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Antioxidant activity of melanin free ink (MFI) extract from the ink sac of *Loligo duvauceli*

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Abstract
The squid processing units produces ink sac as processing waste. Squid processing discard can be utilized to obtain the valuable component. In the present study, ink sac from *Loligo duvauceli* was used to obtain the melanin free ink (MFI). The MFI was characterized for antioxidant properties by analysing Diphenyl-1-picryl hydrazyl free radical scavenging activity (DPPH FRSA), Ferric reducing antioxidant power (FRAP) activity and inhibition of β-carotene bleaching assay. DPPH FRSA activity of 63.21% was observed at 5 mg/ml concentration. The MFI had FRAP and inhibition of β-carotene value of 0.545 and 0.858, respectively. DPPH FRSA, FRAP activity and β-carotene activity increased significantly with increase in MFI concentration. The result indicates that the MFI which can be obtained from the squid ink sac, a waste from squid processing unit can be used as natural antioxidant in many food products to retard the lipid oxidation and shelf-life of the food can also be enhanced.

Keywords: *Loligo duvauceli*, antioxidant activity, squid ink, melanin free ink

Introduction
Cephalopods are soft bodied, bilaterally symmetrical animals with a well-developed head and a body that consists of the muscular mantle cavity that houses the internal organs. Cephalopod includes squid, cuttlefish and octopus. Cephalopod forms an important fishery in India. The total landing of squid, cuttlefish and octopus is reached about 119299, 85655 and 15890 tonnes respectively [1]. During the processing of cephalopods the viscera along with the ink sac are generated as by-products. These processing or by-product waste has low market value and can create serious pollution and disposal problem if it is not managed properly. Ink sac can be used to produce bioactive component and can have application in functional food ingredient.
Cephalopod ink is composed of secretions from ink gland and the funnel organ. Inking of cephalopod is a defence mechanism against predation. The ink contains melanin, carbohydrate, protein and lipid as major constituents. It also contains dopamine, taurine and epinephrine as constituents of ink. An enzyme named tyrosinase present in squid ink is the major reason for the shielding against microbes. The ink has a black colour, which may limit its application. The removal of melanin, a black pigment, prior to the utilization not only increases its appeal but also widens its application [2].
People have used cephalopod ink for many commercial and practical purposes over a period of time, particularly in medicine, cuisine and art applications [2]. The by-product from squid can be used as a very good source of potential bioactive compounds [3]. Cephalopod ink has been used as a food preservative on meat in Japan to increase the shelf life. Cuttlefish ink has wide applications in homeopathic medicine. Cuttlefish ink was believed to exhibit the antiseptic effect on 'Ika-shiokara', which is a cured cuttlefish meat product in Japan [4]. It has been shown that, the product treated with ink had an extended shelf life [5]. Squid and cuttlefish inks can be incorporated in the food industry, especially in a baking industry as a natural food colouring and a functional ingredient [6]. Three different types of MFI extracts such as pure squid ink, squid ink with 5 times dilution and squid ink with 10 times dilutions from *Loligo* sp. showed the antioxidant activity [7]. The water soluble melanin fractions obtained from squid *Ommastrephes bartrami* under alkaline conditions with Mw above 10 kDa exhibited *in vitro* antioxidant activity [8]. MFI from splendid squid (*Loligo formosana*) had DPPH, ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) of 179.6, 957.8 and 171.2μmol TE/g protein, respectively [3].

Lipid oxidation is a deleterious process occurring in food stuffs and leads to rancidity and Reduced shelf-life in foods [9]. Oxidation affects the nutritive value of food, in addition to the reason for the loss of flavours and the formation of toxic by-products. To inhibit these adverse effects of lipid oxidation in food, antioxidants can be used. In industrial practices, synthetic antioxidants have been used as food additives to prevent peroxidation. Synthetic antioxidants may show side effects are a potential health hazard which cannot be ignored. The uses of certain synthetic antioxidant were restricted in several countries because of possible undesirable effects on human health [10, 11]. Therefore, there is a growing interest on natural antioxidants, especially derived marine resources, possibly from the by-products of seafood processing, can be another alternative antioxidant for food application [3].

