International Journal of Applied Environmental Sciences
ISSN 0973-6077 Volume 18, Number 1 (2023), pp. 15-25

https://dx.doi.org/10.37622/IJAES/18.1.2023.15-25

Utilization of Squid Gladius (*Doryteuthis opalescens*) as a Source of Bioactive Compounds: A Waste to Wealth Approach

A.Sree vidhya, M.Asha Berlin, and Dr. R. Leena

Department of Zoology and Research Centre,
Scott Christian College (Autonomous), Affiliated to Manonmanium
Sundaranar University, Tirunelveli, Tamil Nadu, India.

Abstract

Chitosan is a linear polymer obtained from chitin through a deacetylation process. Chitosan has several excellent properties including non-toxicity, adsorption, biocompatibility, and biodegradability. Chitosan was synthesized from squid pen waste through demineralization, deproteinization, and deacetylation. The results of the study show that squid pen is composed of the following: protein 5.26 ± 0.25%, lipid 0.32 ± 0.02%, carbohydrate 0.12 ± 0.007%, moisture 8.9 ± 4.10% and ash 0.45 ± 0.21%. The chitosan showed maximum activity against *Pseudomonas sp* giving 3.31 ± 9.94 mm inhibition zone.

Keywords: *Doryteuthis opalescens*, chitosan, protein, ash, *Pseudomonas*.

Introduction

Natural bioactive substances have the least side effects compared to synthetic products. Most antibiotics are derived from terrestrial organisms, but it is the marine world that is providing the pharmaceutical industry with the next generation of drugs. Biomolecules from seemingly simple marine organisms such as blue-green algae, sponges, and squid provide new ideas to inspire drug development.

Chitosan is a linear polysaccharide that is produced from the deacetylation process of chitin. Chitosan has excellent properties such as non-toxicity, adsorption, biocompatibility and biodegradability. Thus it can potentially be used in many fields such as medicine, wastewater treatment, agriculture, and the food industry. Therefore, chitosan has numerous properties such as antifungal, antimicrobial, antitumor, antioxidant, immune stimulating, anti-inflammatory, and antidiabetic effects. It has

been widely developed for various biomedical applications such as tissue engineering, bone, nerve, skin, wound healing, and burn wound treatment.

The squid pen chitosan is classified as β-from (Elieh-Ali-Komi and Hamblin, 2016). Chitosan can be converted into COS with antioxidant and antimicrobial activities and can be used as a preservative in some foods, especially lipid oxidation or perishable foods. Low molecular weight chitosan (2.8-87.7kDa) has significantly improved solubility and functional activity over high molecular chitosan (604-931 kDa) (Laokuldilok *et al*.2017).

The extraction efficiency of bioactive compounds depends on, among other factors, the extraction solvent (Ngo *et al*., 2017). Most solvents used to extract bioactive compounds from cephalopods are aqueous solution of ethanol, methanol, acetone, and hexane; for example, ethanolic extracts of squid *(Loligo duvauceli*) ink exhibited higher antioxidant activity than hexane extracts (Fatimah and Rabeta, 2017).

The squid pen by-product generated by the squid processing industry is a transparent structure located on the dorsal side of the squid mantle and is approximately the same length as the mantle (Yang *et al*., 2014). The majorsquid species found in Indian coastal waters are *Loligo duvaucceli*, *Sthenoteuthis oualaniensis*, and *Thysanoteuthis rhombus*. Dry squid pen contains mainly protein (52 to 75g/100g) and chitin, a low amount of lipids, lipoproteins, and some minerals (Susana Cortizo *et al*., 2008). Squid pens are an inexpensive by-product of squid processing typically sold at US$0.3/kg (chen *et al*., 2012).

The present study reports the biochemical characterization, antibacterial activity, antioxidant potential, larvicidal potential and protease activity of the chitosan extracted from the pen of squid *(Doryteuthis opalescens*)

Methodology

Collection of squid pen

Squid pen (*Doryteuthis opalescens*) was collected from a fish market in Marthandam, Tamilnadu. The squid pen was washed thoroughly with water and sun dried. The dried material was homogenized in a laboratory mixer, ground and preserved in air tight containers until further processing.

