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Extraction and Characterization of Therapeutic Potential of the Ink and Tissue Extract of Doryteuthis Singhalensis from the Gulf of Mannar

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Abstract

The marine environment is a rich source of unique bioactive compounds with promising pharmaceutical potential. Cephalopods, particularly cuttlefish, produce biologically active substances in their tissues and ink that may serve as novel antimicrobial agents. The present study investigates the antimicrobial properties of crude ink and tissue extracts of *Doryteuthis singhalensis* against selected pathogenic bacterial strains. Extraction was performed using polar and non-polar solvents, followed by antimicrobial assays, SDS-PAGE protein profiling, and GC-MS analysis to characterize the bioactive constituents. Results revealed significant antibacterial activity, particularly in methanolic extracts and identified several bioactive compounds, including carveol, phenolic derivatives, steroids and astaxanthin. These findings highlight the potential of cuttlefish-derived compounds as candidates for novel antimicrobial drug development.

Key Words

Pharmaceutical, Cephalopods, Cuttlefish, SDS-PAGE and GC-MS.

1. INTRODUCTION

The marine environment covers nearly 70 % of the Earth's surface and represents one of the most biologically diverse ecosystems on the planet. Oceans harbor an extraordinary range of life forms, and nearly two-thirds of all known phyla are exclusively marine. This vast biodiversity has made marine ecosystems a prolific source of structurally unique and biologically potent natural products with applications in pharmaceuticals, nutraceuticals, cosmetics and agrochemicals (Faulkner, 2002). Marine organisms produce diverse secondary metabolites shaped by extreme ecological conditions such as high salinity, pressure and intense competition, resulting in compounds with remarkable pharmacological activities. To date, thousands of marine-derived natural products, including terpenes, polyketides, alkaloids, peptides and polyketides, have been reported with antimicrobial, antiviral, antitumor and anti-inflammatory properties (Blunt *et al*., 2023).

Among marine organisms, invertebrates, particularly molluscs, have gained attention due to their rich repertoire of bioactive compounds. Molluscs, the second-largest invertebrate phylum, possess innate defense mechanisms that yield metabolites with antimicrobial, cytolytic, antitumor, and immunomodulatory activities. Cephalopods, a highly evolved molluscan class including cuttlefish, squids and octopods, are notable for their advanced nervous systems and effective chemical defenses. In addition to their nutritional value, cephalopods produce ink, a complex secretion composed of melanin, proteins, enzymes, biogenic amines and antioxidants, which functions in predator deterrence. Cuttlefish ink

has been traditionally used in medicine and is known for its antimicrobial, antioxidant and antitumor properties (Palumbo *et al.,* 2003).

The increasing threat of antimicrobial resistance has intensified the search for novel antimicrobial agents from marine sources. In this context, cephalopods represent an underexplored reservoir of bioactive proteins and peptides. Therefore, the present study evaluates the antimicrobial potential of ink and tissue extracts of the cuttlefish *Doryteuthis singhalensis* (Ullah et al., 2022). This work aims to contribute to marine natural product research and highlights the therapeutic potential of cephalopod-derived bioactive compounds.

2. REVIEW OF LITERATURE

Bioactive compounds from the marine habitat have been represented as the greatest underexploited source of potentially active pharmaceutical agents. They produce a variety of metabolites, some of which can be used for drug development (Chellaram and Prem Anand, 2010). Marine invertebrates offer a good source of potential antimicrobial drugs (Bansemir *et al*., 2006; Mayer *et al*., 2007; Jayaraj *et al*., 2008). The presence of antimicrobial activity in mollusca has been reported from the Portonovo region (Prem Anand *et al*., 1997). Antibacterial activities of oysters, *Crassostrea virginica* and mussels *Mytilus edulis* and *Geukensia demissa* was carried by Anderson and Beaven, (2001).

