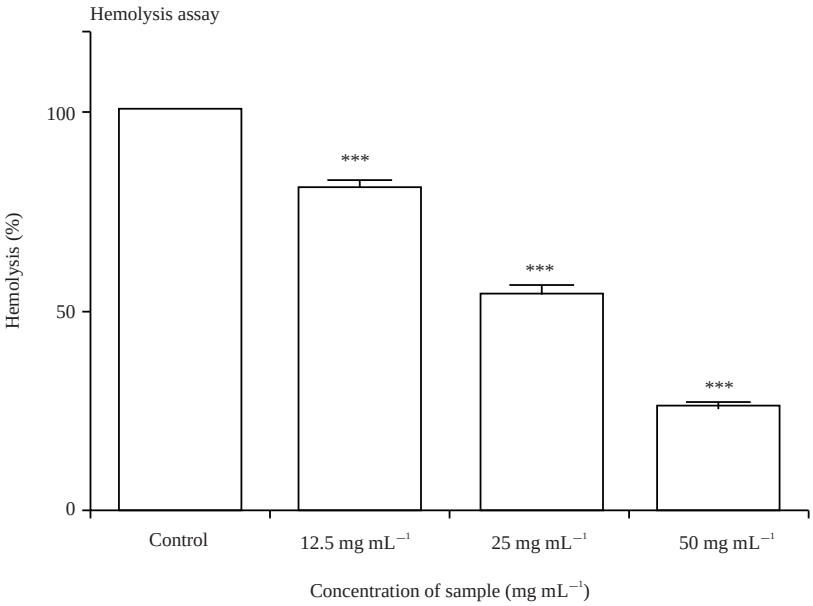

Fig. 6: Figure represents the hemolysis activity of MRSA

Control showed 100% hemolytic activity than the extract-treated groups, hemolytic activity reduced as extract concentration increased, data are expressed as Mean±SD, treated groups showed high significance compared to the induced group and ***(p<0.0001) as compared to the control group

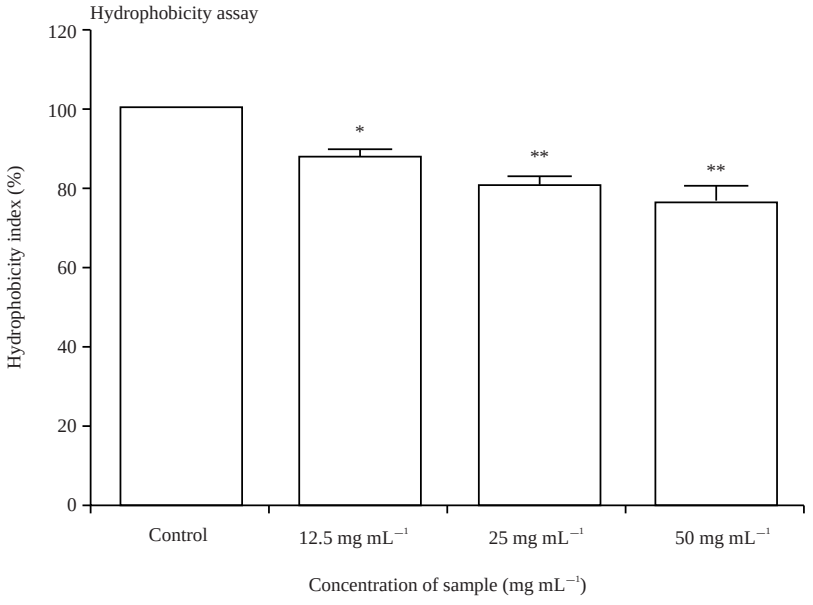

Fig. 7: Graph represents the surface hydrophobicity of MRSA

C. tinctorius L. hydroalcoholic extract reduced hydrophobicity of MRSA compared to control, data are expressed as Mean±SD, treated groups showed moderate significance compared to the control and ** (p<0.01)

Hemolysis activity: The cytotoxicity analysis of extract to human erythrocytes and the ability to inhibit hemolysis caused by MRSA was represented in Fig. 6. The supernatant of extract treated with bacteria showed reduced hemolysis in a dose-dependent manner and 100% inhibition of hemolysis induced by MRSA was observed in control.

Surface hydrophobicity: The hydrophobicity index of bacteria is considered to be an important physicochemical property controlling bacterial adhesion. The surface hydrophobicity of MRSA reduced significantly on treatment with extract in comparison control which confirms the prevention of biofilm formation through inhibition of bacterial adhesion (Fig. 7).