Extraction of chitin and chitosan

The grounded material was taken in a 1000 ml beaker and soaked in boiling 4% NaOH for one hour. Squid pen samples were cooled at room temperature for 30 minutes.

Demineralization

The squid pen was demineralized using a 1% HCl solution. Samples were soaked for 24 hours to remove the minerals. The remaining particles were cleaned with distilled water.

Deacetylation

Chitin obtained in the demineralization step was deacetylated in a 50% NaOH solution and then boiled at 100°C for 2 hours on a hot plate. The sample was cooled at room temperature for 30 minutes. Samples were then washed with 5% NaOH and filtered. The sample was thoroughly washed with water to remove excess water and dried. The samples were then left uncoated and dried in an oven at 110° C. for 6 hours. The final dry material is chitosan.

Biochemical characterization

Estimation of protein

Proteins were evaluated according to the method of Lowry *et al*. (1951). A sample weighing 0.5 g was ground in a mortar with the addition of 10% TCA to precipitate proteins and centrifuged at 5000 rpm for 10 minutes. The supernatant was decanted and the precipitate was analyzed. Solution A was prepared by adding 75 ml of 0.1N. NaOH solution and 2 g of sodium carbonate. Solution B was prepared by adding 0.05 g of copper sulfate and dissolving it in 5 ml of distilled water. Solution C was prepared by adding 1 ml of Solution B to 49 ml of Solution A. Then, 0.6 ml of folin ciocalteu reagent was added rapidly with immediate stirring. The solution was incubated at room temperature for 20 minutes and read at 620 nm against the corresponding blank.

$$\text{Protein content} = \frac{\text{OD of sample}}{\text{OD of standard}} \times 0.1$$

Estimation of lipid

Lipids were evaluated according to the following method (Folch *et al*., 1957). 0.5 g of chitosan was weighed out and homogenized with a mixture of chloroform-methanol (2:1). 0.5ml sodium chloride was added to remove the non-lipid contamination and centrifuged. The upper phase and middle precipitate were removed. The extracted lipids were weighed and calculated.

Estimation of carbohydrate

Carbohydrates were measured colorimetrically (Dubois *et al*., 1956) using the Antron reagent. 0.5 g of chitosan was taken and ground well with 2 ml of TCA. The content was centrifuged at 4000 rpm for 5 minutes. Then, 5 ml of anthrone reagent was added. The solution was kept in a boiling water bath for 10 minutes and cooled. A blank containing 1 ml glucose was prepared. The absorbance was read at 620 nm.

Estimation of moisture

Moisture content was determined gravimetrically (Blacke and Hartge 1965) 0.5 g of chitosan was placed in an oven at 110°C and dried for 3 hours. Weight was calculated using the formula

$$\text{Moisture content (\%)} = \frac{\text{Intial weight} - \text{After drying weight}}{\text{Intial weight}} \times 100$$

Estimation of ash

Ash content was determined by a laboratory muffle furnace method. 0.5g of sample was put into a crucible and put into a muffle furnace and was maintained at 575±10°C for 6 hours. After coolling, the ash present was calculated as

$$\text{Ash (\%)} = \frac{Weight\ of\ Ash\ (g)}{Weight\ of\ sample\ (g)} \times 100$$

Larvicidal Activity

To analyze the effectiveness of chitosan as a potential larvicide. The III and IV instar larvae of culex and *Aedes* mosquitos were exposed to varying concentrations of the prepared chitosan extract. The mortality rate was recorded every 3 hours until 24 hours.

Antibacterial Activity

Antibacterial activity was measured by the well diffusion method. 25 ml of molten Mueller-Hinton agar was poured into a sterile petri dish. Plates were allowed to solidify after 18 hours of growth. 100 µl of pathogenic bacteria were transferred to the plate and cultured using a sterile L-rod spreader. A 5 mm diameter hole was made in the agar after adding the pathogenic microorganisms for 5 min with a sterilized cork borer. Test samples were dissolved in sterile saline and placed in wells. Saline was used as a negative control and azithromycin (30 µg/ml) was used as a positive control. Plates were incubated at 37° C. for 24 hours. Antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using a squid pen in the antibiotic zone.

