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Research Article
Bioactive Properties of Ink Gland Extract from Squid *Loligo duvauceli*

Immaculate Jeyasanta and Jamila Patterson

Suganthi Devadason Marine Research Institute, 44-Beach Road, 628001 Tuticorin, Tamil Nadu, India

Abstract

Background and Objective: Indian squid *Loligo duvauceli* is common major sources of seafood available in Tuticorin. However, the ink of these cephalopods is considered as the waste product. In the present study, the *L. duvauceli* ink was taken as a raw material which is generated as a byproduct in the processing industry, has low market value and has the potential to cause environmental pollution. **Materials and Methods:** The ink samples were screened for phytochemical, functional properties, proximate, antibacterial and antioxidant activities by standard methods. **Results:** Large numbers of compounds are present in the *L. duvauceli* ink are amino acids, carbohydrates, phenols, flavonoids, alkaloids and saponins whereas, tannins, steroid, glycosides are present in a small amount and anthraquinones, fixed oils and fats are absent. It had moisture, protein, lipid, ash and carbohydrate content were 79.25±0.051, 10.67±0.005, 0.19±0.01, 0.15±1.78 and 0.91±0.29%, respectively and this study showed that squid ink had the highest proximate composition and nutritionally preferred. The functional property of ink had pH (6.52±0.03) and water solubility index (16.8±0.004%) and the study revealed that ink can be used as a natural emulsifier in food applications. This investigation showed the methanol extract of ink exhibited significant activity against pathogens *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeroginosa*. The partially purified form of *L. duvauceli* ink also tested for antioxidant activity such as enzymatic antioxidants, nitric oxide scavenging activity and total antioxidant activity. **Conclusion:** This study suggests that *L. duvauceli* ink extract exhibit great potential for antioxidant activity and may be useful for their nutritional and medicinal functions. These results concluded that the *L. duvauceli* ink is a novel potential product and it has to further characterize to improve the pharmacological active marine natural products.

Key words: *Loligo duvauceli* ink, phytochemical, proximate composition, functional properties, antibacterial and antioxidant activity

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Corresponding Author: Immaculate Jeyasanta, Suganthi Devadason Marine Research Institute, 44-Beach Road, 628001 Tuticorin, Tamil Nadu, India

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In recent years, great attention has been paid to study the bioactivity of natural products due to their potential pharmacological utilization. Approximately 5000 species of sponges, 11000 species of corals, jellyfish and sea anemones, 9000 species of segmented worms, 100,000 species of annelids, polychaetes, snails, clams and octopus, 6000 species of sea stars and sea cucumber and 200 species of sea squids are present in the marine environment[1]. Among the various phyla represented in the marine environment, mollusca is the second largest phylum next to arthropod consisting of around 100,000 species all over the world in almost all types of habitats. From the above-mentioned groups more than 6,500 biologically active compounds have been isolated[2]. From the beginning, the number of compounds isolated from various marine organisms virtually soared and now exceeds 10,000 with hundreds of new compounds still being discovered every year[3]. Huge innovation opportunities exist in using an underutilized/waste/by-product to create new healthy products to consumers.

Cephalopods are common and important fisheries in terms of domestic consumption and economic values[4]. The class Cephalopoda contains the nautili cuttlefish, squids and octopods with internal shells or without shells is placed in the subclass coleoidea. Squid, cuttlefish and octopus are major seawater catch other than fishes and prawns. Cephalopods form a major item in marine fishery export from India, with increasing demand from various parts of the world. Cephalopod landings increased steadily in the last 40 years, from 1617 t in 1970 to 1, 65,394 t in 2010. They are mainly exported, so large amounts of waste and unutilized materials are produced (e.g., squid ink sacks). Cephalopods are famous for their defenses from their fast jetting escape movements, to changes in coloration that can be cryptic, disruptive or startling, to arm autotomy, toxic venom and by inking[5,6]. All cephalopods, with the exception of the Nautilidae and the species of octopus belonging to the sub-order Cirrina, are able to release ink. Squid and cuttlefish ink was produced at the end process of maturation in a viscous, colorless medium[7]. Each species of cephalopod produces slightly differently colored inks; generally, octopuses produce black ink, squid ink is blue-black and cuttlefish ink is brown. Cephalopod inks are chemical secretions produced by and released from the ink sac, which is not a homolog of the ink glands of gastropods but rather a modified hypobranchial gland[8,9]. Ink sacs have attracted considerable attention, both casually and scientifically[10]. Humans have used cephalopod ink for many practical and commercial purposes over the millennia, particularly in medicine, cuisine, art as well as in even broader applications[10]. This ink has been used since ancient times in Rome and Greece to treat baldness and kidney stones, also it is used to treat the hormonal imbalances in women in homeopathy and it is still used as a food preservative on meat to increase the shelf life in Japan. Cephalopod ink has wide application in homeopathic medicine, in food products (mainly Japan), antiseptic effect, antibacterial activity, antitumor activity etc. and their utilization in India was very limited[11].