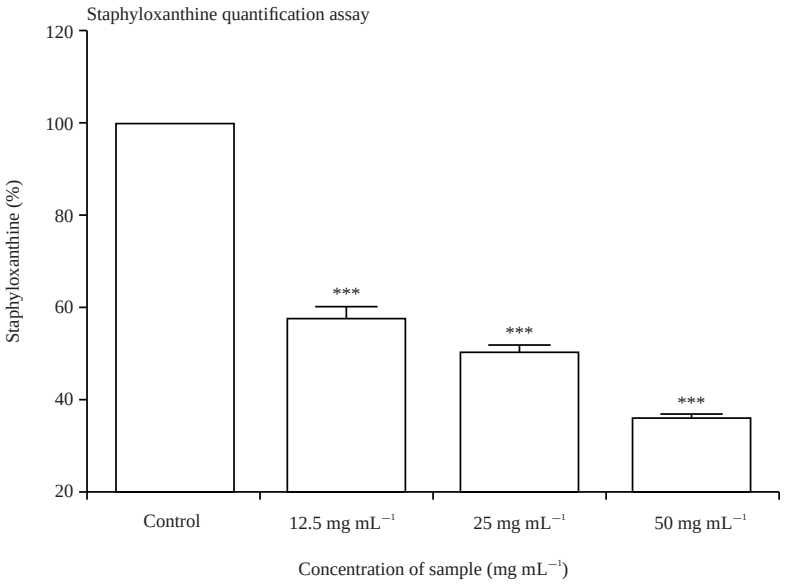

Fig. 8: Figure indicates the quantity of staphyloxanthin in MRSA

Control showed maximum amount of staphyloxanthin compared to treated group the quantity of staphyloxanthin reduced with increasing concentration of extract, data are expressed as Mean±SD, treated groups showed high significance compared to the induced group and ***($p<0.0001$) as compared to the control group

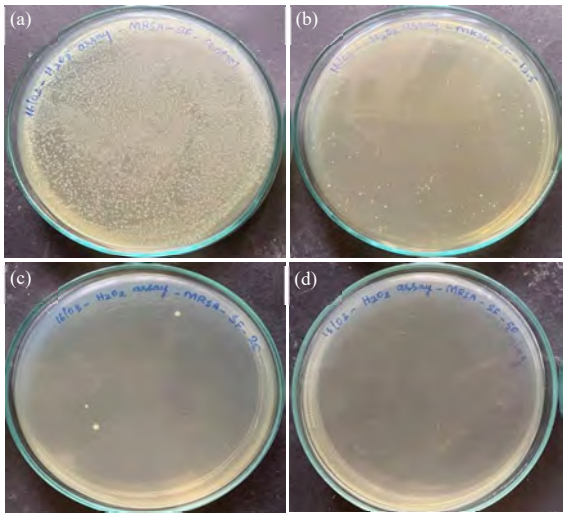

Fig. 9(a-d): Number of colonies obtained In hydrogen peroxide resistance assay, (a) Control, (b) 12.5, (c) 25 and (d) 50 mg mL^{-1} of extract

Quantitative analysis of staphyloxanthin: Staphyloxanthin production was analysed by cell pellets separated from extract-treated MRSA suspension and compared with untreated cells as shown in Fig. 8. A significant decrease in the staphyloxanthin production by MRSA was observed with an increase in the concentration of extract.

Evaluation of hydrogen peroxide resistance: The resistance toward hydrogen peroxide decreased with the inhibition of staphyloxanthin production which acts as an antioxidant and scavenges oxygen radical (O_{2-}) and Hydrogen Peroxide (H_2O_2). The extract treated MRSA were more susceptible to H_2O_2 than untreated cells. Fig. 9a-d.

Analysis of virulence gene expression: MIC concentration of the extract (12.5, 25 and 50 mg mL^{-1}) showed bactericidal effect and decreased expression of genes mecA (Fig. 10a) and spA (Fig. 10b) involved in biofilm formation, involved in the

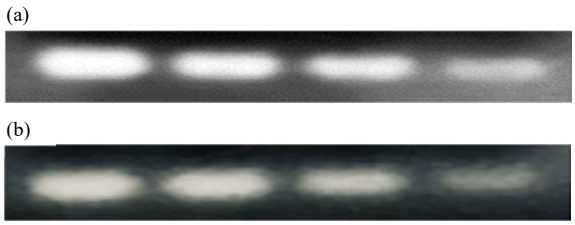

Fig. 10(a-b): Biofilm-related gene expression of MRSA with hydroalcoholic *C. tinctorius* L. extract, (a) MecA gene and (b) spA gene
Lane 1: Control, Lane 2: 12.5 mg mL^{-1}, Lane 3: 25 mg mL^{-1}, Lane 4: 50 mg mL^{-1} of extract and downregulation of the mecA antibiotic resistance gene of MRSA and spA virulence gene was observed in a dose-dependent manner

pathogenesis of MRSA with an increase in the concentration of extract. These results represent the extract as a potential antibiofilm agent that regulates biofilm formation in the budding stage itself.

