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# Bioprospective studies of pigmented ink from *Sepioteuthis lessoniana* and its molecular identification using CO1 gene

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## Abstract

**Background** The present-day world is teeming with numerous diseases due to the changing environment. The enormous growth of population has overburdened the prevailing resources of drugs; hence, drug manufacturers are in the lookout to develop effective and safe drugs in the pharmaceutical field. Marine environment is well known for its secondary metabolites, having a high potential in the research world of medicines. Several successful researches have explored the bioactivities of the marine organisms. In this regard, this study highlights the bioprospective activities of squid ink and identification of the organism using CO1 gene marker.

**Results** In the present study, anti-inflammatory activity evaluated by human red blood cell (HRBC) membrane stabilization assay revealed protection of human blood cells in hypotonic solution confirming anti-inflammatory property of squid ink extract. Bovine serum protein denaturation method for investigating in vitro anti-arthritis activity proved that the ink extract has appreciable inhibitory effect on denatured proteins. The in vitro antioxidative property of the squid ink disclosed remarkable free radical scavenging activity. The squid ink exhibited potent antibacterial activity against three microbial pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. From the molecular study using CO1 gene sequencing, it was found that the given species of squid showed 100% similarity with a species in NCBI GenBank and it was identified to be *Sepioteuthis lessoniana*.

**Conclusions** It is evident from the study that squid ink extract is a good source of anti-inflammatory, antioxidative, anti-arthritis and antimicrobial agents which would replace the existing cost of effective investigations intending to purify these active compounds and its identification of new molecular skeleton can give idea to the development of either the base or a new drug itself in the future.

**Keywords** *Sepioteuthis lessoniana*, Anti-inflammatory activity, Anti-arthritis activity, Antioxidant activity, Antimicrobial activity

## Background

Secondary metabolites are natural compounds which are produced by living systems that give particular species their characteristic features. These metabolites have

significant roles in the research fields of pharmaceutical industry. The marine environment is one of the main sources for the production of these bioactive compounds, and they are biologically and chemically diverse in nature. Most of the marine organisms possess bioactive compounds with multifunctionalities. The marine-derived natural products are unique chemical compounds that have the potential to be developed as new therapeutic drugs for future use. In this connection, numerous bioactive compounds have been extracted, characterized and purified from various marine organisms like bacteria,

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algae, dinoflagellates, tunicates, sponges, soft corals, bryozoans, cephalopods and echinoderms in the recent years (Donia & Hamann, 2003; Haefner, 2003; Chen et al., 2010; Jismi et al., 2018; Agustini et al., 2019; Utami et al., 2021; Karthikeyan et al., 2022; Shaban, 2022).

Cephalopod molluscs (squid, cuttlefish and octopus) are one of the commercially important invertebrates with high neural advancement. Among the cephalopods, squid belongs to the order Teuthida and comprises of about 304 species. The main squid species distributed in Indian waters are *Sepioteuthis lessoniana*, *Loligo duvauceli*, *Loligo uyii*, *Loligo edulis*, *Loligo singhalensis*, *Loliolus investigatoris*, *Symplectoteuthis oualaniensis* and *Thysanoteuthis rhombus*, among which *Sepioteuthis lessoniana* species is normally found in Palk Bay and Gulf of Mannar waters (Mohamed, 2008). Cephalopods are also one of the economically important species, and their distinctive feature is inking behavior. During defensive mechanism, they release the colored ink consisting of melanin compounds. Studies have reported that squid ink extract which is responsible for the anti-inflammatory activity consists of melanin pigment (90%), proteins (5.8%) and carbohydrates (0.8%) (Mimura et al., 1982). The ink of cephalopod species also contains several other compounds that are capable of disrupting the predator's chemical senses, but evidences are not fully recorded (Caldwell, 2005).

In recent years, different types of diseases are emerging massively in an uncontrolled manner. Most of the diseases' primary symptom is the condition of pain, which is associated with actual or potential tissue damage. The free radicals are the important macromolecules which lead to cell damage in the body system. A deleterious process called oxidative stress can arise when cells cannot adequately destroy excess of free radicals formed and the process can be counteracted by producing antioxidants. Squid ink contains a number of constituents particularly high in antioxidants and helps to protect cells against these free radicals (Nadarajah et al., 2017). Another study reported that squid ink has positive effects on broiler chickens in terms of their growth performance, antioxidant activity as well as immune modulation (Liu et al., 2011). Another report defines squid ink as a multifunctional marine bioactive material which promotes thromboxane production, kills cancer cells and elevates leukocyte number (Sasaki et al., 1997). Moreover, it has antioxidant, anti-radiation, anti-retrovirus and antibacterial properties (Fahmy & Soliman, 2013). A broad spectrum of antibacterial activity was reported for aqueous ink extract of *Loligo duvauceli* and *Sepia pharaonis* against nine human pathogens by Patterson and Murugan (2000). Nowadays, the use of commercial drugs for the treatment of various infectious diseases has

become highly resistant to human pathogenic microorganisms, particularly antibacterial drugs. Moreover, cost of production of these synthetic drugs is also high and they cause adverse effects when compared to naturally derived bioactive drugs.

