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Antioxidant activity of methanol extract and partially purified form of *Loligo duvauceli* ink

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Abstract
The ink sac of *Loligo duvauceli*, generated as a byproduct during processing, has low market value and has the potential to cause environmental pollution. Antioxidant and antibacterial activities of crude and partially purified form of *Loligo duvauceli* ink has been largely studied. The in vitro free radical scavenging activity of crude and partially purified form of *Loligo duvauceli* ink was conducted by using DPPH and Nitric oxide assay. The percentage of inhibition was calculated. The IC 50 value was calculated by plotting a graph, the percentage of inhibition against the concentration. From the IC 50 value the partially purified form shows higher free radical scavenging activity compared to the crude form of *Loligo duvauceli* ink.

Keywords: *loligo duvauceli*, free radical scavenging activity, DPPH, nitric oxide

1. Introduction
Our marine world constitute large number of organisms, they are well known for their bioactive components for their defense against predators and for the protection of their young ones. The wide diversity of marine world their biologically active compounds their chemical structure and the way of action and their reproducibility and application have been increased day by day towards their medicinal potential. During recent years the many biopotential compounds have been extracted, purified and characterized from large marine organisms like algae, dinoflagella sponges, soft cereals and cephalopod [1, 3]. Among the marine organisms the cephalopod constitute in large amount and they are well known for their defense mechanisms against predators by easily jetting escape movement by changing their colour which leads to puzzling mysterious release of toxic venom and inking. The main class of cephalopod include the squid and cattle fish. In India the cephalopod resources are utilized mainly for export. The cephalopod are well known for their ink jetting mechanism against their enemies. The main chemical component of their ink is melanin and mucous. The ink in cephalopod which is a chemical secretion and produced and released from their ink sac, which is not similar to ink gland of gastropod but it is modified hypo brachial gland [4], which is defense organ against their predators.

In our body several oxidation process are taking place. These oxidation process are chemical reaction that are involved in the transfer of electrons or hydrogen to an oxidizing agents. As a result of oxidation process in our body free radicals are generated and these can initiate chain reaction, in the cells these chain reaction will leads to cell damage or cell death. Large numbers of free radicals are created as a result of biochemical reactions in our body, these are associated with many diseases like cancer and heart diseases. Antioxidants are the compound which has the ability to slow down or prevent oxidation process under the influence of reactive oxygen species or atmospheric oxygen the free radical chain reaction the antioxidant terminates the chain reaction by removing the free radicals and prevents further oxidation process [5]. Antioxidants are sometimes reducing agents like vitamin, polyphones and thiols. Antioxidants which are required in small quantity have the ability to inhibit or slow down oxidation of easily oxidizable materials [6]. Antioxidants are mainly functional, enzymatic and no enzymatic, antioxidants which are able to react with lipid radicals are termed as primary antioxidant, vitamins phytochemicals and, minerals are included in this category. The phenol compounds which are terminate the chain reactions by capturing free radicals are termed as secondary antioxidants. The primary and secondary enzymes included in the enzymatic antioxidants where as the flavonids, vitamins their derivatives and cofactors are included in the no enzymatic category. In food science antioxidants are termed as any substance which is present in food in low concentration compared to oxidizable substrate which slows down or inhibit the adverse effect of oxygen and nitrogen species in physiological functions in human. Various studies reported that the molecule which are presents in mollusks and cephalopod possess antioxidant activity. In present study the free radical scavenging activity in *Loigo duvauceli* in its form and methanol extract and partially purified by two methods DPPH and Nitric oxide assay were investigated and the total phenol content also studied.

2. Materials and methods
Loligo duvauceli were collected in fresh condition just after their landing by small trawlers from Beypore and brought in to laboratory cleaned and washed with fresh water to remove impurities. The ink glands were dissected and ink was collected by gently squeezing the gland and the ink was air dried.

