Reaction was inhibited by the addition of 250 µL of trypsin followed by centrifugation. The supernatant was collected, and absorbance was observed at 210 nm. Acetyl salicylic acid was used as a positive control. The experiment was carried out in triplicates and percent inhibition of protease inhibition was calculated.

%Protease inhibition = 100 − ([A1-A2]/A0 × 100)

where A1 is the absorbance of the sample, A2 is the absorbance of the product control, and A0 is the absorbance of the positive control.

Fourier transform infrared spectroscopy

The ethanolic extracts of the squid ink were freeze dried, and Fourier transform infrared (IR) spectra were recorded in Shimadzu IR Affinity-1S.

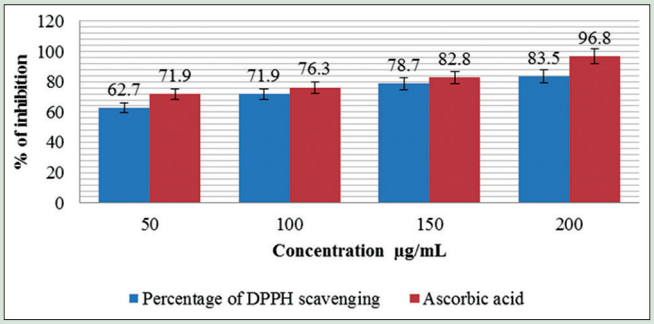

Figure 1: DPPH radical scavenging assay of *Loligo vulgaris*

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 fishes is prevalent among the local communities from the immemorial. In the absence of such traditional knowledge, the categorizing of the fishes according to their medicinal value is Herculean task as the varieties of fishes are more.

Squid ink is very rich in important nutrients. The proximate analysis of squid ink extracts for major biochemical contents were estimated. The carbohydrate content was found to be 4.81%, protein level 14.52%, and lipid level 0.82%. The percentage of carbohydrate and lipid content is very less and protein level is similar according to Nutrition Information of Squid Ink (http://www. fitbit. com/foods/Squid+Ink+Fettuccine+Pasta+Noodles/63658).

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.[23] DPPH-free radical scavenging assay is an easy, rapid, and sensitive method for the antioxidant screening of animal extracts.[24] The DPPH radical scavenging activities are based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals by converting them to the nonradical species.[25] DPPH is a radical having an odd electron and reacts with hydrogen donated from antioxidant. The DPPH radical obtain one more electron and the absorbance decreases.[26] In the present study, the *L. vulgaris* extracts has high DPPH scavenging capacity, which increased with increasing concentration [Figure 1] The DPPH assay was carried out at different concentrations of *L. vulgaris* extracts, such as 50, 100, 150, and 200 µg/mL. DPPH assay did not show any significant difference at 50 and 100 µg/mL concentrations in *L. vulgaris* sample; however, it was significant for 150 and 200 µg/mL for the extracts DPPH is a relatively stable-free radical. DPPH radical react with suitable reducing agents, the electrons become paired off, and the solution losses color stoichiometrically depending on the number of electrons taken up. Hence, this assay provides information on reactivity of test samples with a stable-free radical. The decrease in the absorbance of the DPPH radical caused by test samples was due to the scavenging of radical by electron donation.

IR spectroscopy is among the most powerful spectroscopic techniques for food analysis since it covers the details on the functional group as well as chemical composition that is contained in the IR spectrum of specific substance.[27] IR spectroscopy is a powerful method for studying molecular structure and intramolecular interaction in biological tissues and cells.[28] Several authors have studied IR spectroscopy on biological substances such as muscle and liver. The IR spectroscopy is a promising technique both to define the biochemical basis of cell viability more clearly with quantitative information about chemical functional groups in cells and to identify those characteristics specific to viable cells.[29] Fourier transform IR spectroscopy (FTIR) allows measurement of the entire spectrum simultaneously, providing a means to collect spectral information accurately and rapidly.[30] The present study was carried out to analyze the functional group of ethanolic extract *L. vulgaris* ink using FTIR [Table 1].

Figure 2 indicates that strong absorbance were observed at peak 3379.29 cm^{-1}, 3163.26 cm^{-1}, 3005.10 cm^{-1}, 2627.05 cm^{-1}, 2252.86 cm^{-1}, 2148.70 cm^{-1}, 1442.75 cm^{-1}, 1375.25 cm^{-1}, 1037.70 cm^{-1}, 918.12 cm^{-1}, 748.38 cm^{-1}, which are characteristic of N–H stretch, –C≡C–H: C–H stretch, =C–H stretch, H–C=O: C–H stretch, C≡N stretch, –C≡C– stretch, C–H bend, C–H rock, C–N stretch, O–H bend, C–Cl stretch and the presence of functional groups such as 1° and 2° amines, amides, alkynes (terminal), alkenes, aldehydes, nitriles, alkanes, aliphatic amines, carboxylic acids, and alkyl halides. In overall, spectral profile is similar except for the variation in intensities of the bands. The most widely used modes in protein structural studies are 1° and 2° amines and amides. The broad band at 3297 cm^{-1} has been assigned that in the present study to O–H stretching, the bands of proteins have made a small contribution to it. The bands observed at 2923 cm^{-1} and at 3250 cm^{-1} are due to the asymmetric and symmetric starching modes of the methylene chain in the membrane lipids.[31] The sharp bands observed at 1653 cm^{-1} are assigned to the in plane C=O stretching vibration (amide) and to the aliphatic amines and carboxylic acid.[32]