Protease activity

Protease was determined according to the method of Anson (1938). 100 ml of the enzyme extract was mixed with the substrate prepared in buffer and incubated at 37° C. for 30 minutes. After 30 min incubation, trichloroacetic acid TCA (10%) was added to attenuate the reaction. This mixture was filtered and the released amino was determined as tyrosine according to the method of Folin and Ciocalteau (1929).

Dye decolourization

Solutions were prepared by adding various concentrations of chitosan. Samples were taken on day 0 at 24-hour intervals for 9 days and the OD of the samples was determined. A 3 ml sample was taken from each flask under sterile conditions. The suspension was centrifuged at 6000 rpm for 15 minutes. Absorption spectra of supernatants were recorded in λ max 500 medium using a spectrophotometer.

$$\text{Decolourization (OD)} = \frac{\text{OD of zero day} - \text{OD of sample}}{\text{OD of zero day}} \times 100$$

Antioxidant activity

The free radical scavenging activity of the methanol extract was measured by using DPPH (1,1 diphenyl 2 picryl -hydrazyl). 0.1N solution of DPPH in methanol was

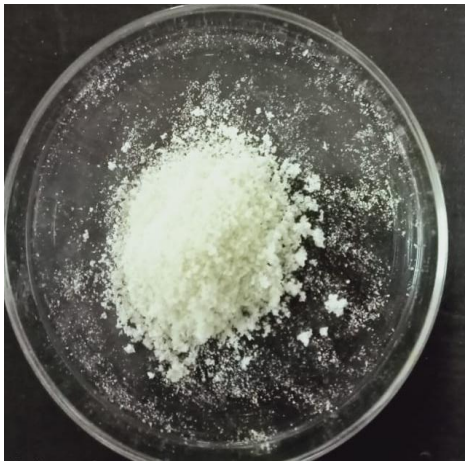
prepared and 1ml of this solution was added to 3 ml methanol extract and reference compound (500 and 1000/ml) at various concentrations. After 30 minutes, the optical density was measured at 517 nm. Ascorbic acid was used as a reference substance. Percentage inhibition was calculated by comparing the uptake of the compound (control) and sample.

$$\text{DPPH Scavenging effect (\%)} = (A_0\text{-}A_1)\ A_0 \times 100$$

Where, A_0 is the absorbance of the control at 30min. A_1 is the absorbance of the sample at 30 min.

Plate 1. *Doryteuthis opalescen*

a) Squid pens



b) Squid pen Chitosan

Plate 2. Extraction of Chitosan

Table 1. Biochemical composition of chitosan

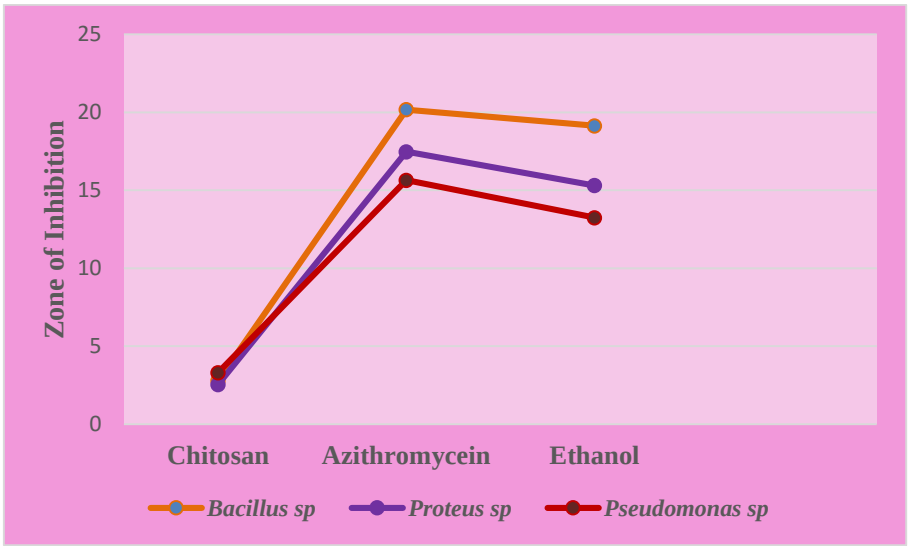
S. No	Assay	± SD
1	Protein	5.26 ± 0.25
2	Lipid	0.32 ± 0.02
3	Carbohydrate	0.12 ± 0.007
4	Moisture	8.9 ± 4.10
5	Ash	0.45 ± o.21