Cephalopod ink has been studied extensively due to its chemical composition, which includes melanin, mucopolysaccharides, proteins, and other bioactive compounds. The ink of the *Sepia* species, such as the common cuttlefish (*S. officinalis*), has been shown to exhibit antimicrobial effects against a range of bacterial strains including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Atta *et al*., 2021). Studies suggest that the ink may contain phenolic compounds and alkaloids that disrupt bacterial cell membranes or inhibit the growth of microorganisms.

Cephalopod skin secretions are produced by specialized cells and contain a wide range of bioactive molecules. These secretions have been found to exhibit antimicrobial effects, particularly in species like the *Octopus vulgaris*. The skin-derived antimicrobial peptides have been noted for their ability to combat various pathogens, including *Candida albicans* and *Bacillus subtilis* (Ullah *et al*., 2022).

The antibacterial effect of the Indian squid, *Loligo duvauceli* was reported by Nirmale *et al*., (2002). Du-Tie-ping *et al*., (2005) studied the antibacterial activity of extract from ink sacs of cuttlefish on bacteria *Staphylococcus aureus* and *E. coli*. Sherief *et al*., (2007) isolated, purified and characterized the antibacterial and anticancer agents from the accessory nidamental gland and ink of the cuttlefish *S. pharaonis.*

3. MATERIALS AND METHODS

3.1. Collection and Preparation of Extract:

In the present study, the animals *D. singhalensis* were collected from the Gulf of Mannar, Thoothukudi coastal region (Long 78° 8" to 79° 30" E and Lat 8° 35" to 9º 25" N) by trawl catch, brought to the laboratory, cleaned and washed with fresh seawater to remove all impurities. The abdomen of cephalopods was cut open and the ink glands and mantle tissue were carefully removed (Plate.1).

3.2. Crude extraction of ink:

The ink was collected by gently squeezing the glands with the spatula and the raw ink obtained from the glands was directly used for extraction. The ink used in the present work was crude and contained almost all the ingredients such as melanin, protein, lipids and glycosaminoglycans, etc.

Extraction was done by using different solvents. 25 ml of the ink was extracted with 75 ml of polar and non-polar solvents (1:3v/v) like hexane, chloroform, benzene, methanol and ethanol in sterile glass bottles. The 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 a vacuum rotary evaporator. Crude extracts were collected and stored at 4°C in glass bottles (Bansemir *et al.,* 2006).

3.3. Crude extraction of tissue:

The skin was removed and the tissues were cut into small pieces. 5g of tissue of cephalopod was weighed and homogenized with 5 ml of different solvents like hexane, chloroform, benzene, methanol and ethanol using a mortar and pestle following aseptic techniques. To this homogenate, 100 ml of solvents were added and incubated at room

temperature for 48 hrs. This extract was centrifuged at 27°C at 10000 rpm for 10 min and the supernatant was collected and concentrated under vacuum in a rotary evaporator at 30°C. The crude extract was analysed for its antimicrobial activity using the standard disc diffusion method (Boscaro *et al.,* 2022)

