Antibacterial effect of squid ink on ESBL producing strains of *Escherichia coli* and *Klebsiella pneumoniae*.


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Received 08 February 2011; revised 07 July 2011

Present study consists a novel therapeutics from natural sources to prevent the emergence and proliferation of resistant microbial populations that can make a significant impact in treating clinically challenging microbial infections. Squid ink has proved to play various primary roles in the world of alternative medicine and has the widest range of therapeutic applications. Present study is designed to report the antibacterial effect of the squid ink against the ESBL producing strains of *E.coli* and *K.pneumoniae*. ESBL strains are isolated from patients with typical urinary tract infections. They have been identified microbiologically and characterized by double disc synergy test and plasmid profiles. Active metabolite of squid ink was extracted using solvents and was checked for its antibacterial activity by agar well diffusion method. MIC value is determined by microbroth dilution method. Results conclude that the hexane extract of the squid ink scored high antibacterial activity against the ESBL producing strains of *E.coli* and *K.pneumoniae*. Present study suggests that squid ink is an enigmatic pigment of therapeutic value in near future for treatment of dreadful infections caused by the ESBL strains.

**Keywords:** ESBL, *E.coli*, *Klebsiella*, squid ink, antibacterial activity

**Introduction**

The relentless threat posed by microbial drug resistance has achieved the dimension of a global pandemic, with a relevant impact in terms of morbidity, mortality and health-care associated costs. A matter of major concern for this type of resistance is the emergence of new β-lactamases such as the extended-spectrum β-lactamases (ESBLs) and the carbapenemases capable of degrading the expanded-spectrum cephalosporins and/or carbapenems. Production of ESBL activity results in a significant narrowing in the available therapeutic options. Infections caused by ESBL producers are associated with higher morbidity and mortality, a higher risk for inadequate treatment and increased health-care associated costs.

Plasmid-borne β-lactamases capable of hydrolyzing penicillins provide the most common mechanism of resistance to β-lactam antimicrobial agents among gram-negative bacteria and their existence is the principal stimulus to the development of β-lactamase inhibitors. It has become apparent that changes in the substrate affinity of the enzyme were related to relatively minor changes in the sequences of the genes encoding the TEM-1, TEM-2, and SHV-1 enzymes with novel enzymes that hydrolyze the third generation cephalosporins. This event has been particularly associated with resistances among *Escherichia coli* and *Klebsiella pneumoniae*. Other species of the family *Enterobacteriaceae* also express ESBL activity.

Reliable detection and eradication of ESBL production is a difficult task for the treating physicians and the microbiologists. Thus it instills the need for an alternative novel therapeutic compound from a natural source. Natural bioactive substances have the least quantum of side effects when compared to synthetic products. Although most antibiotics have been derived from the terrestrial products it is the marine world that provide the pharmaceutical industry the next generation of medicines. The biochemical metabolism of seemingly marine organisms such as blue green algae, sponges and squids are inspiring new ideas for drug development.

Squids belong to the cephalopod family and the south Indian species *Loligo duvauceli*, as a defensive ploy secrete a black pigmented ink 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\(^8\). The potential antibacterial activity of the squid ink has already been reported against biofilm bacteria\(^9\). A protein extracted from cuttlefish ink in very low concentration has been shown to inhibit the growth of \textit{Staphylococcus aureus}\(^10\). Oxidized squid oil is reported to be anti-bacterial in action\(^11\). Tyrosinase an enzyme present in squid ink is known to play a key role in the defense against microbes\(^12\). Recent scientific studies have revealed the preservative and antioxidant values of ink from \textit{Sepia officinalis}\(^13\). Ink from \textit{Sepiella inermis} was studied for its anti-retroviral activity\(^14\). Present study is to explore the antibacterial activity of the ink from the south Indian squid \textit{ Loligo duvauceli} against the ESBL producing strains of \textit{E.coli} and \textit{K.pneumoniae}.

### Materials and Methods

Crude extraction of squid ink

Fresh squids were caught from the south west coast of Chennai and were decapitated by the fishermen. The squids were fixed in 4% formalin (4 mL formalin in 96 mL of water) in a clean glass zoological specimen bottle. Squids were washed with pure tap water and the postero-ventral aspect was cut open with sterile scissors and the exposed intestine and other parts were removed carefully (Figure 1). The ink sac was observed and was dissected with a sterile blade taking care that the ink was not excreted. After dissection the surface of the gland was sterilized with ethanol and was blotted with sterile cotton. The ink duct was cut with sterile scissors and the sac was gently squeezed and the excreted ink was collected in sterile brown bottles. The ink was stored at 4°C until use.