So far only a little marine-derived products presently in the market, numerous novel compounds from marine basis are currently in scientific trials for drug development. While the marine world deals a tremendously rich resource for novel compounds, it also represents a great challenge that requires a multidisciplinary approach to bring the marine chemical diversity up to its therapeutic potential. Among the squids, the Indian squid (*L. duvauceli* Orbigny, 1848) is the dominant species, catching about 97% from Indian waters. Thomas and Kizhakudan[12] reported that males and females are found to be in equal proportion. It matures from the size ranges of 4-28 cm for males and 4-18 cm in females. Juveniles of squids are less than 4 cm in size. Females were dominant during January, March, May and December, whereas males were dominant in other months. The overall male to female ratio was 1:1.3. It is forming a notable fishery along Tuticorin coast throughout the year with a peak from June to November. The ink was generated as a byproduct in a cephalopod processing industry, has low market value and has the potential to cause environmental pollution. So, *L. duvauceli* ink was selected for the present study to explore the bioactive potentials of the ink. In a view to improving the utilization of squid ink first we must know the constituents of the ink. The aim of this study was to analyze the phytochemical, functional property, proximate constituents of *L. duvauceli* ink and its antimicrobial and antioxidant property.

MATERIALS AND METHODS

Sample collection and processing: Fresh squids (n = 30) were collected from the Thirespuram fish landing center of Tuticorin, South east coast of India during August, 2018 and were stored at 4°C while transported to the laboratory and the squid was identified as *L. duvauceli* using the keys given by Budelmann *et al.*[13]. The samples were selected based on same size and physical appearance and kept in a large container with ice cubes to maintain freshness of squid. On reaching the laboratory it was washed in tap water and then in sterile

water, it was then posteroventrally dissected and ink glands were manually removed from the viscera of squids. The ink glands were placed in a clean plastic container with a thin layer of approximately 2.5 cm. The ink was pressed and squeezed out from the ink glands by using a spatula. After that, the ink was frozen at -18°C for 3 h until it freezes completely. During processing, the ink was dried using hot air oven until it is free from water content and using the dry ink powder the extract was prepared. A total of 50 g of dried ink powder was subjected to pulverization using mortar and pestle. The 25 g of squid ink powder was mixed with 75 mL of methanol in sterile glass bottles by parallel extraction method. The ink was slowly mixed with methanol using a sterile glass rod and refrigerated at 4°C for 72 h and shaker at room temperature for 8-10 h. The methanol extract was centrifuged and collect the supernatant. The crude methanol extracts were collected and weighed and the sterility was checked under UV light for about 2 h. The extract was stored at 4°C in brown bottles. The *L. duvauceli* ink extract was partially purified by silica gel column (60-120 mesh silica gel). The partially purified ink extract was used for further analysis.

Qualitative estimation of phytochemicals: The preliminary phytochemical evaluations of methanol extract of *L. duvauceli* ink was carried out the following ways[14].

Test for protein

Biuret test: Take a small quantity of the dispersion of the sample in a test tube and add 2 mL of NaOH solution and add 4-5 drops of 1% $CuSO_4$ solution and warm the mixture for about 5 min. Bluish violet coloration indicates the presence of protein.

Test for carbohydrates

Molisch's test: Dissolve a small amount of ink extract in 4 mL distilled water and filtered. The filtrate was subjected to Molisch's test (5% α naphthol in ethanol) formation of reddish brown ring indicate the presence of carbohydrate.