The present work aims to study various biological properties of squid ink extract so that better insight on this obscure marine product can be obtained and thereby lead to application of the ink in the development of drugs for therapeutic applications.

## Methods

### Collection of samples

Squid samples (12 numbers) were collected with the help of local fishermen from the sampling site at Munambam near Vypeen Island, Ernakulam, Kerala, India (Long. 76° 10' E and Lat. 10° 10' N), and transferred to the laboratory under freezing conditions. The specimens were identified morphologically and anatomically on the basis of international databases and approved taxonomic references by using FAO identification keys (FAO, 1984). After morphological examination, muscle tissue samples were collected from each specimen and fixed in absolute ethanol and stored at -20 °C for further molecular identification studies.

### DNA extraction

The genomic DNA was extracted from the muscle tissue of squid samples with minor modifications in the protocol of DNA extraction (Sambrook et al., 1989). The ground muscle tissue was mixed with 500 µl of TNES buffer (50 mM Tris-HCl pH 8.0, 20 mM EDTA, pH 8.0 and 2% SDS) and 5 µl of Proteinase-K (20 mg/ml). The sample was then homogenized with occasional mixing. The tubes were incubated in water bath at 55 °C for 2 h. The sample was then placed in ice for 10 min, and thereafter, 250 µl of 6 M saturated NaCl was added into it. The sample was again chilled for 5 min. The sample mixture was then centrifuged at 8000 rpm for 15 min, and the supernatant (500 µl) was carefully transferred into a fresh tube and centrifuged again. The supernatant was discarded, and the pellet of DNA was stored for further analysis. The purity of DNA was checked using UV spectrophotometer (Shimadzu UV-1601).

**Table 1** List of primers

Name	Sequence	Sequence length	Orientation
LCO1490	5' GGT CAA CAA ATC AAA AGA TAT TGG 3'	682	Forward
HCO2198	5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'	639	Reverse

### Polymerase chain reaction and DNA sequencing

The extracted DNA was used as the template DNA for amplification of cytochrome oxidase 1 (CO1) gene. The CO1 gene was amplified by a pair of universal metazoan

as the positive control. After 30 min of incubation, the discoloration of the purple color was measured at 518 nm using a spectrophotometer (Shimadzu UV-1601). The ability of squid ink to scavenge DPPH radical was calculated as per the following formula by comparison with the control.

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$$\text{Percentage inhibition (\%)} = (\text{OD of control} - \text{OD of sample}) / \text{OD of control} \times 100.$$


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primers (Folmer et al., 1994) (Table 1) using a thermocycler (Biorad). The PCR reaction was carried out with 20 ng of DNA template in a volume of 25  $\mu\text{l}$  of 10X buffer (with 1.5 mM  $\text{MgCl}_2$ ) including 1  $\mu\text{l}$  of each primer (conc: 10 ppm), 200  $\mu\text{l}$  dNTPs and 1.5U Taq polymerase DNA enzyme. PCR thermal cycling started with initial denaturing step at 94  $^\circ\text{C}$  for 3 min followed by annealing at 54  $^\circ\text{C}$  for 30 s and extension for 1 min at 72  $^\circ\text{C}$  with a final extension for 10 min at 72  $^\circ\text{C}$ . The PCR product was then run on 1.5% agarose gel electrophoresis, and the samples with clear band pattern were selected for further sequencing analysis. The sequences were identified using the BLAST algorithm at the NCBI web site (National Center for Biotechnology Information, <https://www.ncbi.nlm.nih.gov>).

### Assessment of in vitro anti-inflammatory activity

The in vitro anti-inflammatory activity was assessed by HRBC (human red blood cell) method (Azeem et al., 2010) with minor modifications. 100  $\mu\text{l}$  of ink extract solution at 50, 100, 250, 500 and 1000  $\mu\text{g/ml}$  concentration in 0.9% saline was mixed with 300  $\mu\text{l}$  of 0.25% NaCl solution, 100  $\mu\text{l}$  of 0.15 M phosphate buffer (pH 7.4) and 500  $\mu\text{l}$  of 10% human RBC. All the test samples were incubated at 56  $^\circ\text{C}$  for 30 min, followed by cooling in water bath for 20 min and centrifuged for 10 min at 1500 rpm. The supernatant was collected from each tube, and absorbance was measured using a spectrophotometer at 560 nm (Shimadzu UV-1601). As a positive control, aspirin was employed in the same concentrations and an isotonic solution of NaCl used as a negative control. The percentage of hemolysis was calculated as follows:

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$$\text{Percentage inhibition (\%)} = (\text{OD of control} - \text{OD of sample}) / \text{OD of control} \times 100.$$


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### Squid ink extract preparation

The freshly obtained squids were dissected, and the ink glands were manually removed aseptically from the viscera. The squid ink was collected from the main ink sac and from the small secondary deposits behind the eyes by using sterile scissors and forceps. The collected ink was mixed well with acetone and distilled water in the ratio 2:1:1. The ink extract thus obtained was stored at 4  $^\circ\text{C}$  for further studies.