Melanin free ink (MFI) extract can be used as functional food ingredient in the fish product to enhance the functionality and to increase the shelf life of the product. Cephalopod production in Karnataka reached about 26129 tonnes [1]. Among cephalopods, Indian squid (*Loligo duvauceli*) is the dominant species in Karnataka. During processing ink sac is produced as processing waste. In the present study the ink sac of Indian squid, *Loligo duvauceli* has been used as raw material to obtain melanin free ink. Hence the effective utilization of processing waste can be achieved by obtaining the MFI. The obtained MFI was characterized for the antioxidant properties such as DPPH FRSA, FRAP and β–carotene bleaching assay.

Materials and Methods

Materials

The squid *Loligo duvauceli* was collected from the landing center, Mangaluru, Karnataka, India and they were transported to the laboratory in iced condition (squid:ice ; 1:1). The ink sac was removed aseptically from the squid and used to obtain the melanin free ink (MFI). Linoleic acid, 2, 2 Diphenyl-1-picrylhydrazyl (DPPH) and ferric chloride were purchased from Sigma Aldrich St. Louis, MO, USA. Ethanol, sodium dihydrogen phosphate, di-sodium hydrogen phosphate, potassium ferricyanide and trichloroacetic acid were purchased from M/s Merck Specialities, Pvt. Ltd. (Mumbai, India). All other chemicals and reagents used in the present study were either analytical grade reagent (AR) or guaranteed grade reagent (GR).

Methods

Extraction of melanin free ink (MFI) from squid ink sac

The *Loligo duvauceli* ink sac was separated by cutting the ink duct and the ink was squeezed out from the ink sac. The squid ink was diluted tenfold using cold deionized water (4 °C). Thereafter, it was subjected to centrifugation at 18,000×g for 30 min at 4 °C using a refrigerated centrifuge (Sorvall Legand XTR centrifuge, Thermo Fisher Scientific, New Hampshere, USA). The supernatant obtained was referred as melanin free ink (MFI).

Composition of squid *Loligo duvauceli*

Proximate composition of squid was analysed by estimating the protein, moisture, fat and ash content by following standard method [12].

Diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging activity

The DPPH free radical scavenging activity of Melanin free ink was determined according to the method described by Yen and Wu [13]. The Melanin free ink solution of known concentration was prepared by using double distilled water. A known volume of 1.5 ml was added to 1.5 ml of 0.1 mM DPPH in 99.50% ethanol and mixed thoroughly by vortex using cyclo-mixer at high speed. The solution was incubated at room temperature in dark for 30 min. The absorbance was measured at 517_{nm} using double beam spectrophotometer. Lower the absorbance of the reaction mixture indicated higher free radical scavenging activity. Appropriate control was maintained using double distilled water. DPPH radical scavenging activity was calculated with the following equation.

$$\text{DPPH free radical scavenging activty } (\%) = 1 - \frac{\text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \text{ x } 100$$

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power of Melanin free ink was determined by the method as described by Oyaiza [14]. Different concentrations of Melanin free ink were prepared by using double distilled water. An aliquot of 1 ml sample was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 30 min and the reaction was stopped by addition of 2.5 ml of 10% (w/v) trichloroacetic acid. Finally, 2.5 ml solution from mixture was drawn and mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution. The solution was incubated for 10 min and absorbance was measured at 700_{nm} using double beam spectrophotometer. Higher absorbance of the reaction mixture indicated higher reducing power.

β-carotene bleaching assay

The antioxidant activity of Melanin free ink was evaluated by the β-carotene bleaching assay method suggested by Koleva *et al.* [15] with some modification. β-carotene-linoleic acid emulsion was prepared by mixing 0.5 mg β-carotene in 1 ml chloroform, 25µl of linoleic acid and 200 µl of Tween-40. Chloroform was evaporated under vaccum at 45 ºC and 100 ml of distilled water were added. 100 µl of sample was mixed with 2 ml of β-carotene linoleic acid emulsion. Then, the mixture was incubated at 50 ºC, for 2 h, in a water bath. Absorbance was monitored at 470_{nm}. Higher antioxidant activity is indicated by higher absorbance.

Statistical analysis

Experiments and analyses were conducted in triplicate. The data presented as mean ± standard deviation. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS, version 21.0). The analysis of variance (ANNOVA) was performed to determine the significant difference between concentrations.