Test for phenols: The methanol extract was spotted on a filter paper, a drop of phosphomolybdic acid reagent was added to the spot and then ammonia vapors was exposed, the blue color in the spot indicates the presence of phenol.

Test for flavonoids

Lead acetate test: To 5 mL ink extract, 1 mL of lead acetate was added, flocculent white precipitate indicates the presence of flavonoid.

Test for tannins

Braestmer's test: Take 2 mL of ink extract to add 10% alcoholic ferric chloride solution. Dark blue or greenish grey colorations of the solution indicate the presence of tannin.

Test for steroid and terpenoid

Liebermann-Burchard test: Take 1 mL of ink extract and add 1 mL chloroform, 2-3 mL acetic anhydride and 1-2 drops of concentrated sulphuric acid. The dark green coloration of the solution indicated the presence of steroid and dark pink or red coloration of the solution indicates the presence of terpenoids.

Test for alkaloids

Wagner's test: The extract was treated with 2 mL of Wagner's reagent. The reddish-brown precipitation indicated the presence of alkaloids.

Test for glycosides

Legal's test: Dissolve the extract in 2 mL of pyridine and sodium nitroprusside solution and made alkaline with sodium hydroxide solution, pink to red color solution indicates the presence of glycosides.

Test for saponins

Foam test: About 1 mL of the extract was diluted with 20 mL of distilled water and shaken transferred to a graduated cylinder and wait for 15 min. A 1 cm layer of foam formation indicates the presence of saponins.

Test for anthraquinones

Borntrager's test: About 2 mL of the extract was heated with 10% ferric chloride solution and 1 mL of concentrated hydrochloric acid. The extract was cooled, filtered and filtrates were shaken with 1 mL of diethyl ether. Which is then extracted with strong ammonia, pink or red coloration of aqueous layer indicated the presence of anthraquinones.

Test for amino acids

Ninhydrin test: Dissolve a 1 mL quantity of extract with 2 mL of water and 1 mL of ninhydrin reagent was added, blue color indicates the presence of amino acid.

Test for fixed oils and fats: Press small quantity of the extract between 2 filter paper, oil stains on the paper indicates the presence of fixed oils.

Functional properties of squid ink

pH: The pH value of ink sample was determined using pH meter. About 2 g of sample was mixed with 10 mL deionized water at 20°C. The pH meter was calibrated first with standard buffer pH 7 and 4.

Water solubility index: Water solubility index was determined according to Amza *et al.*[15]. About 2.5 g of the sample and 30 mL of distilled water were added to a tube and mixed vigorously by vortex. Then, the suspension was incubated in a water bath at 60°C for 20 min. The samples were then centrifuged at 4000 rpm for 10 min. The supernatant was collected in a pre-weighed beaker, whereas, the residue was dried and weighed after the water was evaporated at 105°C for 24 h. The percentage of the residue with respect to the amount of squid ink powder used in this analysis was taken as water solubility. Water solubility index was calculated as the equation below:

$$\text{Water solubility index (\%)} = \frac{\text{Weight of the supernatant (g)}}{\text{Initial weight of sample (g)}} \times 100$$

Antibacterial activity of squid ink: The antibacterial activity was determined by the agar well-diffusion method using *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The overnight grown bacterial culture was transferred to sterile petri plate with Mueller Hinton agar medium (Hi-Media) and was spread with a sterile spreader to create a lawn. Wells of 5 mm were punched into the previously seeded Mueller Hinton agar plates using sterile cork borer. About 200 µL of the ink extract was placed in the wells and allowed to diffuse for 2 h at 4°C and then plates were incubated at 37°C for 24 h. The activity was determined by measuring the diameter of the inhibition zones for each well and expressed in millimeter.

Biochemical analysis of squid ink: The protein and lipid contents of squid ink were determined according to the method of Lowry *et al.*[16] and Folch *et al.*[17]. Moisture and ash content of cuttlefish ink was determined by the standard AOAC method[18]. Carbohydrate content was determined by the method of Hedge and Hofreiter[19] using anthrone and hydrochloric acid.