### Assessment of in vitro antioxidant activity

The effect of squid ink extract on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was determined using DPPH radical scavenging activity method (Brand-Williams et al., 1995). The samples were allowed to react with the stable

### Assessment of antimicrobial activity

The micro-broth dilution test was carried out for estimating the antimicrobial activity of squid ink extract against freshly grown cultures of Gram-positive bacteria—*Staphylococcus aureus*, and Gram-negative bacteria—*Escherichia coli* and *Pseudomonas aeruginosa*. The susceptibility panel was 96-well microtiter plates, prepared by dispensing 25, 50, 75 and 100  $\mu\text{l}$  of different concentrations of ink extract, 100  $\mu\text{l}$  bacterial culture and nutrient broth (75, 50, 25  $\mu\text{l}$ ) added to each well, and the total suspension was made up to 200  $\mu\text{l}$ . The positive control was inoculated with bacterial suspension, and the negative control contained only the extract and nutrient broth. After incubation at 37  $^\circ\text{C}$  for 24 h, the optical density of each well was recorded using spectrophotometer at 620 nm (Shimadzu UV-1601).

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$$\text{Percentage inhibition (\%)} = (\text{OD of control} - \text{OD of sample}) / \text{OD of control} \times 100.$$


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DPPH radical in methanol solutions. The reaction mixture consisted of 10  $\mu\text{l}$  extract, 0.48 ml methanol and 0.5 ml of methanol solution of DPPH. The methanol solution served as the blank, DPPH in methanol without the extract served

### Assessment of anti-arthritis activity

For the evaluation of anti-arthritis activity, the inhibition percentage of protein was evaluated by using the in vitro bovine serum protein denaturation method

(Rahman et al., 2012). The ink extract of various concentrations such as 50, 100, 250, 500 and 1000 µg/ml was taken and mixed with bovine serum albumin (0.5% w/V BSA), respectively. Initially, samples were incubated at 37 °C for 20 min and later the temperature was increased to incubate the samples at 57 °C for 3 min. After cooling, 2.5 ml of phosphate buffer was added to the above solutions, followed by the measurement of absorbance using UV–visible spectrophotometer at 255 nm. And the test sample results were compared with a standard reference drug (positive control) diclofenac sodium in the range of concentration 50–1000 µg/ml and as a negative control distilled water was also taken.