The squid ink of contains a rich array of chemical secretions to escape from predators; it contains many constituents of bioactive compounds and antimicrobial properties.[33] 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,[34] reported the ink of cephalopods exhibit antimicrobial activity. Purified extract of the cuttlefish, *S. lessoniana* ink showed antibacterial activity against *S. aureus*.[8] Smiline *et al.*[35] studied that the methanol extracts from the ink of *L. duvaucelii* exhibited activity against several bacteria. The methanol extract from ink of *L. duvaucelii* showed 18 mm zone of inhibition against the bacteria tested except *E. coli* and *K. pneumoniae*. These findings corroborate the results of the present study. In the present investigation, crude ink of *L. duvauceli* at 200 µl showed highest activity against *E. coli* (28 mm), *K. pneumoniae* (22 mm), *P. aeruginosa* (21 mm), and *S. aureus* (24 mm) [Table 2]. This results very similar to the Patterson and Murugan.[10]

Hemolysis may be said to exist when the normal rate of red blood cell destruction is increased. Many of the biologically active compounds

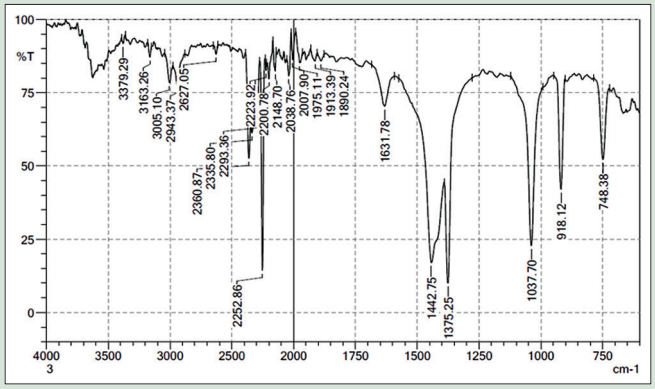

Figure 2: Fourier transform infrared spectroscopy analysis of *Loligo vulgaris* ink

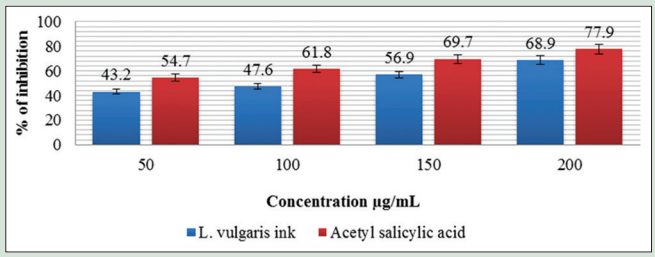

Figure 3: Inhibition of protein denaturation of *Loligo vulgaris* ink

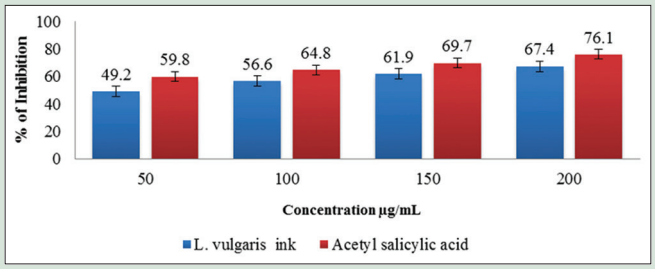

Figure 4: Inhibition Antiproteniase activity of *L. vulgaris ink*

were found be responsible for hemolysis of the cell membrane. It has been proved that squid ink samples showed a better result which acted on the RBC's and destructed them when compared to other test samples. Hemolytic activity was performed earlier[36] and was used as a reference work where the bioactive compounds from marine source have been studied. The breakdown of hemoglobin begins with oxidative ring rupture at the α-methene group of the porphyrin fraction of the molecule. In this study the hemolytic assay has been performed with human blood samples, B + ve group blood showed maximum 128 HU. A similar result was reported in the mucus of two other fish species, *Channa punctatus* and *Cirrhinus mrigala*.[37] Further, Uthayakumar *et al.*[38] observed that the mucus secretion of *Mastacembelus armatus* possesses a potent hemolytic activity against sheep and cow blood cells.

There are certain problems in using animals in experimental pharmacological research, such as ethical issues and the lack of rationale for their use when other suitable methods are available or could be investigated. Hence, in the present study, the protein denaturation and anti-proteinase action bioassay were selected for *in vitro* assessment of anti-inflammatory property of ethanolic extraction of *L. vulgaris* ink. The acetyl salicylic acid was used as a standard drug for inflammation which dose-dependent ability to thermally induced protein denaturation.[21] As a part of the investigation the anti-inflammatory activity of *L. vulgaris* was studied. It was effective in protein denaturation at different concentrations 50, 100, 150, 200 µg/mL, respectively. The *L. vulgaris* were observed as [Figure 3] squid ink of 68.9 µg/mL and acetyl salicylic acid of 77.9 µg/mL, respectively.

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors.[39] In the present study, the antiproteinase ability of ethanolic extraction of *L. vulgaris* was investigated at different concentration. The ethanolic extracts of *L. vulgaris* have shown squid ink of 67.4 µg/mL and acetyl salicylic acid of 67.4 µg/mL [Figure 4].

CONCLUSION

Squid Ink has a diversity of benefits to offer us through industrial and medical applications. They are major potential benefits are identifying antimicrobials to treat products used in food, cosmetics and healthcare and developing drugs for use as antimicrobials, anti-inflammatory and anti-oxidants properties. It offers promise for identifying new, prospective drugs.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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