Table 2. Larvicidal activity

S. No	Chitosan dosage(mg/l)	Mortality rate (Hours)				
		3	**6**	**12**	**20**	**24**
1	**0.5**	1	2	6	8	10
2	**1.0**	1	3	5	9	10
3	**1.5**	4	6	8	10	-
4	**2.0**	5	7	10	-	-
5	**2.5**	6	9	10	-	-

Table 3. Antibacterial activity

S. No	Pathogens	Zone of inhibition		
		Chitosan	**Control**	
			Azithromycin	**Ethanol**
1	***Bacillus* sp**	2.79 ± 0.01	20.17 ± 0.148	19.13 ± 0.02
2	***Proteus* sp**	2.55 ± 0.03	17.47 ± 0.015	15.31 ± 0.015
3	***Pseudomonas* sp**	3.31 ± 9.94	15.64 ± 0.009	13.24 ± 0.081



Graph 1. Antibacterial activity

Table 4. Protease activity

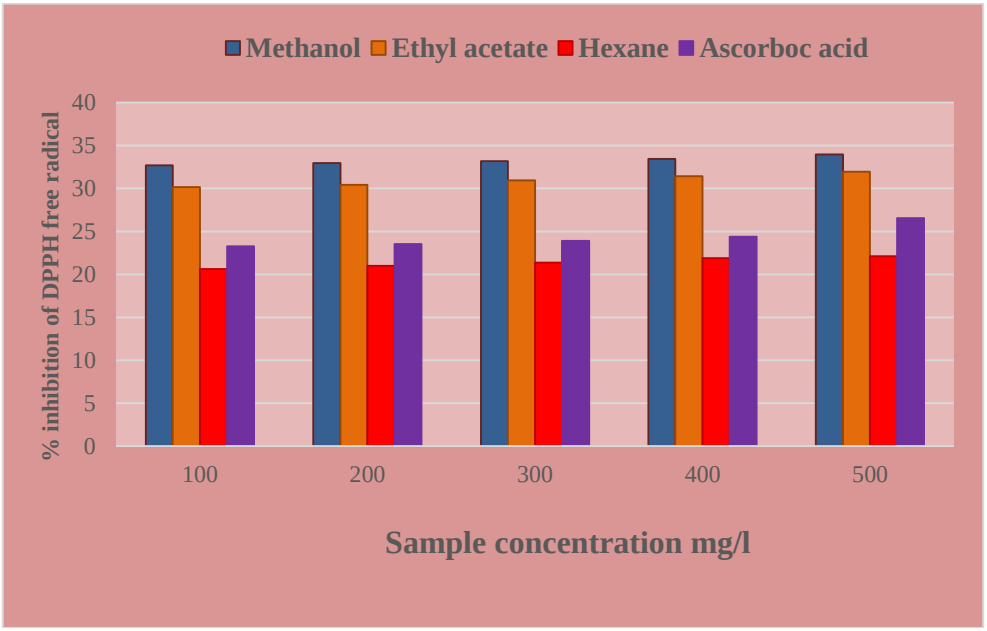
S. No	Sample	Activity of protease
1	**A**	0.643 U
2	**B**	0.639 U
3	**C**	0.652 U