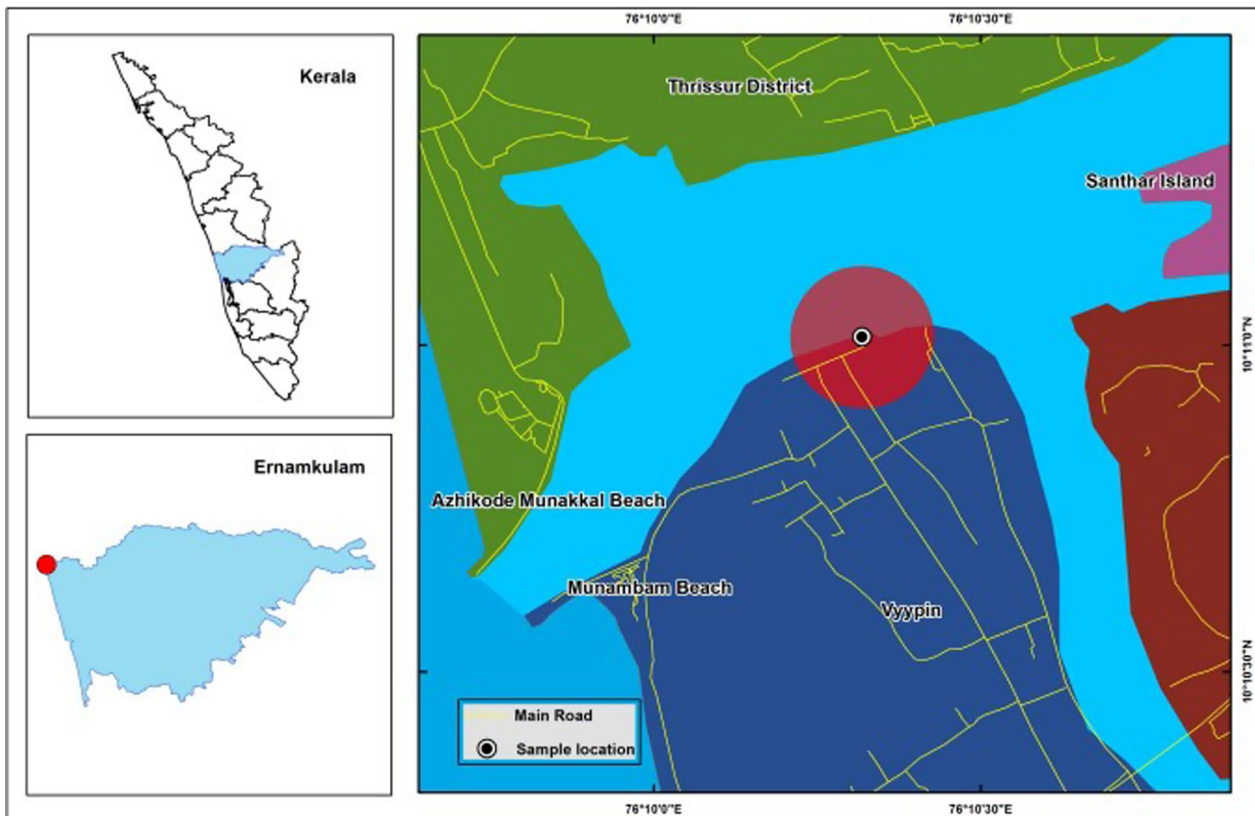
**Fig. 2** *Sepioteuthis lessoniana*

## Results

### Morphological identification

The squid samples collected from Munambam near Vypeen Island, Ernakulam, Kerala, India (Fig. 1), were identified as *Sepioteuthis lessoniana* (Fig. 2) as per the FAO Species Catalogue Vol. 3, Cephalopods of The World (FAO 1984). The key identification features of *Sepioteuthis lessoniana* as per FAO guidelines include:

mantle long, robust, its width about 40% of length. Fins very large, their length over 90% up to nearly 100% of mantle length, their width up to 75% of mantle length; the greatest width occurs posterior to the midpoint of the fins. Tentacular clubs long, expanded; median manal suckers enlarged; rings with 14–23 sharp teeth. Arm



**Fig. 1** Map showing collection site at Munambam

sucker rings with 18–29 sharp, triangular teeth; tentacles long, robust; left arm IV hectocotylized along distal 1/3–1/4 of arm.

**Molecular identification**

The identification of the mitochondrial DNA CO1 gene sequence (633 bp) through NCBI GenBank BLAST analysis inferred that the sequence showed 100% similarity with *Sepioteuthis lessoniana*. The NCBI presently has 738 accessions available for *Sepioteuthis lessoniana*, and its Taxonomy ID is 34570.

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ACTTTTCTTATTTTGGTATTTGAGCAGGA
TTAGTTGGTACCTCACTAAGATTAATAATT
CGAACCGAATTAGGTAAACCCGGCTCATTA
CTAAATGATGACCAATTATATAATGTTGTA
GTTACTGCACACGGTTTTATTATAATTTTC
TTTATAGTTATACCTATTATAATTGGAGGC
TTTGGTAACTGACTTGTCCTCTCATACTA
GGAGCACCTGATATAGCATTTCCACGAATA
AATAATATAAGATTCTGATTGCTACCTCCA
TCACTAACACTCCTTTTAGCGTCCTCAGCA
GTTGAAAGAGGAGCCGGTACAGGATGAACC
GTCTATCCGCCCTCTCAAGTAACCTGTCT
CATGCTGGACCTTCAGTTGATCTTGCTATC
TTCTCACTACATTTAGCTGGTATCTCTTCT
ATCCTAGGAGCAATTAACTTTATTACAACC
ATTATTAATATACGATGAGAAGGTTTACTT
ATAGAACGCTTACCTTTATTTGCCTGATCT
GTCTTTATTACTGCTATCTTACTCCTTCTA
TCATTACCTGTTTTAGCGGGAGCCATTACA
ATATTACTTACAGACCGAACTTTAATACC
ACTTTCTTTGACCCAAGAGGTGGGGGAGAC
CCTATTCTATATCAACACTTATTTTGATTT
TTTGGCACATAGAAAAGATAAA
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**Fig. 3** Ink extract from *Sepioteuthis lessoniana*

**Mitochondrial DNA CO1 gene sequence of *Sepioteuthis lessoniana***

See Fig. 3.

