Efficacy of Methanolic Extract of *Hemidesmus indicus* Root in Conjunction with Amoxicillin and Clindamycin Against Biofilm Forming Methicillin-Resistant *Staphylococcus aureus* of Bovine Origin

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**SECTION I- ABSTRACT**

The methanolic extract of *Hemidesmus indicus* root (MHIR) was evaluated for therapeutic potential against biofilm forming methicillin resistant *Staphylococcus aureus* (MRSA). MRSA organisms (n=13) were isolated from milk samples of mastitic cows of Chhattisgarh state in India and characterized for antibiogram profile, virulence markers and biofilm forming ability. Antimicrobial potential of MHIR alone; and in combination with amoxicillin and clindamycin was evaluated by microdilution susceptibility test and microtiter plate assay against biofilm forming MRSA. MRSA isolates exhibited almost cent percent resistance to methicillin, cefoxitin, and amoxicillin but sensitive to linezolid, imipenem, clindamycin and tetracycline. Phenotypic expression of virulence determinants viz. coagulase, DNase and α-β, β and γ haemolysins was observed in thirteen MRSA isolates; however biofilm formation was observed in twelve isolates. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum biofilm inhibitory concentration (MBIC) of clindamycin (1.55±0.25 µg/ml, 0.104±0.008 mg/ml and 1.1±0.16 µg/ml) was significantly lower (P>0.05) than MHIR (281.25±31.25 µg/ml, 90.67±16.63 mg/ml and 177.08±18.58 µg/ml) and amoxicillin (53.39±9.06 µg/ml, 4.17±0.39 mg/ml and 62.5±9.42 µg/ml). These two antibiotics in combination with MHIR exhibited additive antibacterial interaction (FIC<sub>index</sub>=0.5-4) and synergistic antibiofilm interaction (FBIC<sub>index</sub>≤0.5). Hence, combination of MHIR with amoxicillin/clindamycin is advocated in treatment of MRSA associated infections.

**Keyword:** MRSA, biofilm, MHIR, amoxicillin, clindamycin

**SECTION II- RESEARCH PROBLEM AND GAP IN RESEARCH**

Despite of active surveillance and ongoing development of new antibiotics, Methicillin Resistant *Staphylococcus aureus* (MRSA) remains a threat to animal and public health. One of the reasons behind unresponsiveness of MRSA towards antibiotics could be attributed to ability of MRSA to form biofilm. Biofilm in turn either restrict the penetration of therapeutic antibiotics thereby subinhibitory concentration of antibiotics reaches to bacteria. Therefore antibiotics often fail to eradicate infections or at the most, it can only suppress the infection leading to possible recurrence of disease. These gaps in the management of MRSA infections still remain to be investigated. Hence the concept behind present work was that if we use combination of antibiotics with suitable antibiofilm agent against MRSA; it would be capable of eradicating MRSA infections completely. Present study was conducted to explore the antibiofilm potential of natural herb *Hemidesmus indicus* in order to offer cost effective as well as safe solutions to emerging threat of MRSA.
SECTION III- INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a potentially important human pathogen and also an emerging concern in veterinary medicine. In Chhattisgarh state, during the past two decades, an alarming expansion of bovine mastitis and other infections caused by MRSA has been recorded. Lack of optimum measures of treatments renders the pathogen to evolve with greater virulence as well as antimicrobial resistance besides producing clusters of high risk population of carrier animals. Ultimately, outcome is the threat to public health due to interspecies transmission of MRSA which creates an alarming situation of possible community spread of MRSA among human population. Multiple drug resistance (MDR) coupled with ability to form biofilm by MRSA is an upcoming threat in treatment of staphylococcal infections in animal and public health [8, 20]. In addition, they possess several enzymes and toxins such as free and bound coagulase, thermostable DNase and membrane-damaging haemolysin that lead to colonization and persistence of MRSA within the host and allow the organism to evade from action of the antimicrobials and immune system [2, 9-10]. Presently, domestic research on MRSA is centered on the search for natural antimicrobial agents like herbal extracts that effectively inhibit biofilm formation to evolve alternate strategy that would completely eliminate MRSA infections. *Hemidesmus indicus* commonly known as *Indian Sarsaparilla, Anantmool, Sariva* and *Ananta* is one of the herbs used in traditional medicine having both antimicrobial and antibiofilm activities [21,24]. The present study was therefore undertaken to evaluate therapeutic potential of methanolic extract of *Hemidesmus indicus* root (MHIR), either alone or in conjunction with antibiotics against biofilm formation by MRSA to explore combinatorial effect.

