Preliminary screening of the antibacterial effect of the pigmented squid ink extracts against methicillin resistant *Staphylococcus aureus* (MRSA)

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This present investigation is to assess the antibacterial property of the squid ink extracts from *Loligo duvauceli* against methicillin resistant *Staphylococcus aureus* [MRSA]. MRSA strain was isolated and was characterized by standard microbiological methods. Genotypic detection of meca gene was done by PCR. Isolated strain was further subjected to antibacterial bioassay with the crude extracts of squid ink obtained using various solvents. Hexane extract scores a promising antimicrobial effect against MRSA, with trace activity by ethyl acetate extract and no activity by other solvent extracts. MIC value was determined as 2.5mg/ml.

**[Key words]:** MRSA, Squid ink, antibacterial, crude extraction

**Introduction**

Methicillin resistant *Staphylococcus aureus* (MRSA) has become a headline concern due to its emergence as a common infection in the new age of antibiotic resistance. MRSA shows resistance to the antibacterial activity of methicillin and other related antibiotics of the penicillin class¹. MRSA is known for causing skin infections and may progress substantially within 24–48 hours of initial topical symptoms. Initial presentation of MRSA starts from small red bumps to large, painful and eventually open into deep, pus-filled boils². MRSA also causes pneumonia, surgical wound infections and bloodstream infections. Majority of MRSA infections occur among patients in hospitals or other healthcare settings; however, it is becoming more common in the community setting. About 75 percent community acquired MRSA (CA-MRSA or CMRSA) infections are localized to skin and soft tissue and usually can be treated effectively. However some CA-MRSA strains display enhanced virulence, spreading more rapidly and causing illness much more severe than traditional healthcare associated infections. MRSA affects vital organs and has led to widespread infection (sepsis), toxic shock syndrome and necrotizing ("flesh-eating") pneumonia. This is thought to be due to toxins carried by CA-MRSA strains. The other forms of MRSA include hospital-acquired or health care acquired MRSA (HA-MRSA or H-MRSA) or epidemic MRSA (E-MRSA). Data from a prospective study from 2005-2008, suggests that 12% of clinical MRSA infections are community-associated, but this varies by geographic region and population³. MRSA has been a leading cause for increasing mortality in recent decades due to its establishment of untreatable necrotizing lesions. Being resistant to most of the routine drugs of treatment, it becomes a serious threat and challenge to the treating physician and microbiologists. Thus it implicates the need for an alternative medication for the eradication and treatment of MRSA and the infections caused by them respectively. Natural bioactive agents have widest range of application with medications formulated to be administered in minute doses, yet safe and without side effects. Interest in obtaining biologically active compounds from natural sources has recently spiked due to their low toxicity, complete biodegradability, availability from renewable sources and in most cases are in low cost.

More recently, marine life forms have proved to be a rich source of novel bioactive agents. The future pharmaceutical industry will rely naturally on the antibiotics that have been derived from the marine world. The marine life forms and their products are inspiring new sources for drug development. From the Phylum of Mollusca hails the squids. The South
Indian squid, *Loligo duvauceli*, secrete a black pigmented ink as a defensive ploy to escape from predation. The squid ink has proved to play various primary roles in the world of alternative medicine and has widest range of therapeutic applications\(^4\). Squid ink has already been reported to possess potent antibacterial activity against forty biofilm bacteria\(^5\). Ink from cuttlefish has reported to possess a potent antibacterial protein in very low concentration which has shown to inhibit the growth of *Staphylococcus aureus*\(^6\). The preservative property of squid ink has also been reported which is due to the presence of oxidized squid oil with anti-bacterial action\(^7\). Tyrosinase an enzyme present in squid ink is known to play a key role in the defense against microbes\(^8\). The ink from other species under the phylum Mollusca have been reported to reveal the anti-retroviral activity\(^9\). Present study is to explore the antibacterial activity of the ink from the south Indian squid *Loligo duvauceli* against methicillin resistant *Staphylococcus aureus*.