**Assessment of in vitro antioxidant activity**

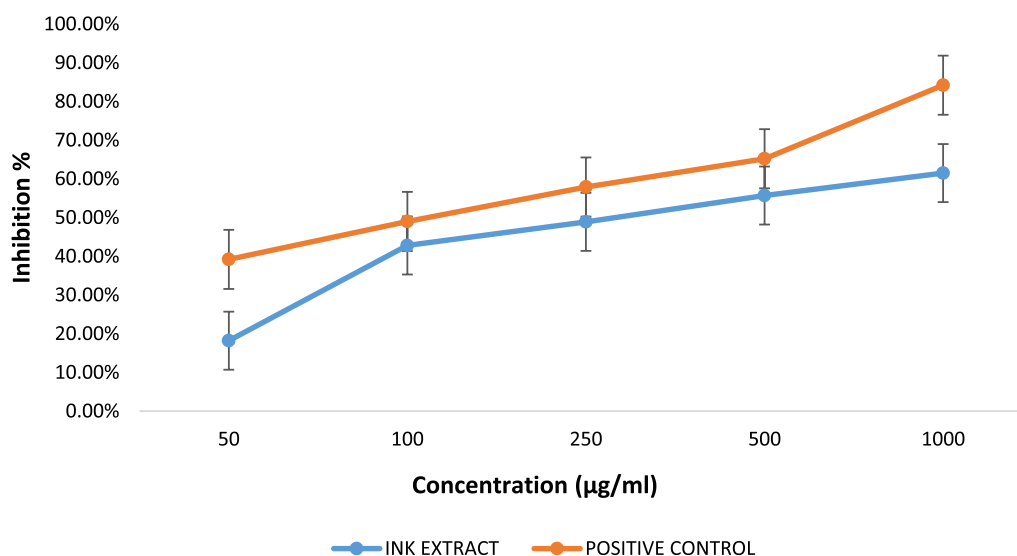
DPPH radical scavenging activity of squid ink extract (Fig. 3) was studied at different concentrations ranging from 50 to 1000 µg/ml (Table 2), and the result is depicted in Fig. 4. The squid ink extract at all concentrations was found to inhibit the DPPH free radicals. In the present study, *S. lessoniana* ink extracts exhibited highest DPPH scavenging activity at a concentration of 1000 µg/ml (61.5%), and in the lowest concentration of 50 µg/ml it was 18.2%. The results of the present study indicated that ink extract obtained from *S. lessoniana* possessed hydrogen donating capability and could exert in vitro antioxidant property.

**In vitro anti-inflammatory analysis**

The anti-inflammatory activity of *S. lessoniana* was studied by HRBC membrane stabilization method. Squid ink extract at all concentrations 50, 100, 250, 500 and 1000 µg/ml concentration showed significant stabilization toward the HRBC membrane (Table 3) and also protected further lysis of membrane. Extract at a concentration of 1000 µg/ml inhibited 84.1% of RBC hemolysis and represented the highest percentage of inhibition. The result indicated that the squid ink extract protection percentage toward the membrane increased with increase in the concentration of samples. Maximum percentage of stabilization establishing significant in vitro anti-inflammatory activity was evident from the results of the analysis (Fig. 5).

**Table 2** In vitro antioxidant activity of ink extract (DPPH radical scavenging activity of different concentrations)

Treatment	Concentration (µg/ml)	Absorbance at 518 nm	% Inhibition
Ink extract	50	1.25	18.2
	100	0.875	42.8
	250	0.782	48.9
	500	0.677	55.7
	1000	0.589	61.5
Positive control	50	0.93	39.2
	100	0.78	49.01
	250	0.643	57.9
	500	0.532	65.2
	1000	0.241	84.2
Negative control	–	1.53	–



**Fig. 4** In vitro antioxidant activity of ink extract (DPPH radical scavenging activity of different concentrations)

**Table 3** In vitro anti-inflammatory activity of ink extract in different concentrations

Treatment	Concentration (µg/ml)	Absorbance at 560 nm	% Inhibition
Ink extract	50	0.568	10.7
	100	0.481	12.06
	250	0.396	32.6
	500	0.252	57.7
	1000	0.148	84.1
Positive control (Aspirin)	50	0.545	14.3
	100	0.329	48.2
	250	0.213	66.5
	500	0.201	68.3
	1000	0.091	85.6
Negative control (NaCl)	–	0.636	–

**Antimicrobial analysis**

In the present study, the antimicrobial property of squid ink extract was tested against three different bacterial pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The growth of pathogenic bacteria was inhibited by the ink extract at different concentrations such as 25 µl, 50 µl, 75 µl and 100 µl (Fig. 6). Squid ink extract exhibited prominent antibacterial activity against Gram-negative bacteria *E. coli* than the other bacterial pathogens. There was maximum percentage inhibition of growth of *E. coli* (92%) than *Pseudomonas* and *Staphylococcus* at a concentration of 100 µl confirming the antimicrobial potential of squid ink extract.

**Anti-arthritis analysis**

The ink extract of *S. lessoniana* was able to inhibit protein denaturation in a concentration-dependent manner, and the inhibitory effect of ink concentrations (50–1000 µg/ml) on protein denaturation is shown in Fig. 7. The crude ink extract demonstrated 98.01% of protein denaturation at a maximum concentration of 1000 µg/ml and 10.1% of inhibition at the lowest concentration of 50 µg/ml (Table 4).