SECTION IV- LITERATURE REVIEW

(a) Screening of *Staphylococcus aureus* and MRSA
La Zonby and Starzyk [1] carried out conventional cultural and staining method for identification of *Staphylococcus aureus* from clinical samples and bacterial isolates showing Gram positive cocci in large irregular clusters, golden yellow pigmented colony on nutrient agar and manitol fermentation on manitol salt agar were screened out as *Staphylococcus aureus*. Kateete et al. [2] identified 32 isolates of *Staphylococcus aureus* by PCR-amplification of the nuc gene. Further they detected coagulase activity using human and sheep plasma, in 29 (91%) and 27 (84%) isolates, respectively and DNase activity on DNase test agar among 24 (75%) isolates. Rajabiani et al. [3] screened MRSA genotypically through detection of mecA gene by PCR in 250 isolates of *Staphylococcus aureus* obtained from human biological specimens in Tehran.

(b) Antibiogram of MRSA
Bauer et al. [4] described the method of disc diffusion test to perform antibiotic sensitivity test of bacterial isolates and CLSI [5] has provided the guidelines for interpretation of antibiotic sensitivity test and microdilution susceptibility test. Krishnan et al. [6] reported MRSA isolates susceptible to vancomycin and linezolid whereas only 9.2% MRSA were susceptible to clindamycin. Resistance towards other antibiotics was 79% (amoxyclav) and 40.2% (gentamicin). Arjyal et al. [7] observed MRSA isolates resistant to gentamicin (50%), cotrimoxazole (25%), erythromycin (50%), and ciprofloxacin (25%) whereas susceptible to linezolid (100%), clindamycin (100%), ciprofloxacin (75%), erythromycin (50%), tetracycline (100%) and cotrimoxazole (75%). Yehia et al. [8] reported resistance of MRSA against all classes of antibiotics, such as β-lactam, polymyxin E, aminoglycosides, cyclic peptides, sulfonamide, quinolone, fluoroquinolone, and oxazolidone.

(c) Screening of virulence determinants and biofilm forming ability of MRSA
Cruickshank et al. [9] and Bodade et al. [10] detected coagulase and haemolytic activity by coagulase test and on 5% Ox blood agar, respectively. Stepanovic et al. [11] screened biofilm formation among bacterial isolates by microtitre plate test and quantitatively categorized isolates as strong, moderate and weak biofilm formers. Rohde et al. [12] and Tristan et al. [13] demonstrated biofilm formation among *Staphylococcus aureus* isolates through detection of biofilm associated genes such as intercellular adhesion genes (icaA and icaD genes) and fibrinogen binding proteins (fib) gene by PCR. Mathur et al. [14] evaluated biofilm forming ability of *Staphylococcus* spp. obtained from clinical samples and observed that out of 88 (57.8%) biofilm formers 14.47%, 39.4% and 46.0% were strong, moderate and weak biofilm formers, respectively. El-Gayar et al. [15] studied 48 biofilm former MRSA isolates by microtitre plate test and observed that 27% were strong, 42% moderate while 31% were weak biofilm former. Serray et al. [16] studied biofilm forming ability of 53 isolates of MRSA recovered from human samples at Marrakech, Morocco. All the 53 strains were biofilm former by microtiter plate test, out of which 21 (39.62%) were found strong biofilm former, 20 (37.74%) strains as moderate, and 12 (22.64%) strains as weak biofilm former. They reported positive correlation between presence of icaD gene and results of microtiter plate test. Furthermore,
only 5.66% isolates were found positive for fib gene. Dakheel et al. [17] reported polysaccharide intercellular adhesion (PIA)- independent as well as PIA-dependent biofilm formation. They further observed that PIA-independent biofilms showed enhanced biofilm formation and DNase reduced biofilm in the majority of isolates tested, with a loss in biofilm biomass up to 84%. Selvabai et al. [18] characterized 114 strains of MRSA and observed that 110 were found biofilm formers by microtitre plate test. Out of 110 isolates, 04 (3.5%), 85 (74%), 23 (20%) and 2 (1.7%) were non biofilm former, weak former, moderate former and strong biofilm former, respectively. Triveni et al. [19] screened a total of 188 clinical isolates of Staphylococcus aureus from pus, urine and blood. Seventy two (38.29%) isolates were biofilm producer which were further characterized as strong (18.08%), moderate (20.21%) and weak/non biofilm formers (61.7%). Shah et al. [20] studied biofilm forming ability of MRSA isolates obtained from mastitic milk by microtitre plate test which revealed that 65%, 20% and 15% isolates were strong, moderate and non biofilm former, respectively.