Antioxidant activity of squid ink extract

Catalase activity: The catalase activity was assayed by the titrimetric method described by Radhakissnan *et al.*[20]. About 1 g of the samples were weighed and homogenized in pre-chilled mortar and pestle by adding 5 mL of chilled phosphate buffer (50 mM, pH 7.0). The homogenate was collected in centrifuge tubes and centrifuged at 15,000 rpm for 20 min at 4°C. The obtained supernatant was sieved through a filter paper and stored in the refrigerator to be used as the extract for the estimation of catalase. The following was done in sequence. Two conical flasks were taken namely control and test. About 5 mL of phosphate buffer (50 mM pH 7.0), 1 mL of hydrogen peroxide and 0.5 mL of water was added to both the flasks. Then 1 mL of 6 N sulphuric acid was added to the control. About 0.25 mL of enzyme extract was added to both the flask. Both the flasks were allowed to stand for 5 min at 0°C. Then 1 mL of 6 N sulphuric acid was added to the test. Finally, the two flasks were titrated against 0.005 M potassium permanganate solution to a pink color end point which lasted for 15 sec. The catalase activity was expressed as µmols min^{-1} mL^{-1}.

Peroxidase activity: The method proposed by Reddy *et al.*[21] was followed for assaying the activity of peroxidase. A 20% homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) from the squid ink that was clarified by centrifugation and the supernatant was used for the assay. To 3.0 mL of pyrogallol solution (0.05 M in 0.1 M phosphate buffer, pH 6.5), 0.1 mL of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5 mL of H_2O_2 was added and mixed. The change in absorbance was recorded every 30 sec up to 3 min in a spectrophotometer (Cary 60 UV-Vis Spectrophotometer). One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

Radical scavenging activity: The nitric oxide radical scavenging capacity of the squid ink was measured by Griess Ilosvay reaction[22]. The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (pH 7.4, 0.01 M, 0.5 mL) and methanolic extract (0.2-1 mg mL^{-1}) and standard tocopherol (0.5 mL) was incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipetted and mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min to complete diazotization. Then, 1 mL of naphthyl ethylenediamine dihydrochloride (0.1%) was added, mixed and allowed to stand for 30 min in diffuse light. The absorbance of the pink colored chromophore was measured at 540 nm against the corresponding blank solutions in a Cary 60 UV-Vis Spectrophotometer. The percentage of nitric oxide scavenging by the different concentrations of the ink extracts and the standard was calculated using the following equation:

$$\text{NO scavenged (\%)} = \frac{A_o - A_1}{A_o} \times 100$$

where, A_o was the absorbance of the control and A_1 was the absorbance of the test samples or standard[23].

Total antioxidant activity: Total antioxidant activities of the ink extract were determined according to the method of Prieto *et al.*[24] Briefly, 0.3 mL sample was mixed with 3.0 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min in a water bath. The absorbance of all the sample mixture was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalence of ascorbic acid.