**Discussion**

Marine environment is a distinct reservoir of life forms containing nearly 250,000 described species (Boeuf, 2011; Malakoff, 1997), and most of the organisms provide extremely diverse and distinctive natural products. These natural products are highly bioactive compounds and have unique structures which are useful in the therapeutic field, and it can be used as a food source. Most of the marine natural products have potent bioactivities, and they have been serving as promising tools in the pharmaceutical industry. *Sepioteuthis lessoniana* is one of the economically important cephalopod species in India. In this study, identification of *S. lessoniana* was confirmed by morphological and molecular techniques, which could be fruitful for advanced research and related studies. In support of the occurrence of *S. lessoniana* in the geographical location of Kerala waters, egg clusters of *S. lessoniana* were noticed in the Vizhinjam coast of Kerala during August to October season (Neethu Raj et al., 2015).

The ink of *S. lessoniana* contains a large number of biologically active constituents with multifunctional

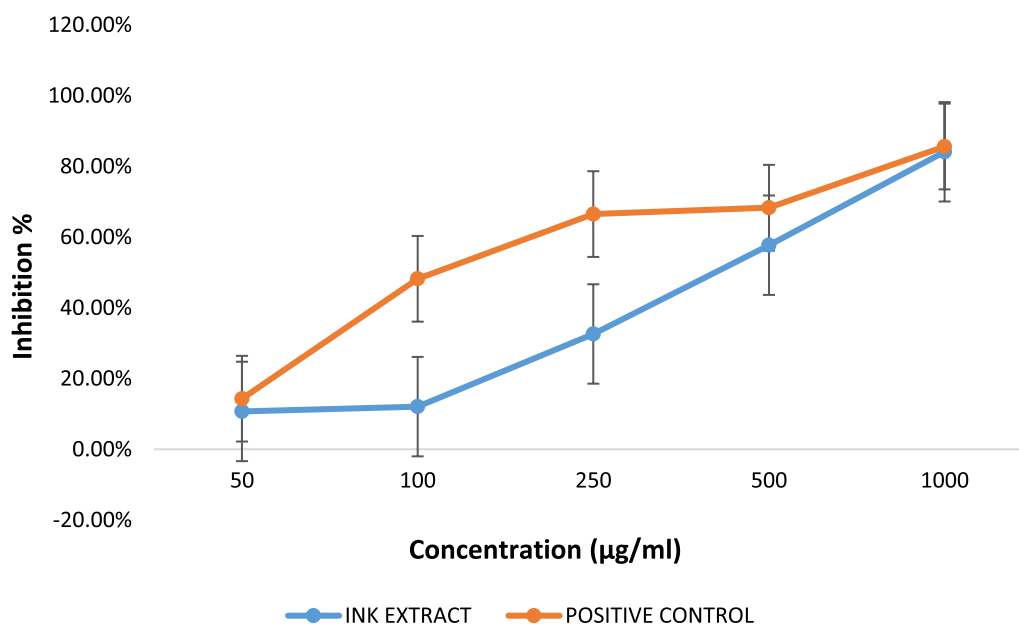


Fig. 5 In vitro anti-inflammatory activity of squid ink in different concentrations