(d) Antibacterial and antibiofilm efficacy of Hemidesmus indicus and antibiotics
Kannapan et al. [21] evaluated antibiofilm potential of methanolic extract of Hemidesmus indicus root against Staphylococcus aureus and found that the minimum biofilm inhibitory concentration of Hemidesmus indicus was 300µg/ml. They reported that the root extract prevented tissue adherence of organisms but didn’t have no effect on preformed biofilm of the test pathogens. Das et al. [22] evaluated antibacterial activity of Hemidesmus indicus root extract against multidrug resistant Staphylococcus aureus and observed that methanolic extract of Hemidesmus indicus was found effective against Staphylococcus aureus by disc diffusion method. Saritha et al. [23] studied mechanism of antibacterial activity of methanolic extract of Hemidesmus indicus root against E. coli and observed that extract at 300 µg/ml concentration was bactericidal. They reported that antibacterial action involved disruption of membrane potential, inner membrane permeabilization, blebbing and leakage of cellular contents. Kannapan et al. [24] evaluated antibiofilm potential of Hemidesmus indicus against biofilm formation by Staphylococcus epidermidis. The minimum biofilm inhibitory concentration of Hemidesmus indicus root extract was found to be 500 µg/ml. Ferran et al. [25] evaluated MIC of amoxicillin and clindamycin against Staphylococcus aureus using micro broth dilution method and recorded MIC of 0.5µg/ml and 0.064 µg/ml, respectively. By comparison with amoxicillin, clindamycin reduced bacterial load to a greater extent in both the suspension (2.76 log_{10} CFU/ml) and biofilm (2.09 log_{10} CFU/ml). Majidpour et al. [26] assessed MIC and MBIC of clindamycin against MRSA strains. MIC value was in the range of 8 to 128 µg/ml. The maximum antibiofilm effects (34-50% biofilm inhibition) were detected at subminimal inhibitory concentration of clindamycin.

(e) Antibacterial and antibiofilm efficacy of Hemidesmus indicus and antibiotics
White et al. [27] studied interactions between antimicrobials against bacterial isolates and compared the efficacy of drug alone and in combination by determining fractional inhibitory concentration index (FIC_{index}). Aqil et al. [28] investigated interaction of ethanolic extract of Hemidesmus indicus stem with tetracycline, chloramphenicol, ciprofloxacin, cefuroxime and ceftazidime against Staphylococcus aureus by disc diffusion method. Synergistic interaction between Hemidesmus indicus and tetracycline/cefuroxime against Staphylococcus aureus was reported. Vignesh Kanna et al. [29] determined antibacterial activity of ethanolic extract of Hemidesmus indicus root against Staphylococcus aureus and observed synergism with doxycycline, ciprofloxacin and amikacin against 61%, 45% and 40% MRSA isolates, respectively.