**Materials and Methods**

Pus and wound swabs were collected from 54 patients with necrotizing skin lesions after obtaining the informed consents. Pus was collected either as swabs or as aspirates and was inoculated to blood agar, chocolate agar and MacConkey agar and was incubated at 37°C / 24 hrs. Isolates were screened for *Staphylococcus aureus* using standard microbiological procedures\(^11\). MRSA was detected for the production of mexitilime using the mexitilime spot expression assay\(^12\). Briefly, the lawn culture of *S. aureus* (ATCC 25923) strain, which is sensitive to oxacillin, was prepared on Mueller-Hinton agar plates with 4% NaCl by swabbing the plates with bacterial culture corresponding to 0.5 McFarland units. This was left to dry for 10 minutes. An oxacillin disc (1µg) was placed in the middle by means of a sterile blunt needle and 4-5 colonies of each clinical strain isolated were picked with the help of a sterile wire loop and were placed as spots around the disc at a distance of 7-8 mm. The plates were then incubated overnight at 37°C and the result was recorded.

The strain was then subjected to PCR amplification for *mec*-A gene for confirming the MRSA strain as described earlier\(^13\). Briefly, genomic DNA was extracted by inoculating a single colony of *S. aureus* in 1.8 ml of Luria Bertani broth (LB broth, Sigma) and incubated overnight at 37°C in a shaker and was centrifuged at 7000rpm for 3 mins. Supernatant was discarded and the pellet was dissolved in 400µl of sucrose TE buffer (Tris-EDTA) and lysozyme (Sigma) was added (from 10mg/ml stock) and kept at 37°C for 30 mins. 100µl of 0.5 M EDTA (pH 8.0) and 60µl of 10% SDS were added. 3µl of Proteinase K (Sigma) (from 20mg/ml stock) was added to the solution and incubated at 55°C for 12 hrs. 250µl of chloroform was added to the solution, centrifuged at 10,000 rpm for 10 mins and extracted twice with phenol:chloroform and once with chloroform: isoamylalcohol (500 µl). The final solution was centrifuged at 10,000 rpm for 10 mins and the supernatant was precipitated with 2.5 volume of 100% ethanol and centrifuged. DNA was spooled out with the cut tips and washed with 70% ethanol followed by air drying. After isolating the genomic DNA, the DNA sample was electrophoresed in 3% of Agarose. The amplification of *mec*-A gene was performed using a 10 µl reaction set-up in sterile 0.2 ml tubes. The following components were added sequentially. The reaction set up used was sterile double distilled water - 4.1 µl, 10X assay buffer -1.0 µl, dNTP’s (0.2mM) - 1.0 µl, Taq polymerase (5U/µl) - 0.2 µl, MgCl2 - 0.7µl and genomic DNA (25ng/µl) - 1.0 µl. Forward primer (300ng/µl) 1.0 µl of 5’-AGTTGTAGTTGTCGGGTTT-3’ and reverse primer (300ng/µl) 1.0 µl 5’-AGTGGAAACGAGTATCATC-3’. The reaction condition was set up as follows: Initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute 20 seconds, extension at 72°C for 1 minute 20 seconds and final extension at 72°C for 5 minutes. The reaction was carried for 35 cycles in an Eppendorf Master Cycler.

Species identification of the squids, dissection of the ink sac and collection of ink was done as per our earlier studies\(^14\), \(^15\), \(^16\). Crude extraction of the active biomolecules from the squid ink was done using solvent extraction method\(^17\). Squid ink was extracted with polar and nonpolar solvents like hexane, petroleum ether, chloroform, butanol, ethyl acetate, acetone, methanol and ethanol in the ratio of 1:3 v/v using parallel extraction method. Mixed ink was refrigerated at 4°C/7 days for crude extraction and each preparation was filtered using Whatman No.1 filter paper and the crude extracts were concentrated under vacuum at 40°C using Heidolph, VE-11 Rota Evaporator. Crude extracts were collected and
weighed. Prior to antimicrobial assay the crude extracts were sterilized by exposure to UV light for 2 hrs. Sterility was checked by inoculating 5 mg of each extract mixed in sterile nutrient broth and was incubated for 2 hrs which was then plated onto Nutrient agar. The extracts were stored at 4ºC in brown glass bottles.