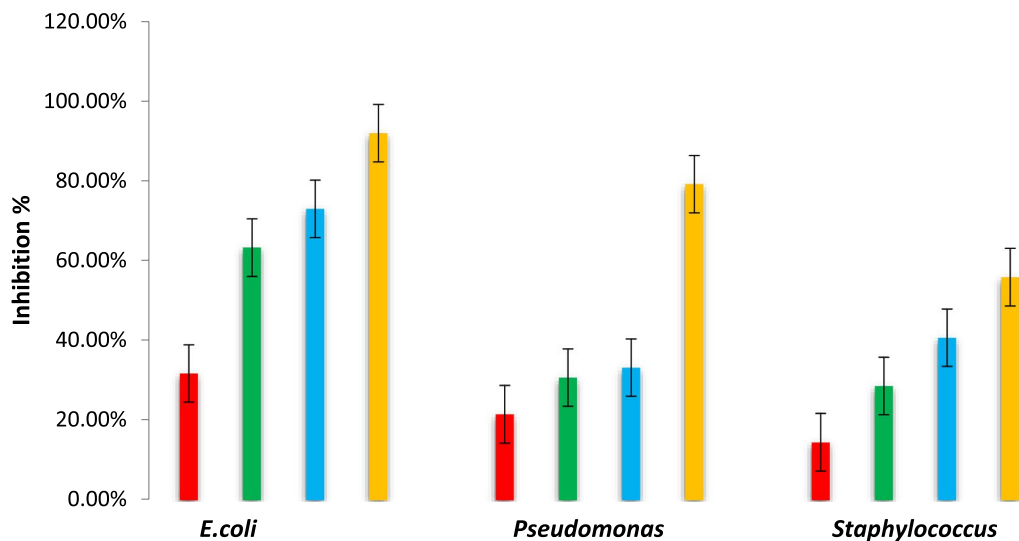
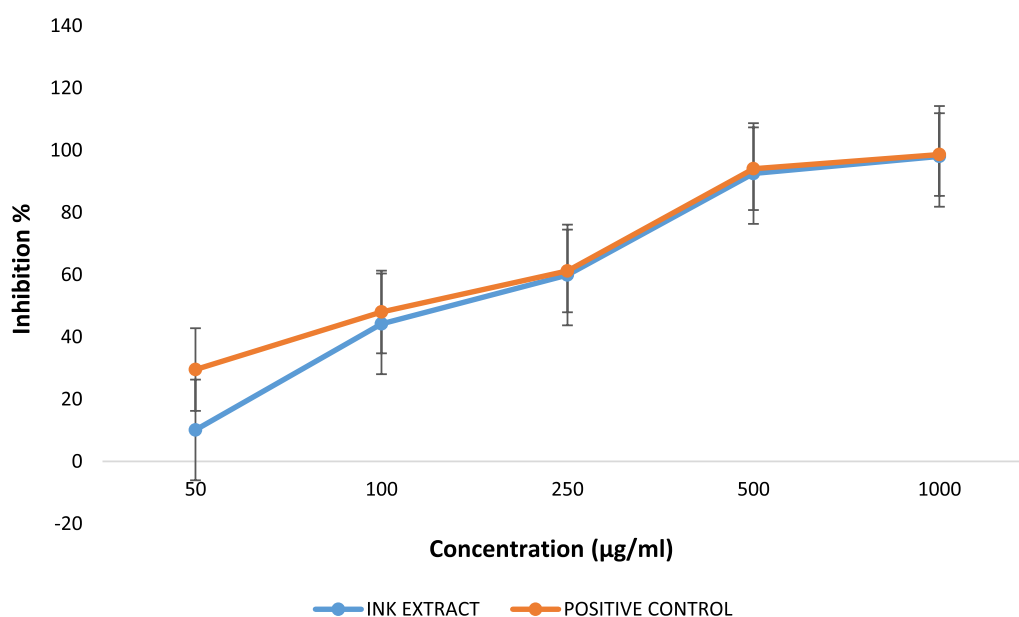


Fig. 6 Growth inhibitory action of ink extract against three pathogens at different concentrations

applications in various fields of science. In the present study, we attempted to evaluate the in vitro antioxidant activity of the ink extract from *S. lessoniana* using the DPPH assay. The free radicals are extremely reactive molecules produced by environmental stress and or radiation. These reactive oxygen species (ROS) are the exogenous factors or are generated during metabolic processes in the form of superoxide anion radical ( $O_2^-$ ), hydroxyl radical (OH), hydrogen peroxide ( $H_2O_2$ ), etc. The DPPH free radical scavenging test is a feasible and

rapid method for the screening of antioxidants in animal extracts (Hagerman et al., 1998). The squid ink extract was screened for this ability, and an increasing trend with DPPH radical scavenging activity was observed. The antioxidants can donate a hydrogen atom or an electron to stabilize the radicals. In this assay, the DPPH has an odd electron and this molecule might have accepted a hydrogen atom donated by the antioxidants present in the squid ink extract and it is reduced to non-radical species and hence the solution loses its color. The absorbance of



**Fig. 7** Effect of ink extract against protein denaturation using bovine serum albumin (anti-arthritis activity)

**Table 4** Effect of ink extract against protein denaturation using bovine serum albumin (anti-arthritis activity)

Treatment	Concentration (µg/ml)	Absorbance at 255 nm	% Inhibition
Ink extract	50	0.408	10.1
	100	0.253	44.2
	250	0.182	59.9
	500	0.034	92.5
	1000	0.009	98.01
Positive control (diclofenac sodium)	50	0.32	29.51
	100	0.236	48.01
	250	0.176	61.2
	500	0.027	94.05
	1000	0.006	98.6
Negative control (distilled water)	–	0.454	–

DPPH radicals decreases due to the scavenging activity of radicals by electron donation. This indicates that when concentration of the test sample increased, the scavenging activity of the ink extract also increased. L-dopa and dopamine have been detected in squid ink which have hydroxyl groups and are capable to donate oxygen, serving as antioxidant agent (Girija et al., 2014; Liu et al., 2011). Another study estimated the DPPH antioxidant assay based on the decolorization of stable free radical in the presence of antioxidants (Kumarasamy et al., 2007). Squid ink consists of several compounds such as melanin, protein, carbohydrate and lipid (Meyskens et al., 2001). The melanin of sepia ink can catalyze  $O^{2-}$  to  $H_2O_2$  and can break the free radical chain triggered by  $O^{2-}$  ion (Chen et al., 2007). Various studies have confirmed that

the melanin and other constituents present in squid ink were also responsible for free radical scavenger function and thereby has substantial antioxidant activity (Liu et al., 2011; Guo et al., 2014; Fatimah & Rabeta, 2018).