SECTION V- METHODS

(a) Screening of Staphylococcus aureus and MRSA
Staphylococcus aureus was isolated from milk samples of mastitic cows by conventional cultural method [1] and confirmed using thermonuclease nuc gene primer (Forward 5'-GGCTGATTGATGTAGATC-3' and reverse 5'-AGCCAAGCCTTGAGAAGA-3') by PCR [2]. Screening of MRSA among Staphylococcus aureus was done through amplification of mecA gene (Forward primer 5'-AAAATCGATGTTAAGGTTGC-3' and reverse primer 5'-AGTTTGCAGTACCGATTGC-3') by PCR [3]. Amplification was carried out with initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 7 min.

b) Antiogram of MRSA
Each of the MRSA isolates was subjected to antibiotic sensitivity test (AST) by disc diffusion method [4-5] using antibiotic discs of methicillin (10 µg), penicillin G (10 U), cefoxitin (30 µg), tetracycline (30 µg), amoxicillin (10µg), gentamicin (10 µg), vancomycin(30 µg), imipenem (10 µg), linezolid (30 µg) and clindamycin (2 µg) procured from Himedia laboratories, Mumbai.
(c) **Determination of virulence factors and biofilm forming ability of MRSA**

Each of the MRSA isolates was examined for presence of coagulase by slide and tube coagulase test [9], DNase activity on DNase test agar [2] and haemolytic activity on 5% Ox blood agar [10]. Further, each of the MRSA isolates was also screened for biofilm formation by microtitre plate test so as to categorize it as weak, moderate and strong biofilm formers [11]. Further all the MRSA isolates were screened for biofilm associated genes including icaA (Forward primer 5'-ACACTTGCTGGGCGAGTCAA -3' and reverse primer 5'- TCTGGAAACCAACATCCAACA -3'), icaD (Forward primer 5'- ATGGTCAAGGCCAGCAGAG -3' and reverse primer 5'- AGATTTTCAATGTTAAGCAA -3') and fib gene (Forward primer 5'- CTACAAGCTAAATTGCCGTCAACAG-3'and reverse primer 5'- GCTCTTGAAGACCATTTCAC -3') by PCR [12-13]. PCR cyclic condition of nuc and mecA gene was followed with slight variation in annealing temperature viz. 58°C for 30 sec (icaA and icaD genes) and 63°C for 30 sec (fib gene).

(d) **Determination of efficacy of MHIR, amoxicillin and clindamycin against MRSA**

(i) **Preparation of MHIR and antibiotics stock solution**

MHIR was prepared as per the standard protocol [21]. One bactericidal antibiotic to which MRSA exhibited higher resistance *i.e.* amoxicillin; and another having bacteriostatic activity and relatively low resistance against MRSA *i.e.* clindamycin were chosen (Table No.1). Stock solution of amoxicillin (Cat. No. CMS646-1g) and clindamycin hydrochloride (Cat. No. CMS9366-5g) was prepared in sterile MilliQ water at a final concentration of 100 mg/ml.

(ii) **Microdilution susceptibility test**

The microdilution susceptibility test was performed as per standard protocol [5] to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of MHIR, amoxicillin and clindamycin against MRSA.

(iii) **Microtitre plate assay**

Minimum biofilm inhibitory concentration (MBIC$_{50}$) of MHIR, amoxicillin and clindamycin against MRSA was determined by microtitre plate assay [21].

(iv) **Checkerboard assay**

Interaction of MHIR with amoxicillin/clindamycin was determined using checkerboard assay performed in 96 well flat bottom microtiter plate with three replicates [27]. Fractional inhibitory concentration index (FIC$_{index}$) and Fractional biofilm inhibitory concentration index (FBIC$_{index}$) was calculated as given below. Interaction was interpreted as synergistic if FIC/FBIC$_{index}$ ≤0.5, additive if >0.5-4 and antagonistic if >4.

\[
\text{FIC}_{\text{MHIR}} = \frac{\text{MIC}_{\text{MHIR}} \text{ in combination}}{\text{MIC}_{\text{MHIR}}}
\]
\[
\text{FIC}_{\text{antibiotic}} = \frac{\text{MIC}_{\text{antibiotic}} \text{ in combination}}{\text{MIC}_{\text{antibiotic}}}
\]
\[
\text{FIC}_{\text{index}} = \text{FIC}_{\text{MHIR}} + \text{FIC}_{\text{antibiotic}}
\]

(e) **Data analysis**

Data analysis was done by the one way analysis of variance using SPSS computer software package (Version 16.0.0.247©2007).