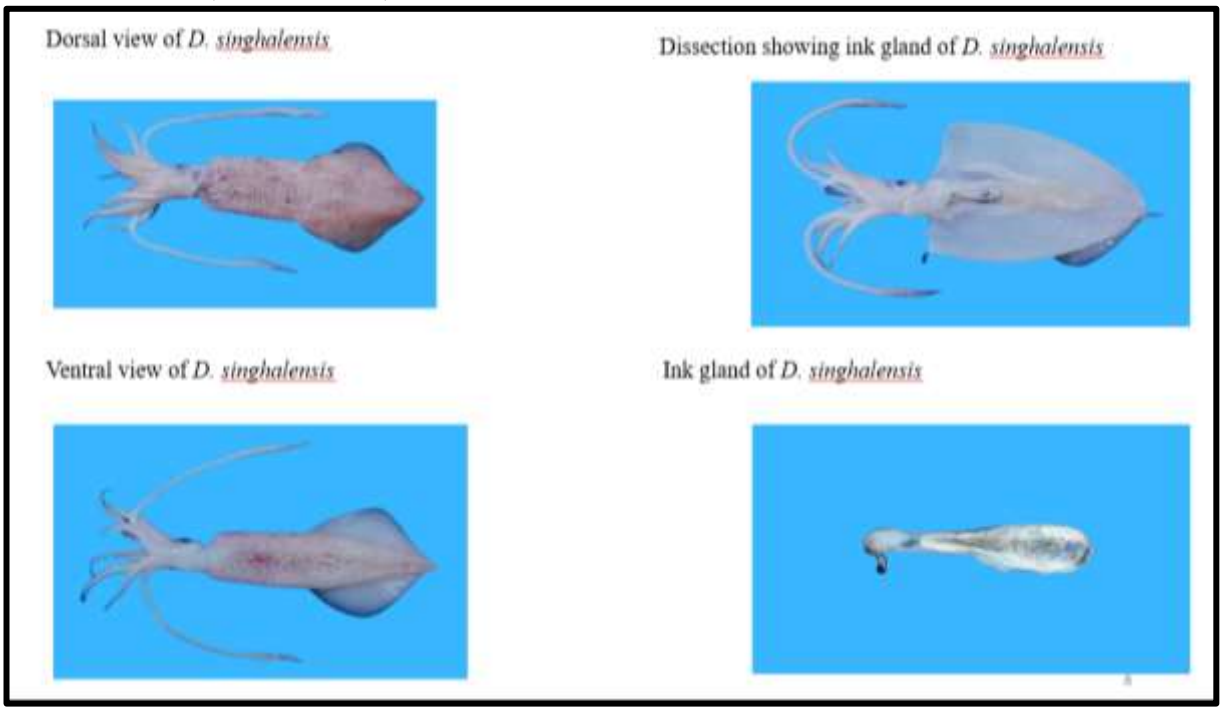
Plate 1. Experimental Animal: *Doryteuthis singhalensis*

3.4. Microbial Cultures:

Five bacterial strains, namely *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pneumoniae*, and *Staphylococcus aureus*, were obtained from the Zoology Department of St. Xavier's College (Autonomous), Palayamkottai.

3.5. Inoculum preparation for bacteria:

Nutrient broth was prepared and sterilized in an autoclave at 15lbs pressure for 15 minutes. All five bacterial strains were individually inoculated into the sterilized broth and incubated at 37°C for 24 hours. Nutrient agar was prepared and poured into sterile Petri dishes. The 24-hour-old bacterial broth cultures were inoculated in the Petri dishes using a sterile cotton swab (Boscaro, 2005).



3.6. Antimicrobial assay:

3.6.1. Agar well diffusion method:

The antimicrobial activity of *D. singhalensis* ink was evaluated using the well diffusion method. Mueller-Hinton Agar (MHA) was prepared, sterilized, and poured into sterile Petri dishes, allowing it to solidify. Bacterial *Klebsiella pneumonia***,** *Escherichia* coli, *Pseudomonas aeruginosa, Streptococcus pneumoniae* and *Staphylococcus aureus* strains were cultured and adjusted to a 0.5 McFarland standard. Using a sterile cotton swab, the microbial suspensions were evenly spread on the agar surface. Wells of approximately 6 mm in diameter were created using a sterile cork borer. Different concentrations of ink extract were loaded into the wells (25, 50,100 µL), along with a control. The plates were incubated at 37°C for 24 hours for bacterial strains and at 25 - 0°C for 24 hours for bacterial strains. After incubation, the antimicrobial activity was assessed by measuring the diameter of the zone of inhibition (ZOI) around each well using a ruler or calliper. The results were recorded, and statistical analysis was performed to determine the significance of the antimicrobial effect of cuttlefish ink compared to the controls (El Masry *et al.,* 2005).