RESULTS AND DISCUSSION

Marine natural products continue to be a structurally diverse and pharmacologically most interesting source of bioactive metabolites. Some of them hold great potential for the development of new and much needed drugs primarily in the treatment of diabetic, inflammatory, cancer, etc. The traditional knowledge regarding the medicinal value of the marine resources is prevalent among the local communities from the immemorial. In the absence of such traditional knowledge, the categorizing of the marine resources according to their medicinal value is Herculean task as the varieties of marine resources are more. Different phytochemical tests have been done for the detection of bioactive compounds[25]. The present study conducted on the *L. duvauceli* squid ink revealed the presence of bioactive compounds. The qualitative analysis of the squid ink showed the presence of protein, phenols, amino acids, flavonoids, alkaloids, saponins and carbohydrates in the methanolic extraction of ink as a higher amount. The steroids, terpenoids, tannins and glycosides were present in a small amount whereas anthraquinones, fixed oils and fats are absent (Table 1). Phytochemicals which are the major constituents, possess many ecological and physiological roles. Animal synthesizes of natural compounds in the form of primary and secondary metabolites. The primary metabolites such as carbohydrates, fat, amino acid and proteins play a vital role in growth and developmental process and the secondary metabolites that are used commercially as biologically active compounds, are generally high value-low volume products (e.g., steroids, quinines, alkaloids, terpenoids and flavonoids) which are used in drug manufactured by the pharmaceutical industries. The presence of these phytochemicals supports the utilization of the ink where they are used to prepare traditional medications for treatment of various ailments[26,10]. Zhong *et al.*[27] identified the cephalopod ink as a potential source of phytochemicals and it is a traditional Chinese medicine listed in the *Compendium of Materia medica*. From the results, it is observed that the protein, amino acid and carbohydrates are present in the extracts of the squid ink. Proteins contributed to the structure and functions of the living cell, they occur as independent units as well as in combination with lipids, nucleic acids, carbohydrates and many other compounds[28]. Flavonoids are reported to possess antioxidant, free radical scavenger, antileukemic, vasodilator and antibacterial properties and are reported to be useful for improving blood circulation in the brain of Alzheimer patients[29]. Flavonoids are important antioxidants with high redox potentials that allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. They can also chelate metals. When consumed regularly by humans, flavonoids have been associated with a reduction in the incidence of diseases such as cancer and heart disease[30]. In the present study, flavonoids found high in *L. duvauceli* ink represented by +++. Alkaloids are reported to be biologically and therapeutically active (e.g., morphine, atropine and quinine) and have numerous medical applications[31]. Alkaloids are known to exert antioxidant activity through a scavenging or chelating process[32]. Scavenging of reactive oxygen species can counteract lipid oxidation *in vitro* and improve the body's antioxidant enzyme activity and decrease peroxide formation *in vivo*[33]. In the present study, alkaloid was luxuriantly present in the methanolic extract of *L. duvauceli* ink. Saponins have been referred to as nature's biological response modifiers because of strong experimental evidence of their inherent ability to modify the body's reaction to allergies, virus and carcinogens. They show antiallergic, anti-inflammatory, antimicrobial and anticancer activity[34]. Saponins have

Table 1: Qualitative evaluation of photochemical content in ethanol extract of *L. duvauceli* ink

Phytochemicals	Results
Amino acids	+++
Proteins	+++
Carbohydrates	+++
Phenols	+++
Flavonoids	+++
Tannins	+
Steroid	++
Terpenoids	++
Alkaloids	+++
Glycosides	++
Saponins	+++
Anthraquinones	-
Fixed oils and Fats	-

+++: Strongly present, ++: Present, +: Weakly present, -: Absent

properties of precipitating and coagulating red blood cells and they also have cholesterol binding properties, formation of foams in aqueous solutions and hemolytic activity[35] and traditionally saponins have been extensively used as detergents and molluscicides, in addition to their industrial applications as foaming and surface active agents and also have beneficial health effects. The presence of flavonoids and saponins indicates the squid ink extracts potentially have antioxidant activities. Phenolic acids are a major group of primary antioxidants[36]. They are composed of one or more aromatic rings bearing one or more hydroxyl groups and can quench free radicals by forming stabilized phenoxyl radicals[37]. Phenols are structural and allelopathic components which are associated with diverse functions including activation of enzymes, nutrient uptake, protein synthesis and photosynthesis[38]. In the present study, the luxuriant presence of polyphenols was detected in methanolic extracts of *Loligo duvauceli* ink.

The glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal. Cardiac glycosides and catecholamine are agents of choice in the treatment of congestive cardiac failure[39] and in the present study glycosides present in ink extract. Tannins inhibit the pathogenic fungi and antimicrobial activity of extracts showed better activity by the presence of tannins[40]. The terpenoids form a group of compounds, the majority of which occur in the plant kingdom, a few terpenoids have been obtained from other sources. More terpenes have been discovered as an efficacious compound in human disease therapy and prevention. Terpenoid compound has been used to treat cancer, malaria, inflammation and a variety of infectious disease (viral and bacterial)[41]. Steroids isolated from marine animal and plants have high medicinal value[42,43]. In the present study, glycosides, tannins, steroids and terpenoids, are moderately present in methanolic extracts of squid ink whereas, and anthraquinones, fixed oils and fats are totally absent in the ink extract. The phytochemical analysis of the present study agrees with the findings of Nisha and Suja[43] in *L. duvauceli* were collected from Beypore fort. These observations on phytochemical compounds support on the usefulness of ink in the traditional medicament and added a note on the phytochemical history. The presence of phytochemicals of interest suggests that further isolation, purification and characterization and any isolated compounds could be used to design new pharmaceuticals.