Crude extraction of the active biomolecules was done using solvents\(^15\). 25 mL of the squid ink was extracted with 75 mL of polar and non-polar solvents (1:3 \(v/v\)) like hexane, petroleum ether, chloroform, butanol, ethyl acetate, acetone, methanol and ethanol in sterile glass bottles by parallel extraction method. Ink was mixed gently with the solvents using sterile glass rods and was refrigerated at 4°C for 7 days for crude extraction. 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, weighed and were sterilized by exposure under UV light for 2 hrs. 5 mg of each extract was mixed in sterile nutrient broth and was incubated for 2 hrs which was then plated onto Nutrient agar for checking the sterility of the extracts. The extracts were stored at 4°C in brown glass bottles.

Identification of ESBL producing strains

Clean catch midstream urine specimens were collected in a sterile wide mouthed, screw capped bottle from 100 patients with typical urinary tract infections. Specimens were processed as per standard microbiological procedures\(^16\) by inoculating the specimens on to MacConkey agar using calibrated loop method with a 4 mm loop which delivers 0.01 mL of the specimen onto the plates. Even distribution of the inoculum was achieved by criss-cross streaking and the plates were incubated at 37°C for 24 hrs. After incubation the significance of a positive culture was assessed in terms of the viable bacteria present by colony count. Thus the specimen showing \(>10^5\) CFU/mL was considered as significant bacteriuria condition. The isolates were then characterized by staining, colony morphology and by specific biochemical tests. All the clinical isolates were subjected to antibiotic sensitivity test by the conventional Kirby Bauer method\(^17\) using the routine drugs of choice.

Extended spectrum beta lactamases (ESBL) producing strains were screened by double disc synergy test\(^18\). Briefly \textit{E.coli} and \textit{K.pneumoniae} strains were screened for ESBL production by placing ceftazidime disc (30 mg) and amoxicillin/clavulanic

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Fig. 1—Postero-ventral section of \textit{ Loligo duvauceli} showing the ink sac
acid (20 + 10 mg) disc by placing them in 25-30 mm apart at the middle of the lawn culture on MHA plates. Following overnight incubation at 37°C aerobically ESBL production is inferred by the expanded zone of inhibition around the ceftazidime disc by the clavulanate. Profiles for resistant plasmids were studied by isolating the plasmid DNA from the ESBL strains using alkaline lysis method. Briefly, 1.5 mL of overnight culture of the organism was taken in a fresh microcentrifuge tube and was centrifuged at 12,000 rpm for 1 min. 200 µl of TE buffer was added to the pellet and the suspension was vortexed briefly. 200 µl of 0.2N NaOH and 1% SDS were added and mixed thoroughly. 300 µl of Sodium acetate ($\text{pH}=4.8$) was then added and the tubes were incubated at 0°C for about 15 minutes. Cell suspension was centrifuged at 12,000 rpm for 15 mins at 4°C and the supernatant was transferred to a fresh tube. 200 µl of Chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 5 mins at 4°C. Aqueous phase was transferred to a clear tube to which double the volume of absolute ethanol was added and incubated in ice for 10 minutes. After centrifugation at 12,000 rpm for 15 mins at 4°C the supernatant was discarded. 1ml of 70% ethanol was added to the pellet and centrifugation was repeated at 12,000 rpm for 15 mins at 4°C. Supernatant was discarded and the pellet was air dried followed by the addition of 50µL of TE buffer. Plasmid profiles were then studied by agarose gel electrophoresis. 2 µl of plasmid preparation was mixed with gel loading dye and the preparation was electrophoresed on a 1% agarose gel.

**Invitro antimicrobial susceptibility assay**

Antimicrobial study was performed by Nathan’s agar well diffusion method. Test organisms used for the study include the isolated ESBL strains from the clinical specimens. ATCC strains of *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 10031) were included as controls. Inoculum was prepared by emulsifying a minimum of four colonies of the test organisms with a sterile loop into sterile Mueller Hinton broth under aseptic conditions and was incubated for two hours at 37°C. After incubation the density of each microbial suspension was adjusted equal to that of $10^6$ c.f.u/ml (standardized by 0.5 MacFarland standards).