3.6.2. SDS-PAGE:

SDS - polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) to qualitatively analyze protein profiles based on molecular weight. Proteins were denatured with sodium dodecyl sulfate

(SDS) and a reducing agent, allowing separation of polypeptide chains solely according to size. A discontinuous gel system consisting of a stacking gel and a separating gel was used (Jayalakshmi *et al.,* 2014).
An 18% resolving gel was prepared using acrylamide, Tris-HCl buffer (pH 8.8), SDS, ammonium persulfate (APS), TEMED, and distilled water, and allowed to polymerize. This was overlaid with a stacking gel prepared in Tris-HCl buffer (pH 6.8). Protein samples were mixed with sample buffer containing bromophenol blue and glycerol, heated at 95 °C for 3 min, and loaded into the wells along with molecular weight markers. Electrophoresis was carried out in Tris-glycine-SDS running buffer at 50 V until the dye front reached the bottom of the gel. After electrophoresis, protein bands were visualized by Coomassie Brilliant Blue staining.

3.6.3. GC-MS Analysis:

GC-MS analysis was performed using an Agilent 7890 gas chromatograph coupled to an Agilent 7000C mass selective detector in the positive ion electron impact (EI) mode. The separation was achieved using an HP-5MS fused silica capillary column, 30m 0.25 mm i.d., 0.25µm film thickness. GC oven temperature was programmed from 60°C to 285°C at a rate of 4.3°C/m in Helium was used as the carrier gas; inlet pressure was 25 kPa; linear velocity: 1 mL/min at 210°C Injector temperature: 250°C and injection mode: split 1:50. MS scan conditions: source temperature, 200°C interface temperature, 250°C E energy, 70 eV; mass scan range, 40-350 amu (Melo *et al*., 2000).

3.6.4. Identification of compounds:

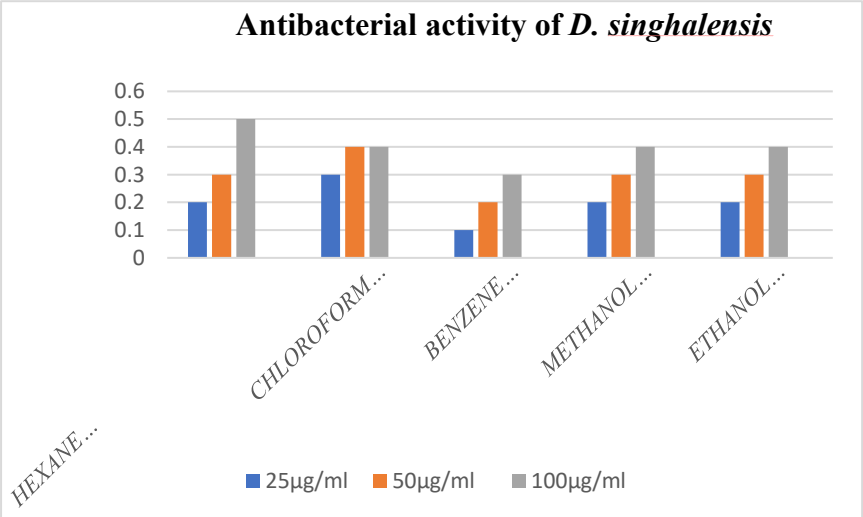
The interpretation of the mass spectrum was conducted using the database of the National Institute of Standards and Technology (NIST) WILEY 8 and FAME, which contains more than 62,000 patterns. The unknown components found in the methanol fraction of the tissue were matched with the spectrum of the known components stored in NIST, WILEY, FAME, and MS library and predicted from Duke's ethno botanical database. The name, molecular weight and structure of the components of the test materials were ascertained (Priya *et al*., 2006).