2.1 Extraction and purification

Crude extraction of the active biomolecules were done using solvents, 25ml of the ink was extracted with 75ml of methanol in sterile glass bottles by parallel extraction method [7] ink was mixed with solvents using sterile glass rod and refrigerated at 4ºc for 72 hours for crude extraction, each preparation was filtered. Crude extracts were collected weighed and were sterilized by exposure under uv light for 2hours. 5mg of extract was mixed in a sterile nutrient broth and was incubated for 2 hours which was placed on to nutrient agar for checking sterility of extracts were stored at 4ºc in brown bottles. The crude ink of *Loligo duvauceli* was partially purified by ammonium sulfate precipitation and dialysis [8]. Ammonium sulfate (20-60%(w/v)saturation) was added to the supernatant fluid at 4ºc which had been obtained after sonication and was adjusted to pH 7.0 with 50mM sodium phosphate buffer. Everything was then left to stand overnight at 4ºc. The precipitate was centrifuged at 15000g for 30 min at4ºc and dissolved in 50mM sodium phosphate buffer (pH7.0) containing 3mM NaN3 and then dialysate Precipitation was induced with 3 volumes of acetones which had been cooled to -10ºc. After 2h the precipitate was centrifuged off at 12000g for 30 min, as above and then driedat4ºc for 24h.Using the crude methanol extract and purified form of the *Loligo duvauceli* ink the total phenol content was calculated.

2.2 Detrmination of total phenol content

The total phenol content (TPC) was determined using Folin-Ciocalteu reagent (FCR) [9] method. Gallic acid was used as a standard. Briefly the solution extract (10ug/ml-70ug/ml) was diluted to 10 ml with distilled water in a volumetric flask. FCR (1ml) was added and mixed well and sodium carbonate (10ml -7%) was added. Make up all the standard up to 25 ml with distilled water. The blank was maintained by taking 10ml distilled water in another volumetric flask. Mixed well and incubated at room temperature for 90 minutes. After incubation read the absorbance value at 750nm in UV spectrophotometer. The total phenol content was determined by comparison with the standard calibration curve of Gallic acid and the results are presented as milligram equivalence of Gallic acid

2.3 Determination of antioxidant activity using DPPH scavenging method

The antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH [10] (2, 2-Diphenyl-1-picrylhydrazyl). The reduction of radicals followed by a decrease in absorbance at 517nm.a stock solution of DPPH (1.3mg/ml in methanol) was prepared such that 75µl of it in 3ml methanol gave an initial absorbance of 0.9. Different concentration of sample extract was prepared and 3ml of DPPH was added. The tubes were covered with parafilm and kept again in dark for 30 minutes. After 30 minutes the absorbance at517nm was measured spectrophotometer UV visible (Systronic double beam UV 2201) and compared to a Gallic acid calibration curve. The percentage of inhibition of DPPH radical was calculated using the following formula

$$I\ \%=\{(\ Ac-A)/Ac\}\times100$$

Where
I= DPPH inhibition (%),
Ac = absorbance of control,
A is the absorbance of test sample
The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC50) was calculated graphically. Tests was also conducted in purified sample and IC50 value also calculated.

2.4 Determination of antioxidant activity by nitric oxide assymethod

The antioxidant activity was measured by nitric oxide assay in terms of nitric oxide generated from sodium nitro preside (SNP) and which is measured by Griess reagent. The sodium nitro pruside in aqueous solution at physiological pH spontaneously generate NO (17, 16), which then interact with oxygen to produce nitrite ion that can be estimated by the use of Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of NO (17, 16). Sodium nitro pruside (SNP) (10mM0 in phosphate buffer saline (PBS) mixed with different concentration of crude methanol extract of *Loligo duvauceli* ink and water and which is incubate at 25ºc for 180 minutes. The samples were then reacted with Griess reagent (1% sulphanilide. 0.1% naphthylethylene diamine dichloride and 3% phosphoric acid). The absorbance of chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthalene diamine dichloride was read at 546 nmand referred to absorbance of ascorbic acid is used as a positive control treated with the same way. The antioxidant activity of purified form of *loligo duvauceli* ink also calculated by the nitric oxide assay method. The result are expressed in microgram of ascorbic acid in dried sample. Each assay was carried out in triplicate, the percentage of inhibition of nitric oxide was calculated by using the following formula

$$I(\%)=[(Ac-A)/A]\times100$$

Where
I is the nitric oxide inhibition,
Ac is the absorbance of control,
A is the absorbance of test sample.
The amount of sample required to decrease the absorbance of nitric oxide by 50 % (IC50) was calculated graphically. IC 50 value of purified form of *Loligo duvauceli* ink was higher as compared to the crude methanol extract of *Loligo duvauceli* ink.