**SECTION VI- RESULTS**

(a) **MRSA isolates**

A total of 17 Staphylococcal isolates that yielded desired amplicon of *nuc* gene (270 bp) by PCR were screened as *Staphylococcus aureus* isolates (Fig. 1-F). Thirteen out of 17 *Staphylococcus aureus* isolates were confirmed as MRSA which produced 533 bp amplicon of *mecA* gene by PCR (Fig. 1-G).

(b) **Antibiogram profile of MRSA isolates**

Antibiogram revealed that 13 MRSA isolates were multiple drug resistant (Table No. 1) and showed resistance to methicillin (100%), cefoxitin (100%), amoxicillin (84.62%), penicillin (76.92%), vancomycin and gentamicin (53.85%). They were found sensitive to imipenem (100%), linezolid (100%), clindamycin (92.31%) and tetracycline (76.92%). Resurgence of penicillin susceptibility was recorded in 23.08% of the MRSA isolates.
Table No.1: Antibiogram of MRSA isolates

<table>
<thead>
<tr>
<th>S.N</th>
<th>Antibiotic disc</th>
<th>AST results of MRSA isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methicillin (10 µg)</td>
<td>R R R R R R R R R R R R R R</td>
</tr>
<tr>
<td>2</td>
<td>Cefoxitin (30 µg)</td>
<td>R R R R R R R R R R R R R R</td>
</tr>
<tr>
<td>3</td>
<td>Amoxicillin (10 µg)</td>
<td>R R R S R S R S R R R R R R</td>
</tr>
<tr>
<td>4</td>
<td>Penicillin-G (10 U)</td>
<td>R R S R S R S R R R R R R S</td>
</tr>
<tr>
<td>5</td>
<td>Vancomycin (30 µg)</td>
<td>S R R S R S R S R S S S S S</td>
</tr>
<tr>
<td>6</td>
<td>Gentamicin (10 µg)</td>
<td>R S S S IS S S S R R IS S S</td>
</tr>
<tr>
<td>7</td>
<td>Tetracycline (30 µg)</td>
<td>S S S S S R S R S S S S S S</td>
</tr>
<tr>
<td>8</td>
<td>Clindamycin (2 µg)</td>
<td>S S S S S S S S S S IS S S S</td>
</tr>
<tr>
<td>9</td>
<td>Linezolid (30 µg)</td>
<td>S S S S S S S S S S S S S S</td>
</tr>
<tr>
<td>10</td>
<td>Imipenem (10 µg)</td>
<td>S S S S S S S S S S S S S S</td>
</tr>
</tbody>
</table>

Note: S- Sensitive, IS- Intermediate sensitive, R- Resistant

(c) Virulence determinants of MRSA isolates: Extracellular enzymes, toxins and biofilm forming ability

Distribution of virulence determinants among MRSA isolates is shown in Fig. 1. All the 13(100%) MRSA isolates were coagulase (Fig. 2-A) and DNase (Fig. 2-B) positive but showed variable pattern of haemolysis (Fig. 2-C & D) viz. α-β (46.15%), γ haemolysis (38.46%) or β (15.38%). Biofilm (Fig.2-E) formation was detected in 12 out of 13 (92.31%) MRSA isolates; out of which 8 (66.67 %,) 2 (16.67%) and 2 (16.67%) isolates were weak, moderate and strong biofilm producers, respectively. Biofilm associated intercellular adhesion genes viz. icaA (Fig. 2-H) and icaD (Fig. 2-I) was detected in 100% and 92.30% MRSA isolates, respectively. None of the MRSA isolates was detected positive for fibrinogen binding protein (fibr) gene.