4. RESULTS

4.1. Antibacterial activity of the tissue of *D. singhalensis* by agar disc diffusion method:

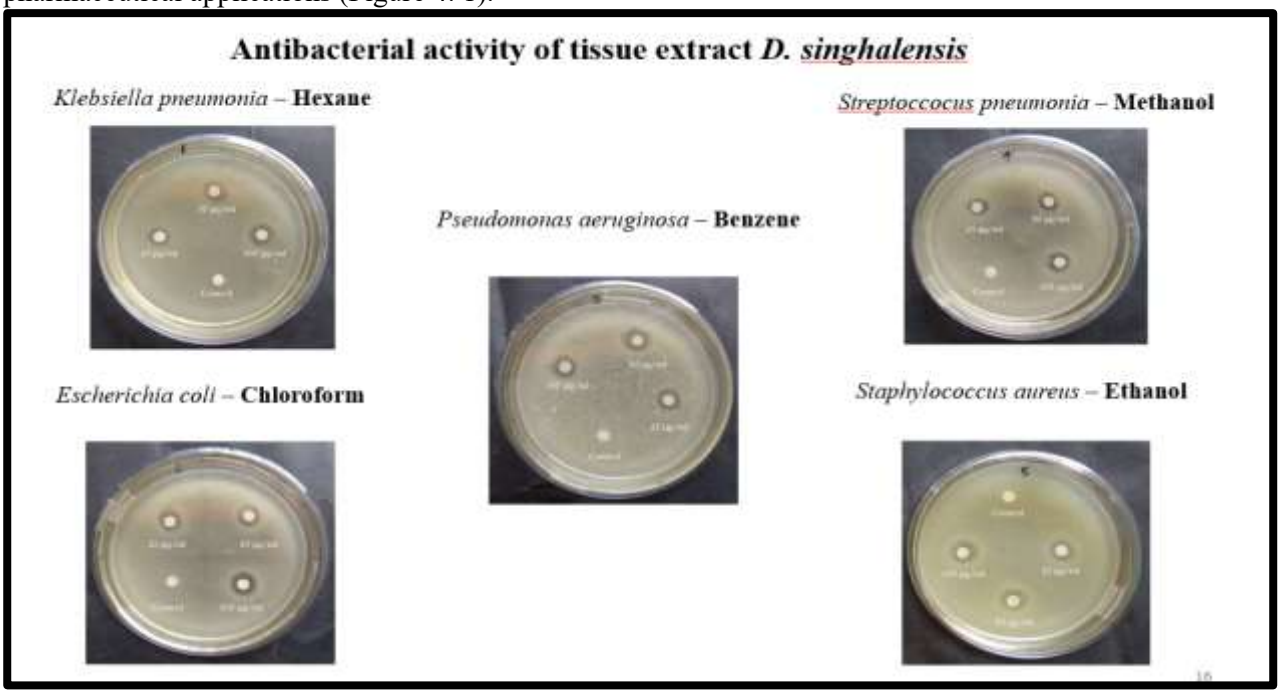
The tissue extracts of *D. singhalensis* showed activity ranging from 0.1mm to 0.5mm. In 25µg/ml, maximum antibacterial activity was recorded against *Klebsiella pneumonia, Escherichia* coli and *Staphylococcus aureus* 0.2mm and a minimum of 0.1mm against *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* in the Hexane, Chloroform, Benzene, Methanol and Ethanol extracts. In 50µg/ml, maximum antibacterial activity was recorded against *Klebsiella pneumonia, Escherichia* coli and *Staphylococcus aureus,* 0.3mm and a minimum of 0.2mm against *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* in the Hexane, Chloroform, Benzene, Methanol and Ethanol extracts, respectively. In 100µg/ml, maximum antibacterial activity was recorded against *Klebsiella pneumonia* at 0.5mm and a minimum of 0.3mm against *Pseudomonas aeruginosa* in the Hexane and Benzene extracts, respectively.

Figure 4. 1: Antibacterial activity of the tissue of *D. singhalensis*



These results clearly indicate that the antibacterial efficacy of *D. singhalensis* tissue extracts is both concentration-dependent and solvent-dependent, with higher concentrations producing stronger inhibitory effects. The observed activity suggests that *D. singhalensis* tissues contain potential antibacterial compounds that could be further explored for pharmaceutical applications (Figure 4. 1).

4.2.