The functional property of squid ink was presented in Table 2. However, insufficient awareness of the functional value of squid ink restricts the full utilization of squid ink in the food industry. In Japan, squid ink has been utilized as a traditional component of food products. pH is a common property that measures the acidity or alkalinity of a solution and the pH ranges from 0-14, where pH 7 is considered neutral. Measuring the pH of each food ingredient may be necessary to assess whether any component can support pathogenic growth. On the basis of this result, the pH value for squid ink was 6.52±0.03, which was slightly acidic and preventing microbial growth. This property is a remarkably important and intrinsic factor affecting the survival and growth of microorganisms in squid ink powders. In addition, pH measurement is useful in evaluating the quality of squid ink powders for further processing[44]. The study reveals squid ink is better ink powder due to low pH value. Solubility is one of the most important functional properties of protein used in liquid food and beverages. The WSI measures the sample protein content that dissolves in water. Squid ink powder showed a water solubility index (WSI) of 16.8±0.004%. Zayas[45] stated that a high WSI value displays good dispersibility of protein molecules and results in the formation of finely dispersed colloidal systems. Protein solubility is affected by pH, solvent type, ionic strength, temperature and processing condition. The solubility degree is increased when the electrostatic repulsion between the molecules is higher than the hydrophobic interaction[46]. The present result suggests squid ink is considered byproducts; they can be incorporated in the food industry, especially in a baking industry as a functional ingredient.

Table 2: Functional properties of *L. duvauceli* ink

Functional properties	*L. duvauceli* ink
pH	6.52±0.03
Water solubility index (%)	16.80±0.004

Table 3: Antibacterial activity of *L. duvauceli* ink extract against bacterial pathogens

Test organisms	Methanolic extract of *L. duvauceli* ink (mm)
E. coli	21
K. pneumoniae	23
P. aeruginosa	20

The squid ink contains a rich array of chemical secretions to escape from predators; it contains many constituents of bioactive compounds and antimicrobial properties[26]. There are previous reports on the antibiotic effects of the fluid from the ink sac of cephalopods and the antibacterial activity in the extracts of gill and ink sac of cephalopods. Sheu and Chou[47] reported the ink of cephalopods exhibit antimicrobial activity. As shown in Table 3 the antibacterial activity of methanol extract of *L. duvauceli* ink was evaluated against 3 bacterial strains such as *E. coli*, *K. pneumoneae* and *P. aeroginosa*. The highest inhibition zone was recorded

against *K. pneumoneae* in the methanol extract of *L. duvauceli* ink at the concentration of 200 µL of was 23 mm for *K. pneumonia*, *E. coli* (21 mm) and *P. aeroginosa* (20 mm). Similarly, Mohanraju *et al.*[48] reported maximum zone of inhibition was observed against *K. pneumoniae* (17 mm) and *E. coli* (13 mm) in *Sepia brevimana* extract. Nisha and Suja[49] reported maximum antibacterial activity of water extract of *L. duvauceli* ink against *E. coli* and *Staphylococcus aureus* was 22 and 15 mm, respectively. Nadarajah *et al.*[50] reported *L. vulgaris* ink extract showed remarkable antibacterial activity as zone of inhibition against *E. coli* (28 mm), *K. pneumoniae* (22 mm), *P. aeruginosa* (21 mm) and *S. aureus* (24 mm). Purified extract of the cuttlefish, *S. lessoniana* ink showed antibacterial activity against *S. aureus*[51]. SmilineGirija *et al.*[52] studied that the methanol extracts from the ink of *L. duvaucelii* exhibited good activity against several bacteria and resulted the methanol extract from ink of *L. duvaucelii* showed 18 mm zone of inhibition against the bacteria tested except *E. coli* and *K. pneumoniae*. Patterson *et al.*[53] observed a broad spectrum of antibacterial activity for aqueous ink extract of the *L. duvauceli* and *S. pharaonis* against nine human pathogens. These findings corroborate the results of the present study. In this study, the antibacterial activity of *L. duvauceli* ink active against *E. coli*, *K. pneumoneae* and *P. aeroginosa* and this study recommends the use of squid ink possessed biopharmaceutical product which shows the antibacterial activity.