3. Result and Discussion

The results of the determination of total phenol was demonstrated in (table 1). The total phenol content of the crude methanol extract of *Loligo duvauceli* was expressed as 0.008mg equivalence of Gallic acid where as the total phenol content of purified sample was found to be 2.65 mg equivalence of Gallic acid (table 2). The DPPH method is widely used to test the ability of the compound to act as free

radical scavengers or hydrogen donors and to evaluate antioxidant capacity. The parameters IC50 (efficient concentration value) is used for interpretation of the results, in the DPPH method IC 50 value is defined as the concentration of substrate that causes 50% loss of the DPPH activity. IC 50 value of methanol extract was found to be28.65 and IC 50 value for partially purified form is found to be 37 indicate the more activity in partially purified form compared to crude extract, which is demonstrated in (table 3) and (fig 1and 2) In nitric oxide assay method the absorbance of the chromophores formed during the diazotization of nitrite with sulphanilide and subsequent coupling with naphthylethylene diamine dichloride was read at 546nm. The parameter IC50 value was used for the interpretation of result, the IC 50 value of methanol extract was found to be22.5and for partially purified form 34 this indicate that activity is increased on partial purification of the ink and (fig 3 and 4).

Table 1: Determination of total phenol content:

Sample. No	Vol of Sample (ml)	Volume of distilled water (ml)	Volume of Folin's ciocaltteu's reagent (ml)	Volume of 7% Na2Co3 (ml)	O.D. Value (After making up to 25 ml)
B		10.0	1.0	10.0	0.000
S1 (10ug/ml)	1.0	9.0	1.0	10.0	0.0312
S2 (20ug/ml)	1.0	9.0	1.0	10.0	0.0682
S3 (30ug/ml)	1.0	9.0	1.0	10.0	0.1011
S4 (40ug/ml)	1.0	9.0	1.0	10.0	0.1286
S5 (50ug/ml)	1.0	9.0	1.0	10.0	0.1532
S6 (60ug/ml)	1.0	9.0	1.0	10.0	0.1828
S7 (70ug/ml)	1.0	9.0	1.0	10.0	0.2180
Crude	1.0	9.0	1.0	10.0	0.13
Purified	1.0	9.0	1.0	10.0	1.230.

Table 2: Total phenol content:

S I no	Sample	Total phenol content (expressed as % milligram equivalence of Gallic acid)
1	Methanol extract	0.008%
2	Partially purified	2.65

Table 3: Determination of free radical scavenging activity by DPPH Assay:

SI No	Methanol extract concentration(mg/ml)	Absorbance value	% of inhibition	Partially purified concentration (mg/ml)	Absorbance value	% of inhibition
1	control	3.26	0.00	Control	2.20	0.00
2	10	3.02	7.36	10	1.80	18.18
3	20	2.54	22.08	20	1.54	30.00
4	30	1.56	52.14	30	1.32	40.00
5	40	1.23	62.26	40	1.02	53.63

Table 4: IC50 value by DPPH method:

S I no	Sample form	IC50 value (mg/ml)
1	Methanol extract	28.65
2	Partially purified	37

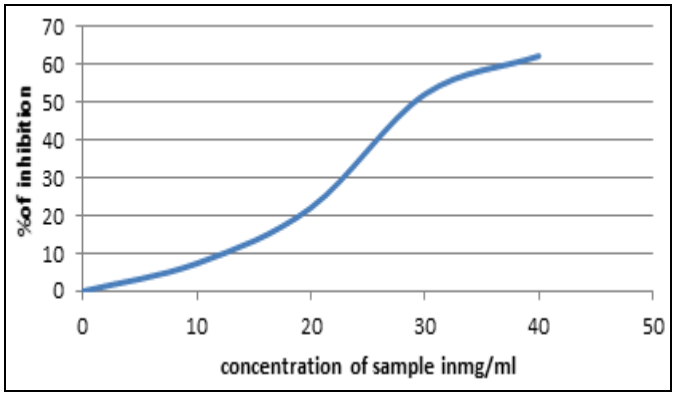

Fig 1: IC 50 value by DPPH method (methanol extract)