The antimicrobial study was performed by agar well diffusion method. Test organisms used for the study include the isolated MRSA strain from the clinical specimens. ATCC strain of *Staphylococcus aureus* (ATCC 25923) was included as standard control. Inoculum was prepared by emulsifying a minimum of four colonies of the test organisms into sterile Mueller Hinton broth under aseptic conditions and was incubated for two hours at 37ºC. After incubation the density of each microbial suspension was adjusted equal to that of 10⁶ c.f.u/ml (standardized by 0.5 McFarland standards). One hundred microlitres (100µl) of the inoculum of each control organism was spread as lawn cultures onto sterile Mueller Hinton agar plates using L-rods to achieve a confluent growth. Agar plates were allowed to dry and wells or cups of 8 mm were made with a sterile agar borer in the inoculated agar plates. 10 mgs of each crude extract were reconstituted in Dimethyl sulphoxide (DMSO) in sterile brown glass bottles for the antimicrobial bioassay. A 50µl volume of each extract was propelled directly into the wells of the inoculated specific media agar plates for the test organism. Plates were allowed to stand for 10 minutes for the diffusion of the extract and incubated at 37ºC for 24 hrs. DMSO and ciprofloxacin (30µg) served as negative and antibiotic controls respectively. After incubation the plates were observed for the zone of inhibition around the wells and the zone size was measured using an antibiotic sensitivity measuring scale (Himedia). Antimicrobial efficacy was graded based on the zone diameter as high activity (> 15 mm), moderately active (10-14 mm), trace activity (5-9 mm) and no activity (< 4 mm).

MIC value for the extract against MRSA was determined by Microbroth dilution method. Serial dilution of the crude extract was done in a 96 well microtitre plate with DMSO in triplicates. The dilution factor was 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/ml. To each dilution 100 µl of the culture broths of the test MRSA strain and control strain were added in their respective wells and the plate was incubated at 37ºC for 24 hrs. After incubation the spectrophotometric analysis was performed and the OD values were recorded. MIC value was deduced in the dilution that showed decreased turbidity and was also confirmed by Microbial Spot Checker board method where 3 µl of each dilution was spotted onto Mueller Hinton agar plates and incubated at 37ºC for 24 hrs. After incubation the spot showing the complete absence of microbial growth indicated the minimum bactericidal dose. One way analysis of variance and Duncan’s multiple range tests were employed to analyze the size of the obtained zone of inhibitions. Differences between means were considered significant when P<0.05.

**Results**

From the 54 study cases, 8 were culture positive with one strain of *S.aureus* (12.5%) phenotypically characterized as methicillin resistant (MRSA). PCR amplification of the same yielded the mec-A gene product with 533 bp amplicon size. This strain was used for the in-vitro antimicrobial assay of squid ink. Based on the zoological taxonomical characters, the squid selected for the study was confirmed by the zoologist as *Loligo duvauceli*. The solvent extraction method was suitable for obtaining the crude extracts from the squid for the antimicrobial assay. All the extracts were found to be sterile after sterility check.

Antimicrobial activity of the crude extracts against MRSA showed that hexane extract showed a zone size of 18 mm for MRSA test strain (Fig 1) thus scoring a high antibacterial activity. The extract was...
Table 1 — Hexane extract of the squid ink showing high antibacterial activity against methicillin resistant \textit{S. aureus} (MRSA)