The measurement of proximate profiles such as protein, carbohydrate, ash, lipid and moisture content is often necessary to ensure that they meet the requirements of food regulations and commercial specifications[54]. As shown in Table 4 the biochemical composition of *L. duvauceli* ink had a moisture content of 79.25%, protein (13.67%), lipid (0.91%), ash (0.25%) and carbohydrate content of 5.59%. The percentage of the results was more or less similar to that of the squid ink was reported by Lopez-González *et al.*[55] and cuttlefish ink reported by Ganesan *et al.*[56]. Thanonkaew *et al.*[57] reported the proximate composition of *Sepia officinalis* ink has 78% of moisture, 5.24% of ash, 18.71% of protein, 0.8% of carbohydrate and 3.33% of lipid content. Nadarajah *et al.*[50] reported that the proximate composition of *L. vulgaris* ink extract was found to be 4.81% of carbohydrate, 14.52% of protein and 0.82% of lipid content. Based on the present and earlier results squid ink possess important nutrients. Food with high protein is generally considered to be nutritionally better and hence, ink from *L. duvauceli* with the highest nutritional content and it is nutritionally preferred.

Table 4: Biochemical composition of *L. duvauceli* ink

Biochemical composition (%)	*L. duvauceli* ink
Moisture	79.25±0.051
Protein	13.67±0.005
Lipid	0.91±0.01
Ash	0.25±1.78
Carbohydrate	5.59±0.29

Table 5: Enzymatic antioxidants in *L. duvauceli* ink extracts

Enzymes	*L. duvauceli* ink
Catalase activity (µmols min^{-1} mL^{-1})	9.600±0.02
Peroxidase activity (change in absorbance min^{-1} at 430 nm)	0.063±0.012

Squid ink has acquired unique space in the biomedical application, particularly high in antioxidants for instance, which as well all know help protect the cells and the heart against damage from free radicals. This means that squid ink might be useful in combating the visible signs of aging, heart disease and various threats to the immune system[25]. In the present study the enzymatic antioxidants such as catalase and peroxidases were studied in squid ink. Catalase is very specific in its reaction, which is the conversion of hydrogen peroxide to water and oxygen. Hydrogen peroxide is a product of a number of oxidation reactions in cells and it can be very damaging to them. One of the functions of catalase is to degrade hydrogen peroxide and thus prevent the accumulation of toxic level of the oxidant. As show in Table 5 the catalase activity of squid ink was 9.6±0.02 µmols min^{-1} mL^{-1} and this can be compared to reports of Chattopadhyaya *et al.*[58], who screened the goat liver and reported catalase activity of about 6.95±0.2 H_2O_2 µmoles min^{-1} mg^{-1} protein collected from Abu Quir bay at Alexandria. Patnaik *et al.*[59] reported fish (*Cirrhinus mrigala*) showed a higher range of catalase activity (20 µmols min^{-1} mL^{-1}), than amphibia (*Bufo melanistictus*), reptile (*Calotes versicolor*), bird (*Gallus domesticus*) and mammal (*Rattus rattus*), as it lives in the most oxygen stress condition. Increase in catalase activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of H_2O_2 produced during cell metabolism and protection against oxidative stress[60-62].

Peroxidase catalyzes the conversion of H_2O_2 to H_2O and O_2, in the presence of the hydrogen donor pyrogallol. The oxidation of pyrogallol to a colored product called purpurogallin can be measured spectrophotometrically at 430 nm with the specified time interval. The intensity of the product is proportional to the activity of the enzyme. In the present study the peroxidase activity was found to be high in *L. duvauceli* squid ink (0.063±0.012). Units of enzyme activities were expressed for peroxidase activity was one

Table 6: Nitric oxide scavenging activity of *L. duvauceli* ink extract

Concentration (mg mL^{-1})	*L. duvauceli* ink extract Inhibition (%)	Standard tocopherol
0.2	20.23±0.003	36.27±0.02
0.4	28.91±0.01	44.15±0.05
0.6	41.25±0.006	56.29±0.29
0.8	50.11±0.45	64.55±0.04
1.0	62.14±0.05	78.43±0.23