(d) MIC, MBC and MBIC<sub>50</sub> of MHIR, amoxicillin and clindamycin against MRSA

MIC, MBC and MBIC<sub>50</sub> of MHIR, amoxicillin and clindamycin against MRSA is shown in Table No. 2. MIC and MBC of MHIR (281.25±31.25 µg/ml and 90.67±16.63 mg/ml, respectively) was significantly (P<0.05) higher than that of amoxicillin (53.39±9.06 µg/ml and 4.17±0.39 mg/ml, respectively) and clindamycin (1.55±0.25 µg/ml and 0.104±0.008 mg/ml, respectively). MIC reduction of test compound was reported when test compounds were used in combination. MIC of MHIR in combination with amoxicillin and clindamycin (Fig. 3) were 145.83±14.1 µg/ml and 177.08±18.6 µg/ml, respectively. Similarly MIC of amoxicillin and clindamycin got reduced to 31.25±6.3 µg/ml and 0.85±0.1 µg/ml, respectively in presence of MHIR. Antibacterial interaction of MHIR and amoxicillin/clindamycin against MRSA was reported to be additive (FIC<sub>index</sub> <0.5-4). MBIC<sub>50</sub> of clindamycin (1.1±0.16 µg/ml) was significantly lower (P<0.05) than amoxicillin (62.54±94.2 µg/ml) and MHIR (177.08±18.58 µg/ml) against MRSA. Likewise MIC; MBIC<sub>50</sub> of MHIR in presence of amoxicillin/clindamycin and MBIC<sub>50</sub> of amoxicillin/clindamycin in presence of MHIR (Fig. 4) was significantly (P<0.05) lower than MBIC<sub>50</sub> of individual compound. MHIR showed synergistic antbiofilm interaction (FBIC<sub>index</sub> <0.5) with amoxicillin (58.33%) as well as clindamycin (83.33%).

SECTION VII- DISCUSSION

Present study demonstrated biofilm formation and production of various virulence factors such as coagulase, DNase and haemolysin by MRSA that play important role in development of bacteremia and sepsis in animals. Ability of MRSA to produce biofilm is the frequent observation [14-20]. Less incidence of strong biofilm producer and higher frequency of weak biofilm formers during present study substantiate the observation of earlier workers [14, 15, 18-19]. The chemical composition of biofilm matrix is attributed to variation in degree of biofilm formation. Biofilm matrix rich in extra cellular DNA undergoes degradation by DNase activity of MRSA isolates causing them to form weak biofilm. On the other hand, PIA dependent biofilm matrix containing poly N-acetylglucosamine remains unaffected by DNase activity of MRSA and thus form strong biofilm [17] which is also supported by concomitant presence of both intercellular adhesion genes (icaA and icaD) in almost all the MRSA isolates.

_Hemidesmus indicus_ inhibits growth of _Staphylococcus aureus_ at log phase through disruption of membrane potential, inner membrane permeabilization, blebbing and leakage of cellular contents [22-23]. The literature also revealed reports on the synergistic potential of _Hemidesmus indicus_ in combination with antibiotics against MRSA [28-29]. Lower
MIC and MBC value of clindamycin than that of MHIR and amoxicillin render clindamycin more efficacious against MRSA. Additive interaction between MHIR and amoxicillin/clindamycin against MRSA could be attributed to inhibition of β-lactamase and disruption of the cell membrane of MRSA by MHIR that augments the action of amoxicillin/clindamycin against MRSA [23]. The percent reduction in MIC (37.04% to 48.15%) was observed when MHIR and antibiotic combinations was used which indicated that the effective dose of antibiotic can be reduced by almost 35 to 50% when the combinatorial regimen of MHIR with either amoxicillin or clindamycin is used without affecting the efficacy of the drug. Amoxicillin inhibited biofilm formation at concentration slightly higher to MIC value whereas MHIR and clindamycin exhibited antibiofilm activities at subminimal inhibitory concentration which is in accordance with the earlier findings [26]. Strong antibiofilm potential of clindamycin could be attributed to its ability to inhibit growth of bacteria at stationary phase inside biofilm [25] whereas MHIR works by inhibiting initial adhesion of staphylococcal organisms on cell surface through poor expression of adhesion genes [21, 24]. When antibiotics and MHIR are used together, planktonic form of MRSA is well exposed to inhibitory concentration of antibiotics which causes efficient inhibition and killing of bacteria resulting into synergistic interaction. There was reduction in MBIC50 to the tune of 62.11 to 90.11% in case of amoxicillin+MHIR combination as well as from 73.64 to 87.49% in clindamycin+MHIR combination that demonstrate the synergistic antibiofilm interaction. Hence, in the treatment by antibiotics against MRSA infection, it is optimistic to target organism’s ability of biofilm formation besides the selective action of the antibiotic.