One hundred microlitres (100 µl) of 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 the each crude extract were reconstituted in Dimethyl sulphoxide (DMSO) in sterile brown glass bottles for the antimicrobial bioassay. A 50 µl volume of each extract was propelled directly into the wells of the inoculated specific media agar plates for each test organism. The plates were allowed to stand for 10 minutes for diffusion of the extract to take place and incubated at 37°C for 24 hrs. DMSO and ciprofloxacin (30 µg) serve 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) (Figure 2).

![Fig. 2—Hexane extract showing a high antibacterial activity against ESBL strains of *E. coli* and *K. pneumoniae*](image_url)
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)\(^{21}\).

**Determination of MIC value**

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of a compound /extract/drug that completely inhibits the growth of the microorganism in 24 hrs. Determination of MIC value for the extract that showed high antibacterial activity was determined by Microbroth dilution method\(^{22}\). Serial dilution of the crude extract was done in a 96 well microtitre plate with DMSO. 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 ESBL strains and control strains were added in their respective wells and the plate was incubated at 37°C for 24 hrs. After incubation the spectrophotometric analysis was performed and the OD values were recorded. MIC value was also confirmed by Microbial Spot Checker board method\(^{23}\) where 3 µl of each dilution was spotted onto Mueller Hinton agar plates and incubated at 37°C for 24 hrs. After incubation the spot showing the complete absence of microbial growth indicates the bactericidal dose or the minimum inhibitory concentration value.

**Results**

Based on the zoological taxonomical characters, the squid selected for the study was confirmed by the zoologist as *Loligo duvauceli*. Dissection was successfully performed and the method followed for obtaining the crude ink was satisfactory. Solvent extraction yielded crude extracts from hexane, ether, chloroform, ethyl acetate butanol, ethanol, acetone and methanol. All the extracts were found to be sterile after sterility check.

Urine specimens yielded *E.coli* (41.3%) and *K.pneumoniae* (10.3%) as the predominant strains. Kirby Bauer method showed 58.3% of *E.coli* and 66.7% of *K.pneumoniae* resistant to most of the routine drugs of choice from the 100 study cases. Double disc synergy method showed 71.4% of *E.coli* and 66.7% of *K. pneumoniae* were ESBL producers. Alkali lysis method showed the presence of plasmids from 85.7% of the strains of *E.coli* and 66.7% of *K. pneumoniae* (Data not shown). All the ESBL producing *E.coli* strains showed the presence of plasmids and 2 ESBL strain of *K. pneumoniae* yielded plasmids. This indicates the role of plasmids for the production of ESBL.

<p>| Table 1—Crude extracts from hexane showing high antibacterial activity (inhibition zone in mm) against the ESBL producing strains of <em>E.coli</em> and <em>K.pneumoniae</em> |
|-----------------|---|---|---|---|---|---|---|</p>
<table>
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<tr>
<th>S. No.</th>
<th>Pathogens under study</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>
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<tr>
<td>1.</td>
<td><em>E.coli</em> [ATCC 25922]</td>
<td>11</td>
<td>12</td>
<td>15</td>
<td>20</td>
<td>11</td>
<td>12</td>
<td>12</td>
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<tr>
<td>3.</td>
<td><em>E.coli</em> [ESBL strain]</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>18</td>
<td>12</td>
<td>11</td>
<td>12</td>
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(A - Acetone, Et - Ether, B - Butanol, H - Hexane, E - Ethanol, M - Methanol, Chl - Chloroform, EthAc - Ethyl Acetate)

Antimicrobial activity of the crude extracts showed that the extracts from hexane scored a high antibacterial activity against the ESBL producing *E.coli* and *K.pneumoniae*. The zone size ranges from a mean value of 18 mm for *E.coli* and 22 mm for *K.pneumoniae*. ATCC strains of *E.coli* and *K.pneumoniae* showed a zone size of 20 mm and 23 mm respectively (Table 1). Extracts from ether, methanol, butanol and chloroform showed trace activity against both *E.coli* and *K.pneumoniae*. Extracts from acetone, ethanol and ethyl acetate did not show any antibacterial activity against *E.coli* whereas they showed a trace antibacterial activity against *K.pneumoniae*.