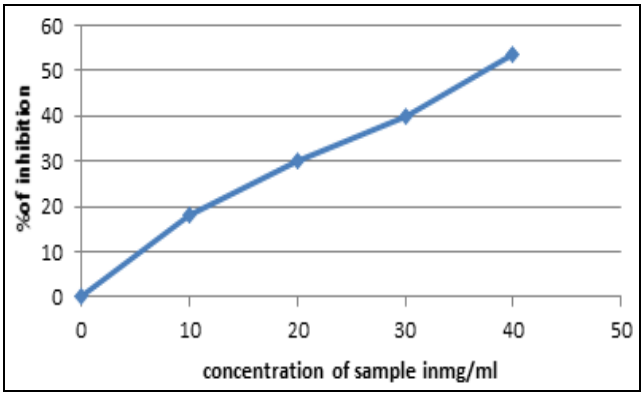

Fig 2: IC50 value by DPPH method (partially purified sample)

Table 5: Determination of free radical scavenging by nitric oxide assay:

SI No	Methanol extract concentration(mg/ml)	Absorbance value	% of inhibition	Partially purified concentration (mg/ml)	Absorbance value	% of inhibition
1	Control	2.80	0.00	Control	3.60	0.00
2	10	2.45	12.5	10	2.84	21.11
3	20	1.52	45.71	20	2.35	34.75
4	30	1.22	56.42	30	2.01	44.17
5	40	1.11	60.35	40	1.50	58.33

Table 6: IC50 value by nitric oxide method:

SI NO	Sample form	IC 50value(mg/ml)
1	Methanol extract	22.55
2	partially purified	34

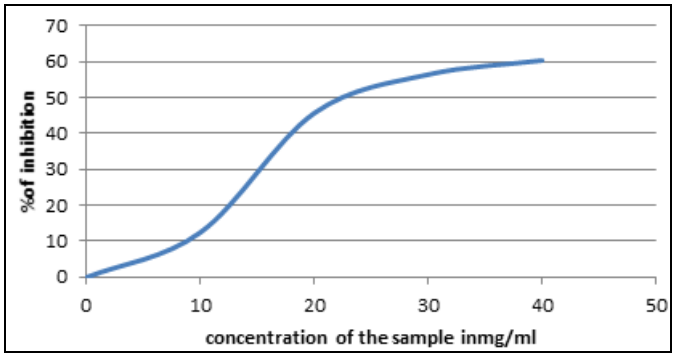

Fig 3: IC50 value by nitric oxide method: (methanol extract)

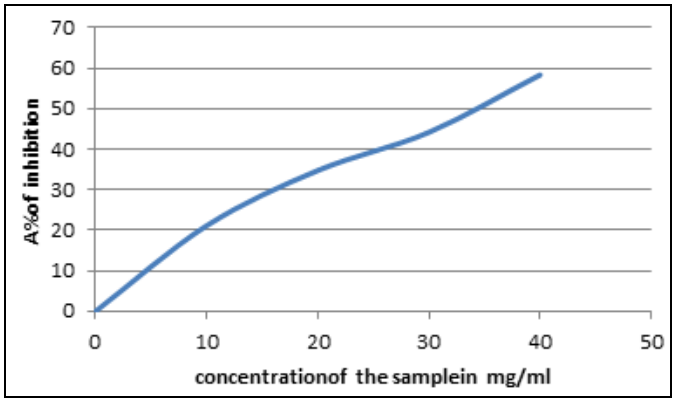

Fig 4: IC 50 value by nitric oxide method: (partially purified)

4. Conclusions

In the present study, the *loligo duvauceli* ink methanol extract and partially purified form were tested with respect to their total phenol content free radical scavenging activity. Extraction done by parallel extractions as methanol as solvent and purification done by ammonium sulfate precipation and dialysis. The existence of phenol content was confirmed by Folin ciocalteu method. The antioxidant activity was measured by the free radical scavenging method DPPH and nitric oxide and was proven too high. This study recommends the use of purified squid ink as a valuable biopharmaceutical product with antioxidant activity. This study concludes by stating that squid inks will definitely considerable attention relation to radicals and oxidative stress cancer prophylaxis therapy and longetivity.

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