Results and Discussion

Composition of squid *Loligo duvauceli*

The proximate composition of squid *Loligo duvauceli* is given in Table 1. In the present study the squid *Loligo duvauceli* showed the protein content of 15.02%. The moisture, fat and ash content of squid were 83.08, 0.74 and 1.13%, respectively. Mehta and Nayak [16] reported the similar result for Indian squid (*Loligo duvauceli*) showed the water, crude protein, lipid and ash contents of 84.6, 14.2, 0.7 and 0.9%, respectively. Sikorski and Kolodzi [17] observed that the gross

chemical composition of the meat of squid mantle and tentacles is similar to that of lean fish. Big squid (*Dosidicus gigas*) living on the Chilean coast, was showed 82.23% moisture, 15.32% protein, 1.31% ashes and 0.87% fat [18]. The characteristic feature of squid proteins with a high protease activity and high water solubility [17].

Table 1: Proximate composition of squid *Loligo duvauceli*

Parameter	Value (%)
Protein	15.02 ±0.455
Moisture	83.08±0.504
Fat	0.74± 0.05
Ash	1.13 ±0.046

Diphenyl-1-picryl hydrazyl free radical scavenging activity (DPPH FRSA)

The Diphenyl-1-picryl hydrazyl free radical scavenging activity (DPPH FRSA) of melanin free ink (MFI) was studied at different concentrations ranging from 1 to 5 mg/ml and the result is presented in Fig.1. The DPPH free radical activity of MFI was found to be concentration dependent. The DPPH FRSA increased significantly (P<0.05) with the increase in MFI concentration. At the concentration of 1 mg/ml, the DPPH FRSA was 10.99%. The maximum activity of 63.21% was recorded at the melanin free ink concentration of 5 mg/ml. DPPH FRSA is often used to evaluate the antiradical activity. DPPH free radical scavenging activity indicates the ability of test substances to donate the electron or proton [19, 20]. DPPH is a radical having an odd electron and reacts with hydrogen donated from antioxidant. The results in the present investigation suggest that the components present in melanin free ink act as electron/proton donors. MFI from squid ink showed the antioxidant activity, the IC 50 value of extract was 0.34 [21]. IC 50 (Inhibitor concentration) value is the effective concentration in sample which could inhibit 50% of DPPH absorbance. The ethanol extract of *Loligo vulgaris* squid ink showed the increase in DPPH activity with increase in concentration. The activity of 62.7% was observed at 50 µg/ml and 83.5% activity at 200 µg/ml concentration [22]. MFI prepared from splendid squid (*Loligo formosana*) showed the DPPH free radical scavenging activity of 179.6 µmol TE/g protein [3]. Squid ink can be used as antioxidant agent as it contains L-dopa and dopamine which have hydroxyl group and capable to be oxygen donor [23, 24]. The results of present study indicated that MFI obtained from *Loligo duvauceli* possessed hydrogen donating capability and could exert antioxidant property.

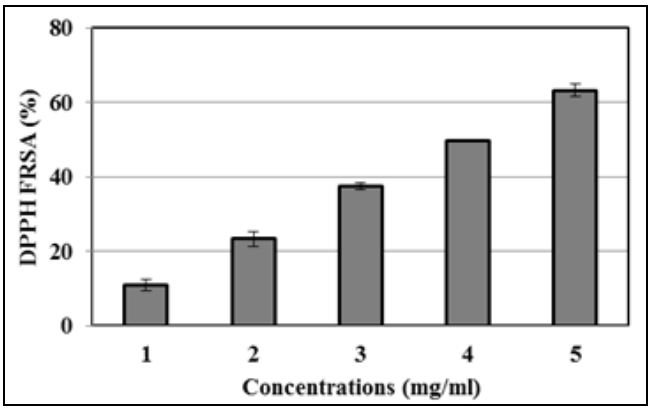

Fig 1: Diphenyl-1-picryl hydrazyl free radical scavenging activity (DPPH FRSA) of Melanin free ink (MFI) extract at different concentration

Ferric reducing antioxidant power (FRAP) activity

The melanin free ink (MFI) concentrations used to analyze the FRAP activity varied between 1 to 5 mg/ml and the result is presented in Fig.2. The FRAP activity of MFI was found to be proportionately related with the MFI concentration. The increase in concentration from 1 to 5 mg/ml increased the FRAP from 0.094 to 0.545 (Optical density at A_{700nm}) and the increase was found to be significant (P<0.05). Results indicate that the components present in MFI were able to reduce the Fe^{3+} (ferric cyanide complex) to the ferrous form. It has been reported that there exists a direct correlation between antioxidant activity and reducing power [25, 26, 27]. Samples with higher reducing power have better abilities to donate electron [28]. FRAP value of distilled water extract of freeze dried and powdered ink of *Loligo duvauceli* was the 929.67 µmol Fe (II) per g of sample, ethanol extract was the 201.00µmol Fe (II) per g of sample and hexane extract was the 79.67µmol Fe (II) per g of sample [29]. MFI from splendid squid (*Loligo formosana*) showed the ferric reducing antioxidant power (FRAP) of 171.2 µmolTE/g protein [3]. The result indicated that MFI was able to act as reducing agent which provided electron for stabilization.