MIC value was determined as an average of 2.5 mg/ml for both *E.coli* and *K.pneumoniae*. Spectrometric analysis yielded a decrease in the measured OD values upon the increasing concentration the hexane extract. Microbial spot checker board method yielded complete absence of the growth at the spot inoculated with the determined MIC value (2.5 mg/ml) of the extract. Previous dilution that showed the visible decrease in the number of colonies was determined as the bacteriostatic dose and was deduced as 1.25 mg/ml for *E.coli* and *K.pneumoniae*.

**Discussion**

The increasing emergence of clinical isolates of the family *Enterobacteriaceae* with ESBL phenotypes remains a major reason for antimicrobial resistance problems\(^{24}\). Prevalence of ESBL enzymes has been increasing in many parts of the world. Infections caused by ESBL producing isolates are difficult to treat, because they confer resistance to all currently available β-lactam agents, except imipenem and in
Among alarming are the high rates of ceftazidime resistance to other classes of antimicrobial agent, such as aminoglycosides and fluoroquinolones. Particularly to other classes of antimicrobial agent, such as the isolation rate of ESBL strains among Gram negative bacilli has been reported earlier with an incident rate of above 60%. This study also reports the correlation of the isolation rate of ESBL strains among E.coli and K.pneumoniae. There has probably been a gene pool in nature for resistance to antibiotics. Resistant pathogens have yielded single and multiple plasmids by alkali lysis method. Findings of the study report the presence of R plasmids from all the ESBL pathogens. It has been reported earlier that the presence of multiple plasmids can result in multi-drug resistance.

As resistance has increased to alarming proportion a safe and cheaper source can always be an alternative to the routine therapeutics. Selection of squid ink is based on the isolation of novel biomolecules from a waste product that can be used from nature’s source. Among the squids the bioactivity of Loligo duvauceli ink has not been reported yet, thus it is selected as a source for the isolation of novel therapeutic agents. Squid ink constitutes the pigment melanin and the process of melanogenesis was well explained in the ink gland of Sepia sp., Squid ink is a complex mixture of organelles, premelanosomes, melanosomes, granules, proteic material (enzymes), glucosamine and phospholipids in suspension. Ink gland has also shown to contain a variety of melanogenic enzymes as tyrosinase, dopachrome tautomerase and peroxidase.

Previous associated studies states that the crude extraction of the ink gland and from other accessory nidalmental gland are achieved using various solvents. In this study the crude extraction is achieved by parallel solvent extraction method. Previous reports suggest that the extracts with alcohols such as ethanol, methanol and butanol yields salt from the ink. Thus the medium activity shown by these extracts could be the effect of salt against the ESBL strains. The trace activity by the other extracts could be due to the presence of trace solvents as the exact evaporation of the extracts could not be achieved with the rotaevaporator. Thus this study elucidates that the hexane extract is effective against the ESBL strains and could be used in the future therapeutic scenario in the treatment of dreadful infections caused by these pathogens. However, the chemical analysis of the hexane extract and the identification of the bioactive constituents are in progress.

**Conclusion**

Microbial development of resistance, as well as economic incentives, has resulted in research and development in the search for new antibiotics in order to maintain a pool of effective drugs at all times. Unless antibiotic resistance problems are detected as they emerge and actions are taken immediately to control them, society could be faced with previously treatable diseases that have become again untreatable, as in the days before antibiotics were developed. Several organizations such as the World Health Organization have recognized the need to implement more concrete drug protocol and novel drugs with biopharmaceutical values and to pair them with access to reliable, safe drugs globally. Thus this study recommends the use of squid ink as a valuable biopharmaceutical product with antibacterial property against the prevailing ESBL producing strains of E.coli and K.pneumoniae. Further analysis of elucidating the structure of the bioactive molecule responsible for the antimicrobial activity is under progress. This study concludes by stating that squid ink will definitely aid in the eradication of these resistant strains in future.

**Acknowledgement**

Authours are thankful to Dr.Mehr Sultana., Department of Zoology, Presidency College, Chennai, Tamilnadu for speciating the squid selected for our study.

**References**