Table 5. Dye adsorption

Days	Percentage of decolourization (%D)			
	0.1	**0.2**	**0.3**	**0.5**
0	0	0	0	0
3	27.12	27.94	27.94	28.76
6	28.21	29.31	29.86	33.97
9	33.69	35.34	36.16	36.98

Table 6. Antioxidant activity

Sample concentration (mg/l)	Percentage inhibition of PPPH free radial			
	Methanol	**Ethyl acetate**	**Hexane**	**Ascorbic acid**
100	32.70	30.18	20.62	23.27
200	32.95	30.44	21.00	23.52
300	33.20	30.44	21.38	23.89
400	33.45	31.44	21.88	24.40
500	33.96	31.94	22.13	24.77



Graph 2. Antioxidant activity

Results

The result of the characterization test of chitosan from squid gladius (*Doryteuthis opalescens*) fish squid pens from table 1 shows that the extracted chitosan has an average protein content of 5.26 ± 0.25% out of the 2 samples analyzed. The lipid content was estimated as 0.32±0.02%. The average carbohydrate content was formed to be 0.12 ± 0.007%. Moisture content was as 8.9 ± 0.03% and the ash content was found to be 0.45 ± 0.21.

Larvicidal activity of the chitosan again *Aedes aegypti* larval instars is given in table 2.100 % mortality was 2.5 mg/l recorded by mg/l chitosan within 9 hours of exposure, whereas 0.5mg/l chitosan should 100% mortality after 20 hours. (Table 2).

The antibacterial activity of the extracted chitosan samples against gram-negative and gram-positive bacteria are shown in table 3 and graph 1. Aqueous extract of chitosan extract was maximum (3.31 ± 9.94) against *pseudomonas* sp., whereas it was for *Bacillus* sp. The ethanolic extract of chitosan gave larger inhibition zones. It was against *Bacillus* sp. And against proteus sp. Azithromycin (positive control) gave the maximum inhibition zones for all three bacterial species tested and the maximum was recorded

The protease activity of the chitosan is given in table 4. The maximum protease activity of 0.652 U was recorded by sample C. 36.95 % of dye decolourization was given by 0.5 g of chitosan after 9 days of treatment, whereas only decolourization was shown by chitosan (Table 5).

The antioxidant activity of extracted chitosan is represented in table 6, which showed a maximum scavenging activity was 33.96 % at 500 mg/l.

Discussion

The waste of fish processing by-products has enormous potential. There is a growing demand to make the most of a large number of by-products of fish processing, not only as a source of untapped biologically active molecules, but also to minimize associated environmental issues (Suresh and Prabhu, 2012). In this study, squid pen was used as a chitosan extraction source. The chitosan was tested for its utilization as an antibacterial, larvicidal, and dye degradation potential. Squid pen protein composition is 5.26 ± 0.25g/100g, carbohydrate is 0.12 ± 0.007g/100g. In a similar study by Remyakumari *et al.* (2018) biochemical profiling of Indian squid (*U. duvauceli*) revealed n importnt composition of essential nutrients such as protein (17.5%) and 0.52% of fat.

Hydroxyradials are the most reactive form of oxygen produced by biological systems and can readily penetrate cell membranes, damaging nearly every molecule found in living cells (Hulcin, 2006). Chitosan extracted in this study showed significant antioxidant activity. An increase in antioxidant activity was noted with increasing concentration. A similar trend was found in Zhao *et al*. (2011).

As for the antibacterial activity, the chitosan showed highest activity against *Pseudomonas* sp. It has an inhibition zone of 3.31 ± 9.94 mm. The zone of inhibition was much smaller compared to the zone obtained for azithromycin (positive control). A similar observation was made by Nirmale *et al.* (2002) while studying the

antibacterial activity of the Indian squid *Loligo duvaceli*. Mochizuki (1979) reported a similar antibacterial effect of squid ink against *S. aureus* (zone of inhibition 10 ± 0.15 mm).