Table 7: Total antioxidant activity of *L. duvauceli* ink extract

Parameter	*L. duvauceli* ink extract
Total antioxidant activity (mg ascorbic acid equivalence g^{-1})	0.537±0.041

unit of enzyme activity was defined as 0.001 changes in absorbance/min, under assay conditions. A significant increase in the peroxidase activity in the blue crab (*Callinectes sapidus*) was reported by Burnette and Flick Jr.[63]. The enzyme peroxidase involves in the decomposition of co-substrates such as phenolic compounds and antioxidants. Certain peroxidase isomers utilize phenolic compounds and H_2O_2 to initiate biosynthesis of secondary metabolites required for the growth, development and differentiation[64]. The increased peroxidase activities were mainly due to increased enzyme synthesis and might be useful for adaptation under conditions requiring prevention of peroxidation of membrane lipids[65]. Peroxidase can also kill microorganisms and destroy chemicals that are toxic to animal cells including H_2O_2, phenols and alcohol. For these reasons, it has been proposed that peroxidase protects cells from microorganisms and toxic chemicals.

Recent research on cephalopod ink has shown that they are an important source of antioxidants[11]. Therefore, in this study, the antioxidant activities were also tested using nitric oxide scavenging activity and total antioxidant activity since the published reports on the antioxidant activity of cephalopod ink was limited. The radical scavenging activity in terms of nitric oxide scavenging activity was studied in the squid ink in the methanol extracts. Sodium nitroprusside in aqueous solution at physiological pH spontaneously produces nitric oxide, which reacts with oxygen to produce nitrite ions, which can be determined through the GriessIllosvoy reaction[22]. Scavengers of nitric oxide compete with oxygen and reduce the production of nitric oxide[66]. In the present study, high nitric oxide scavenging radicals were observed in the ink and the activity increased with increasing concentration and the values were much lower than that of standard tocopherol (Table 6). This was higher compared to the results of Nisha and Suja[49] in methanolic extract of squid ink was found to be 22.5% and for partially purified form 34% this indicates that activity is increased on partial purification of the ink. The total antioxidant activity estimated by the formation of green colored phosphate (Mo IV) from phosphomolybdenum (Mo IV) complex at acidic pH. As shown in Table 7 the total antioxidant activity of the methanol extracts of squid ink was higher 0.537±0.041 mg ascorbic acid equivalence/g. The results were in concordance with the results of Ponnusamy *et al.*[67], who evaluated the total antioxidant activity (1.402±0.002 mg ascorbic acid equivalence/g) from the tissue extracts of cephalopods collected from the Kalpakkam coastal area.

CONCLUSION

Squid ink has a diversity of benefits to offer us through industrial and medical applications. The methanolic extract of squid *L. duvauceli* ink proved to be a reservoir of bioactive constituents and may be recommended as a pharmaceutical importance, which could be used in various diseases in the future. They are major potential benefits are identifying antimicrobials to treat products used in food, cosmetics and healthcare and developing drugs for use as antimicrobials and anti-oxidants properties. It offers promise for identifying new, prospective drugs. In conclusion, it is to be stressed that the ink of squid is available abundantly as a waste material while processing squid for its export as food. If more attention is given on the isolation of bioactive compounds it may pave the way for the development of new drugs from cephalopods processing industrial waste.

SIGNIFICANCE STATEMENT

Cephalopoda is under the phylum of Mollusca and most of the cephalopods, excluding nautiloid, have ink sacs and thus produce ink. In this study the cephalopod ink was tested for their bioactive property and the research proved that cephalopod ink contains different nutraceutical properties which are thrown away as by-product by most of the processing industries and consumers. Compared to synthetic products, natural bioactive products have the fewest side effects and the marine environment can be a good source of natural bioactive products. The study clearly showed that this ink is a great source for decreasing various health problems and can be used widely in pharmaceuticals. Compared to synthetic products, natural bioactive products have the fewest side effects and the marine environment can be a good source of natural bioactive products. In the near future, it is hoped that this can cut the wastage of the ink industrially. Appropriate awareness on its variety of medicinal and therapeutic properties will make the ink an attractive object for preparing functional food and alternative medicine.

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