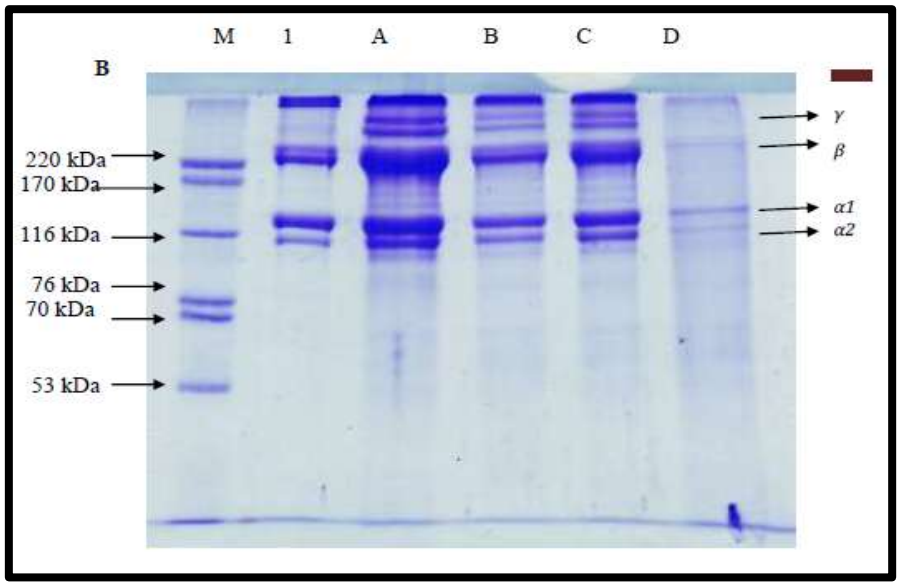


Protein Profiling:

SDS - PAGE analysis of the protein extract from *D. singhalensis* demonstrated the presence of several clearly resolved protein bands with molecular weights ranging from approximately 53 kDa to 220 kDa. The presence of multiple distinct bands indicates that the extract comprises a heterogeneous mixture of proteins, highlighting the organism's biochemical complexity.

Proteins in the lower molecular weight range (53 -70 kDa) may correspond to enzymes, antimicrobial proteins, or immune-associated molecules that are typically involved in innate defense mechanisms in both marine and terrestrial organisms. These proteins are frequently reported to exhibit antibacterial and antifungal activities through mechanisms such as disruption of microbial cell membranes, inhibition of essential enzymatic pathways, or interference with protein synthesis (Figure 4.2).

Figure 4.2: SDS - PAGE analysis of the protein extract from *D. singhalensis*



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4.3. GC-MS analysis:

The methanol extract of the whole-body tissue of *D. singhalensis* showed significant antimicrobial activity. Hence, this fraction was subjected to GC-MS analysis to characterize the compound responsible for antimicrobial activities.

GC-MS analysis of the body tissue of *D. singhalensis* exhibited five peaks (Figure 4.3) with the retention times ranging from 3.96 to 32.34 min. all five compounds were characterized as Carveol, Dasy carpidan-1-Methanol, acetate (ester), Calcitriol, Ursodeoxycholic acid, Desoximetasone. Among the identified compounds Carveol, Dasy carpidan-1-Methanol, acetate (ester), Calcitriol, Ursodeoxycholic acid, Desoximetasone, have roles in Fragrance in cosmetics, Flavor in foods, Anti-inflammatory, Antimicrobial, Antioxidant, Used for Vitamin D deficiency, bone diseases, Pre-dialysis and dialysis patients, Postmenopausal osteoporosis, reduce cholesterol absorption, Anticancer, Chemo preventive, used in skin diseases, Cure psoriasis (Table 1 and 2).