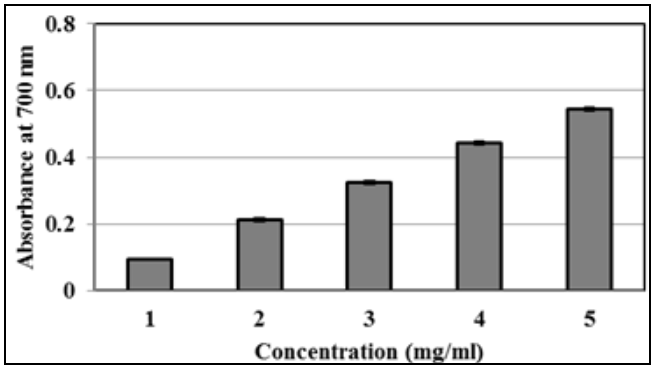

Fig 2: Ferric reducing antioxidant power (FRAP) activity of Melanin free ink extract at different concentration

β- Carotene bleaching assay

The β carotene model system was used to establish the antioxidant activity of melanin free ink (MFI). The β-carotene assay estimates the ability of test substance to function as an antioxidant in a lipid water interface. The assays were carried out as a function of MFI concentration ranging from 1 to 5 mg/ml and results were expressed in terms of absorbance presented in Fig.3. The antioxidant activity of MFI significantly (p<0.05) increased with increase in concentration. At the concentration of 1 mg/ml showed the absorbance value of 0.312 and at the concentration of 5 mg/ml showed absorbance value of 0.858. Result showed that, the presence of melanin free ink could delay the extent of *β*-carotene bleaching by, neutralizing the linoleate free radical formed in the system during oxidation of linoleic acid. MFI from splendid squid (*Loligo formosana*) showed the antioxidant activity in β-carotene linoleate model system. MFI at the level of 500 mg/L showed the highest activity, followed by 200 and 100 mg/L, respectively [3]. In the presence of MFI, β-carotene bleaching was retarded, mainly due to the chain-breaking inhibition of lipid peroxidation by neutralizing linoleic free radical formed. The ability to prevent the bleaching of β-carotene was more likely governed by their amphiphilic properties of amino acid compositions of peptides in MFI. When antioxidative compounds were oriented at linoleic acid/water interface, the antioxidative

effect could be maximized [30]. Thus, MFI could prevent oxidation of lipids in the emulsion system. The present results of β–carotene bleaching assay indicate that the melanin free ink is a good inhibitor of hydro-peroxide formed by the oxidation of linolenic acid oil emulsion system.

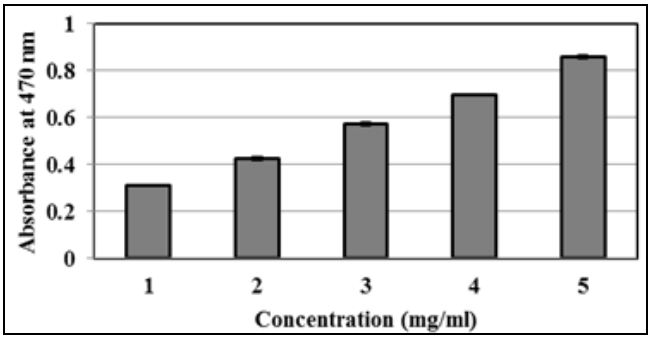

Fig 3: β-carotene bleaching value of Melanin free ink extract at different concentration

Conclusion

Squid processing units produces ink sac as waste. Many studies reported the antioxidant properties of squid ink. The effective utilization of squid ink can be achieved by obtaining the melanin free ink (MFI) from squid ink sac. In the present study *Loligo duvauceli* ink sac was used to obtain the melanin free ink (MFI) and the results showed that the MFI possessed antioxidant properties. Hence the MFI can be used as natural antioxidant to retard the lipid oxidation in food product.

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