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>A</th>
<th>Et</th>
<th>B</th>
<th>H</th>
<th>E</th>
<th>M</th>
<th>CHL</th>
<th>EthAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA [Clinical isolate – test strain]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>\textit{S. aureus} [ATCC 25923]</td>
<td>11</td>
<td>12</td>
<td>12</td>
<td>20</td>
<td>11</td>
<td>12</td>
<td>12</td>
<td>12</td>
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<table>
<thead>
<tr>
<th>[Zone size in mm]</th>
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<tr>
<td>(A - Acetone, Et - Ether, B - Butanol, H - Hexane, E - Ethanol, M - Methanol, CHL - Chloroform, EthAc - Ethyl Acetate)</td>
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</tbody>
</table>

also active against the ATCC control strain of \textit{S. aureus} with 20 mm of zone of clearance. Extract from ethyl acetate showed a trace activity against MRSA test strain with a zone size of 12 mm. The extracts from other solvents did not show any antibacterial activity against the MRSA strain. Trace antibacterial activity was shown by other extracts against the ATCC strain of \textit{Staphylococcus aureus} (Table 1). MBC value was determined as 5 mg/ml for MRSA. Spectrometric analysis yielded a decrease in the measured OD values upon the increasing concentration of the hexane extract. Microbial spot checker board method yielded complete absence of the growth at the spot inoculated with the determined MBC value. The previous dilution that showed the visible decrease in the number of colonies was determined as the minimum inhibitory dose for the extract and was deduced as 2.5 mg/ml for \textit{S. aureus}.

Discussion

MRSA or methicillin resistant \textit{Staphylococcus aureus} is the major threatening species causing severe necrotising lesions of skin and soft tissues. MRSA is especially troublesome in hospitalized patients with open wounds and invasive devices. Patients with weakened immune systems are at greater risk of infection than the general public. For many years it has been a major cause for nosocomial infections in many countries. The proportion of methicillin resistance among clinical isolates of \textit{S. aureus} is alarming. MRSA shows resistance to methicillin and with other related penicillin group of drugs. Major resistance shown by MRSA against these antibiotics is a great concern for the treating clinicians. Previous reports suggest the prevalence of MRSA strains at a rate of 35.4% from Chennai\textsuperscript{22}. This study reports the occurrence of MRSA strain with an incident rate of 12.5%. Heterogenous nature of methicillin resistance is an inherent limitation of the accuracy of susceptibility testing\textsuperscript{23}. mec-A detection tests based on PCR or DNA hybridization correctly identify the most heterogeneous strains and should be considered the gold standard for methicillin resistance. Based on this the MRSA strain has been confirmed by the detection of mec-A gene by Polymerase Chain reaction (PCR). mec-A gene product is responsible for the alteration of the penicillin binding proteins – 2, the target structure of penicillin\textsuperscript{24}. Comparison of the conventional method of MRSA detection and the molecular method is thus a must to do procedure in the present scenario. Isolation of MRSA strain emphasizes the occurrence of nosocomial and community acquired pneumonias in our society\textsuperscript{25}. Rapid detection of MRSA, by mec-A gene based PCR appears to be promising. Since variations among the method exist and no acceptable guidelines are formulated a combination of conventional methods and PCR should be the choice of detection of MRSA. Findings of the study report that \textit{Loigo duvauceli}'s ink constitutes bioactive molecules that are effective against the methicillin resistant \textit{Staphylococcus aureus}. High activity is observed with the hexane extract against MRSA with a good MIC and MBC value. The other extracts from alcohols such as ethanol, methanol and butanol that showed trace antibacterial activity that might be due to the presence of residual salt\textsuperscript{26}. Trace activity by the ethyl acetate extract could be due to the presence of trace solvent as the exact evaporation of the extract could not be achieved with the rotary evaporator. Promising antibacterial activity of squid ink extracts has been reported earlier against ESBL producers of \textit{E.coli} and \textit{K.pneumoniae}\textsuperscript{27}. GC-MS evaluation of the antimicrobial bio-constituents has been evaluated\textsuperscript{28}. Assessment of the anti-HBV and anti-HCV like property has also been analyzed by in-vitro and in-silico studies\textsuperscript{29, 30}. Squid ink extracts also showed antibacterial property against four caries pathogens\textsuperscript{31}. In addition, we have patented a novel protein from squid ink with antimicrobial potential\textsuperscript{32}. This present investigation is an added report on the efficacy of hexane extract against the MRSA strain and could be used in the future therapeutic scenario in the treatment of dreadful infections caused by this group of pathogens. However, further research on squid ink extracts are under progress related to toxicity and animal trials.

References

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