DISCUSSION

Antimicrobial drug resistance has created alarming healthcare issues all over the world leading to a poor impact on current treatment[13]. Biofilm related infections cause severe illness and poor recovery after antibiotic treatment[14]. Thus, there is a need for antibiofilm agents that inhibits bacterial adhesion during the initial stage of infection. *Carthamus tinctorius* L., commonly called safflower used in traditional medicine contains phytochemicals that possess pharmacological activities like the neuroprotective, cardioprotective, anticoagulant, antioxidant, purgative, analgesic, antipyretic and an antidote for poisoning. It was used for the ailment of painful menstrual problems, postpartum haemorrhage and osteoporosis[5]. The current investigation explored the antimicrobial activity of safflower against MRSA by disruption of biofilm and modulating virulence factor and gene expression.

There was no investigation of the antibacterial activity of hydroalcoholic extract of *Carthamus tinctorius* against antibiotic-resistant bacteria. The antibacterial ability of extract was screened based on the zone of inhibition compared with standard chloramphenicol. Even in 1.6 mg mL^{-1} of extract, the zone of inhibition was 6mm which was near to chloramphenicol with 30 mcg/disc showing 10 mm. It showed an increase in the zone of inhibition with a two-fold increase in drug concentrations. It represented high antibiotic potential at a low dose compared to antibiotics[15]. MIC showed 50% inhibition of MRSA at 25 mg mL^{-1} and MBC was observed at 50 mg mL^{-1} with 100% inhibition. Hydroalcoholic extract yields better phenolic compounds and flavonoids from plant parts[16] and secondary metabolites which may be the reason for antibacterial action[17-19].

Based on the time-kill kinetic assay, extracts showed more than a 3-fold reduction in colony count after 24 hrs of incubation in different concentrations. Thus, there was a significant increase in the rate of killing of bacteria which relies on the concentration of extract and duration of exposure[20].

The formation of biofilm is the major bacterial virulence factor involved in microbial dominance[17]. MRSA treated with extract showed inhibition of biofilm formation when compared to the untreated culture. Inhibition of biofilm holds the promise of reducing colonization on surfaces and epithelial mucosa formed by microbes[18].

The toxin released by MRSA induced hemolysis in RBC, bacterial adhesion and resistance against phagocytosis[19]. In the current study, hemolysis reduced with increased dosage of extract may be due to a controlled molecular mechanism of multifactorial virulence like HLA which in turn blocks the pore[21]. In addition, we found that staphyloxanthin production and its resistance against H_2O_2 reduced on treatment with hydroalcoholic extract may be due to the presence of flavonoids similar to other studies[22,23].

Hydrophobicity influences the virulence and adhesion capacity of bacteria[24]. Reduction in hydrophobicity by extract resulted in a decreased ability of biofilm formation of MRSA. Several studies have shown a decrease in biofilm formation of *Staphylococcus aureus* along with a decrease in hydrophobicity on treatment with any antibiofilm or antibiotic agents[25,26]. The result portrayed the direct mechanism involved in hydrophobicity and biofilm formation.

Modulation of mecA and spA gene expression related to resistance and biofilm formation was observed on treatment with the hydroalcoholic extract. spA, one of the MSCRAMMs (Microbial Surface Component Recognizing Adhesive Matrix Molecules) which binds to the conserved Fc region of immunoglobulin IgG, allows immune evasion and biofilm formation[27,28]. MRSA biofilm formation is inhibited with an

increased concentration of extract through downregulation of the mecA and spA gene. Thus, hydroalcoholic extract *Carthamus tinctorius* plays a dynamic role to control MRSA by preventing antibiotic resistance and biofilm formation.

CONCLUSION

The present study confirmed the antibacterial activity of hydroalcoholic extract of *Carthamus tinctorius* against Methicillin-Resistant *Staphylococcus aureus*. The inhibition of antibiotic-resistant bacteria may be due to phenolic and flavonoid compounds in the hydroalcoholic extract. These results provide the ability of plant extract to act on molecular targets involved in the virulence of MRSA by exhibiting antibiofilm activity and antibiotic potential. Further studies need to be carried out to explore the activity of the extract against molecular mechanisms involved in biofilm formation.

SIGNIFICANCE STATEMENT

The current work investigates the antibacterial effect of hydroalcoholic extract of *C. tinctorius* against Methicillin-Resistant *Staphylococcus aureus*. The significant antibacterial and antibiofilm activity against MRSA was confirmed by MIC, MBC, time-kill assay, biofilm inhibition and decrease in hydrophobic index, respectively. This study provides alternative treatment for antibiotic-resistant bacteria and further studies need to be carried out to confirm the molecular mode of action.

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