Fig No.1. Distribution of virulence determinants in MRSA. (A) Occurance of Coagulase, DNase and haemolysin (B) Biofilm forming ability of MRSA (C) Distribution of biofilm associated genes.
Fig No. 2: Characterization of MRSA (A) coagulase positive reaction on slide test (B) Clear halo around bacterial colony indicates DNase activity on DNase test agar (C) α (partial)-haemolysis on Ox blood agar at 37°C (D) α-β haemolysis on Ox blood agar after cold treatment at 4°C (E) Intensity of biofilm formation categorized as strong: column S8-S9, weak :column S1-S2, S4-S7 and S10-S11 and non biofilm former :S3 (F-I) PCR amplified product showing amplicon size of 270 bp, 533 bp, 188 bp and 198 bp of nuc, mecA, icaA and icaD genes on agarose gel electrophoresis.
Fig No. 3: Checkerboard assay for determination of antibacterial interaction against MRSA (A) Interaction between MHIR and amoxicillin (B) Interaction between MHIR and clindamycin
Fig No. 4: Checkerboard assay for determination of antibiofilm interaction against MRSA

(A) Interaction between MHIR and amoxicillin

(B) Interaction between MHIR and clindamycin
Table No. 2: MIC and MBIC<sub>50</sub> of MHIR, amoxicillin and clindamycin against MRSA

<table>
<thead>
<tr>
<th>MRSA sample</th>
<th>MIC (µg/ml)</th>
<th>MBIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>MHIR</td>
<td>Amx</td>
</tr>
<tr>
<td>1</td>
<td>250</td>
<td>62.5</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>7.81</td>
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<tr>
<td>3</td>
<td>250</td>
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<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>500</td>
<td>125</td>
</tr>
</tbody>
</table>

Mean value: 281.25±31.3  53.39±9.1  1.55±0.1  31.25±6.3  0.85±0.1  177.1±18.6  62.5±9.4  1.1±0.2  6.18±1.02  0.29±0.04

Note: MHIR: methanolic extract of *Hemidesmus indicus* root; Amx: amoxicillin; Cld: clindamycin; Amx(+MHIR): of amoxicillin in presence of MHIR; Cld(+MHIR): of clindamycin in presence of MHIR.
SECTION VIII- CONTRIBUTION

Present research findings can contribute largely to the society and farmers. Major contributions from present works included:

The tetracycline in spite of long and consistent use in animals, still remain as a part of cost effective treatment for poor animal owners.

Potentiation of amoxicillin by MHIR against MRSA brought a new hope in mastitis treatment as it could be helpful in upturn of clinical efficacy of amoxicillin.

Considering the efficacy of clindamycin, it could be administered either alone or in combination with MHIR through intramammary infusion for the treatment of bovine mastitis. In addition, clindamycin could be the best choice in treatment of staphylococcal and MRSA infections in monogastric animals such as dogs and cats.

SECTION IX- CONCLUSION

MRSA under present study exhibited showed almost cent percent resistance to penicillin, methicillin, cefoxitin, vancomycin and amoxicillin whereas sensitive to linezolid, imipenem, clindamycin and tetracycline. MHIR showed both antibacterial as well as antibiofilm activity against MRSA. Amoxicillin/Clindamycin in presence of MHIR yielded significantly (P<0.05) lower MIC, MBC and MBIC50 value than individual compound against MRSA. Based on findings, MHIR + amoxicillin/clindamycin combination therapy could be recommended for treatment of MRSA infections.

Justification of Paper to support Atma Nirbhar Bharat

Use of MHIR in combination with amoxicillin provides effective, economical and safe approach in, treatment of MRSA infections particularly in cases of bovine mastitis.

Renaissance of tetracycline sensitivity towards MRSA offers an alternative and cost effective treatment in mastitis management of dairy herd particularly in large population.

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