The HRBC membrane stabilization method was selected to determine the anti-inflammatory activity of the squid ink extract, as the erythrocyte membrane is analogous to lysosome membrane (Murugasan et al. 1981) and its stabilization implies that the extract may as well stabilize lysosomal membranes. The uncontrolled mechanism of inflammatory process leads to systemic inflammatory response by the circulation of pro-inflammatory mediators and ultimately causes death (Pavlov et al., 2003). In this assay, the hypotonic solution induced the lysis of human RBC membrane,



since the erythrocyte membrane represents the lysosomal membrane components (Kothari et al., 2017). The hypotonicity-induced lysis of membrane was stabilized by the ink extract, and it was taken as a measure of in vitro anti-inflammatory activity. The stabilized lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil (Baumann & Gauldie, 1994).

In the present study, squid ink extract inhibited the growth of three different bacterial pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Natural antimicrobial agents have the efficacy to fight against the increasing antibiotic-resistant microorganisms. Numerous studies have been previously conducted for the evaluation of antimicrobial activities of different squid inks (Venkatesan et al., 2014; Nicomrat & Tharajak, 2015; Lu et al., 2016; Fatimah & Rabeta, 2018). Nirmale et al. (2002) suggested that the freeze-dried and precipitated ink of the Indian squid *Loligo duvauceli* has good antibacterial effects (Nirmale et al., 2002). In support of the present study findings, one of the studies verified that the methanol extracts from the ink of *L. duvauceli* exhibited growth inhibitory activity against several bacteria (Smiline Girija et al., 2008). Mochizuki (1979) reported that purified extract of *S. lessoniana* ink showed antibacterial activity against *S. aureus* (Mochizuki, 1979). Fluhr et al (2001) and Takigawa et al (2005) reported that 9-octadecanoic acids/oleic acids content in squid ink raw extract could kill the bacteria directly and maintain an acidic pH condition for the bacteria (Fluhr et al., 2001; Takigawa et al., 2005). Studies have also established that the squid ink extract contains oleic acids which can stick to the bacterial cell membranes and cell wall precursors, damaging the cell wall structures (Kenny et al., 2009).

The crude ink extract was able to show that protein denaturation could be prevented and that *S. lessoniana*'s ink extract has anti-arthritis properties. It was reported that denaturation of protein is one of the causes of rheumatoid arthritis due to the production of auto antigens in certain rheumatic diseases (Mizushima & Kobayashi, 1968; Vane & Botting, 1995). Brown et al. also reported that certain arthritic diseases occur due to the formation of auto-antigens and may be due to in vivo denaturation of proteins (Brown & Mackey, 1968). The anti-arthritis agents in squid ink may function in the same line by suppressing the different types of inflammatory mediators in inflammatory process. It is therefore deduced that the ink extract of *S. lessoniana* was able to prevent the protein denaturation

and has significant therapeutic value as probable anti-arthritis agent.

Further elaborative studies are needed to elucidate other bioactive mechanisms of squid ink extract that could contribute in future industrial and medical applications.

## Conclusions

It is evident from the study that squid ink extract is a good source of anti-inflammatory, antioxidative, anti-arthritis and antimicrobial agents which would replace the existing cost of effective investigations intending to purify these active compounds and its identification of new molecular skeleton can give idea to the development of either the base or a new drug itself in the future. Considering the importance and paucity of information in this line, further research shall pave the way for the application of the extract as novel potent drug for human administration, as this is the need of hour.

## Abbreviations

FAO	Food and Agriculture Organization
DNA	Deoxyribonucleic acid
TNES	Tris NaCl EDTA SDS
HCl	Hydrochloric acid
mM	Millimolar
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
M	Molar
μl	Micro liter
CO1	Cytochrome c oxidase I
PCR	Polymerase chain reaction
PPM	Parts per million
dNTPs	Deoxynucleoside triphosphates
U	Unit
Taq	<i>Thermus aquaticus</i>
BLAST	Basic local alignment search tool
NCBI	National Center for Biotechnology Information
DPPH	2,2-Diphenyl-1-picrylhydrazyl
HRBC	Human red blood cell
BSA	Bovine serum albumin
ROS	Reactive oxygen species

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## Author contributions

MSS contributed to conceptualization, methodology, writing—original draft, investigation and formal analysis. BT was involved in formal analysis and review and editing. KSS contributed to conceptualization, supervision, formal analysis and review and editing. All authors have read and approved the manuscript.

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Not applicable.

## Availability of data and materials

The manuscript has no associated data.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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