Figure 4.3: Chromatogram of the column extract of *D. singhalensis* by GC-MS

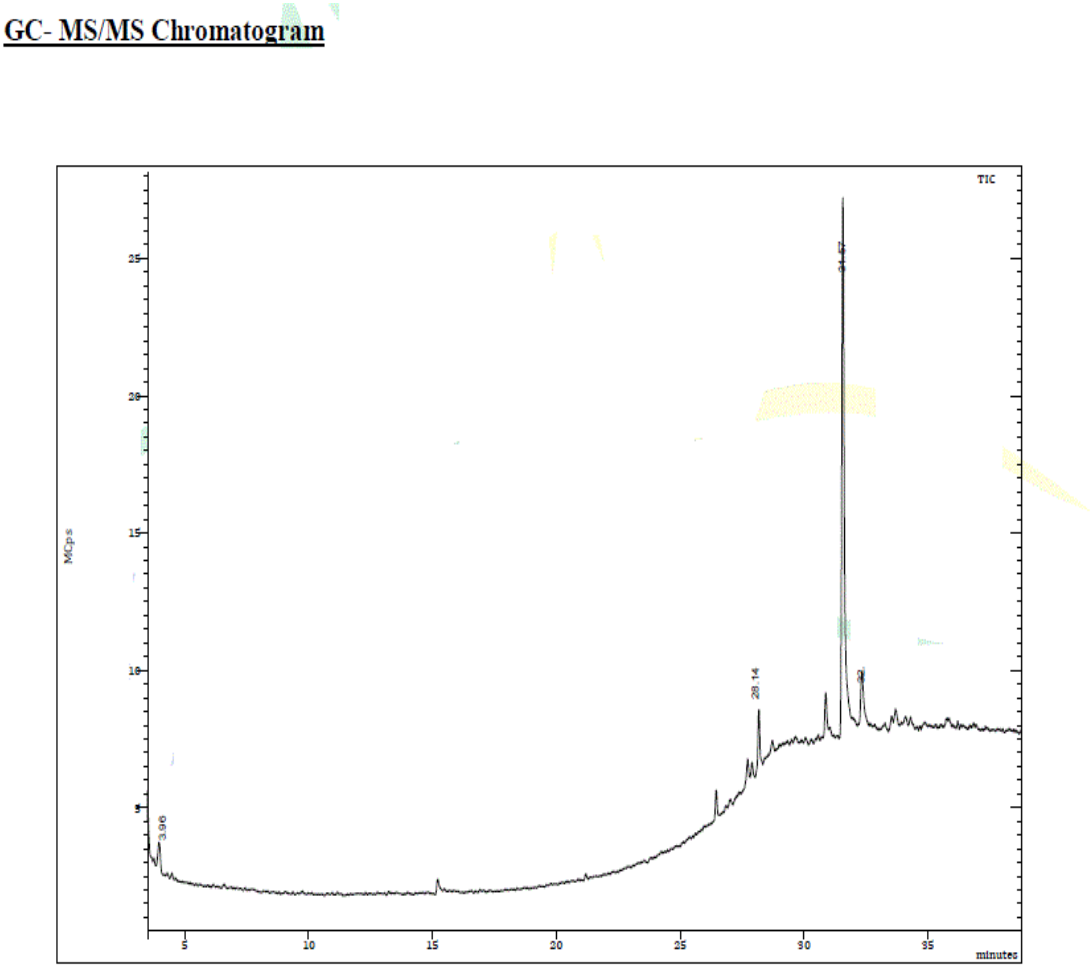

Table 1: Compounds identified in the methanol extract of the tissue of *D. singhalensis* by GC-MS

S.NO	RT	Name of the compound	Molecular formula	Molecular weight	Peak Area %
1	3.96	Carveol	$C_{10}H_{16}O$	152	3.02
2	27.73	Dasy carpidan-1-Methanol, aoetate (ester)	$C_{20}H_{26}N_2O_2$	326	4.50
3	28.14	Calcitriol	$C_{27}H_{44}O_3$	416	13.62
4	31.14	Ursodeoxycholic acid	$C_{24}H_{40}O4$	392	71.00
5	32.34	Desoximetasone	$C_{22}H_{29}F0_4$	376	7.86

Table 2: Activity of components identified in the methanol extract of the tissue of *D. singhalensis* by GC-MS

S.No	RT	Compound Name	Molecular Formula	MW	Peak area %	Compound Nature	**Activity
1	3.96	Carveol	$C_{10}H_{16}O$	152	3.02	Monoterpenoid alcohol	Fragrance in cosmetics, Flavor in foods, Anti-inflammatory, Antimicrobial
2	27.73	Dascarpidan-1-methanol, acetate (ester)	$C_{20}H_{26}N_2O_2$	326	4.50	Alkaloid compound	Antimicrobial, Anti-inflammatory, Antioxidant
3	28.14	Calcitriol	$C_{27}H_{44}O_3$	416	13.62	Vitamin D	Used for Vitamin D deficiency, Bone diseases, Pre-dialysis and dialysis patients, Postmenopausal osteoporosis
4	31.57	Ursodeoxycholic acid	$C_{24}H_{40}O_4$	392	71.00	Bile acids	Reduce cholesterol absorption, Anticancer, Antimicrobial, Anti-inflammatory,

							Chemo preventive
5	32.34	Desoximetasone	C_{22} $H_{29}FO_4$	376	7.86	Corticosteroid	Used in skin diseases, Cure psoriasis, Antimicrobial, Anti-inflammatory

5.

DISCUSSION

The present study demonstrates that the antibacterial and antifungal activities of ink and tissue extract from *D. singhalensis* are strongly influenced by the solvent used for extraction, concentration, and test organism. Solvent polarity plays a crucial role in extracting bioactive metabolites, which explains the observed variations in antimicrobial efficacy among extracts prepared with hexane, chloroform, methanol, ethanol and benzene. Similar solvent-dependent variations in antimicrobial activity have been reported earlier, emphasizing that disc diffusion outcomes are influenced by both extract diffusion and intrinsic potency of bioactive compounds (Kelman *et al*., 2006).

Ink extracts of *D. singhalensis* exhibited broad-spectrum antibacterial activity against all tested bacterial strains, with notable inhibition against *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* at higher concentrations. These findings are consistent with earlier studies on cephalopod inks, which reported pronounced antibacterial activity against human pathogens (Patterson and Murugan, 2000; Chacko and Patterson, 2005). In *D. singhalensis*, methanolic ink extracts showed comparatively higher activity than non-polar extracts, indicating the presence of polar antimicrobial constituents.

The tissue extracts, particularly methanolic extracts, also demonstrated measurable antibacterial activity in both species. The mantle tissue of *D. singhalensis* and the whole-body tissue of *S. brevimana* showed inhibition against both Gram-positive and Gram-negative bacteria, corroborating previous reports on antimicrobial compounds derived from cephalopod tissue (Mohanraju *et al.,* 2013; Ramasamy *et al.,* 2011). The relatively moderate inhibition zones observed in the present study may be attributed to crude extract composition and diffusion limitations.

SDS-PAGE analysis revealed a wide range of low- to high-molecular-weight proteins (≈53 - 220 kDa) in the ink of both species, suggesting the involvement of proteinaceous compounds in antimicrobial activity. Similar protein profiles with antimicrobial and antitumor properties have been reported from cephalopod inks, including bioactive proteins such as ST94 and Lolduvin-S (Naraoka *et al*., 2000; Smiline Girija *et al*., 2011). These findings strengthen the hypothesis that ink proteins contribute significantly to antimicrobial defense mechanisms.

GC - MS analysis of methanolic tissue extracts identified several bioactive compounds, including Carveol, Astaxanthin, Ursodeoxycholic acid, and Desoximetasone, which are known for antimicrobial, antioxidant, anti-inflammatory, and anticancer properties. The detection of such compounds aligns with growing evidence that marine molluscs are rich sources of pharmacologically relevant secondary metabolites (Blunt *et al*., 2018; Carroll *et al*., 2023).

Overall, the results highlight the therapeutic potential of cephalopod ink and tissue extracts as sources of novel antimicrobial agents. Further purification, structural characterization, and mechanistic studies are necessary to identify lead compounds and evaluate their efficacy against multidrug-resistant pathogens.

6. CONCLUSION

This investigation confirms that *D. singhalensis* is a promising source of bioactive compounds with antimicrobial potential. The crude ink and tissue extracts, particularly methanolic fractions, demonstrated significant antibacterial activity and contained diverse bioactive molecules identified via GC-MS. These findings provide a strong basis for developing cephalopod-derived pharmaceuticals and underscore the importance of marine biodiversity in drug discovery.

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