Conclusion

In this study, it is found that the chitosan from squid pen (*Doryteuthis opalescens*) can be a source of bioactive compounds capable of antibacterial activity, antioxidant activity, protease activity, and larvicidal activity. On the basis of the results obtained, chitosan from the squid gladius with presumed antioxidant properties may be used as a source of antioxidants and a possible food supplement or an ingredient in the pharmaceutical industry.

Acknowledgement

The authors are thankful to the Tamil Nadu State Council for Science and Technology (TNSCST) for providing fund for the project under the Student Project Scheme during the academic year 2021-2022.

References

[1] Aleman, A., Perez-Santin, E., Bordenave-Juchereau, S., Arnaudin, A., Gomez-Guillen, M.C., and Montero, P., 2011, "Squid gelatin hydrolysates with antihypertensive, Anticancer and antioxidant activity", Food Research International., 44 (4), 1044-1051.

[2] Anson, M.L., 1938, "The estimation of pepsin, trypsin, papain, cathepsin with hemoglobin", Journal of General Physiology., 22, 79-89.

[3] Aubourg, S.P., Torres-Arreola, W., Trigo, M., and Ezquerra-Brauer, J.M., 2016, "Partial characterization of jumbo squid skin pigment extract and its antioxidant potential in a marine oil system", European Journal of Lipid Science and Technology., 118 (9),1293-1304.

[4] Arias-Moscoso, J.L., Maldonado-Arce, A., Rouzaud-Sandez, O., Marquez-Rios, E., Torres-Arreola, W., and Santacruz-Ortega, H., 2015, "Physico chemical characterization of protein hydrolysates produced by autolysis of protein hydrolysates produced by autolysis of jumbo squid (*Dosidicus gigas*) by products", Food Physics., 10 (2), 145-154.

[5] Doan, C.T., Tran, T.N., Nguyen, V.B., Tran, T.D., Nauyen, A.D., and Wang, S.L., 2020, "Bioprocessing of squid pens waste into chitosanase by *pencibacillus Sp*. TKU047 and its application in low-molecular weight chitosan oligosaccharides production", Polymers.,12, 1163.

[6] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F., 1956, "Colorimetric method for determination of sugars and related substances", Analytical Chemistry., 28 (3), 350-356.

[7] Esparza-Espinoza, D.M., Santacruz-Ortega, H.C., Chan-Higuera, J.E., Cardenas-Lopez, J.L., Burgos-Hernandez, A., Carbonell-Barrachina, A., and Ezquerra-Brauer, J.M., 2021, "Chemical structure and antioxidant activity of cephalopod skin ommochrome pigment extracts", Food Science and Technology., 42, 1-8.

[8] Elieh-Ali-Komi, D., and Hamblin, M.R., 2016, "Chitin and chitosan: Production and application of versatile biomedical nanomaterials", International Journal of Advanced Research., 4, 411-427.

[9] Folch, J., Lees, M., and Sloane Stanley, G.H., 1957, "A simple method for the isolation and purification of total lipids from animal tissues", J Biol chem., 226 (1), 497-509.

[10] Gomathi, P., Nair, J.R., and Sherief, P.M., 2010, "Antibacterial activity in the accessory nidamental gland extracts of the Indian squid, *Loligo duvauceli* orbigny*"*, Indian Journal of Marine Science., 39 (1), 100-104.

[11] Huang, J., Chenu, W., Hu, S., Gong, J., Lai, H.W., Liu, P., mei, L., and Mao, J., 2013, "Biochemical activities of 6-carboxy β-chitin derived from squid pens", Carbohydrate Polymers., 91, 191-197.

[12] Huang, J., Xie, H., Hu, S., Xie, T., Gong, J., Jiang, C., Ge, Q., Wu, Y., Liu, S., Cui, Y., Mao, J., and Mei, M., 2015, "Preparation characterization and biochemical activities of N-(2 carboxyethyl) chitosan from squid pens", Journal of Agricultural and Food Chemistry., 63 (9), 1-8.

[13] Jeyasanta, I., and Patterson, J., 2020, Bioactive properties of ink gland extracts from squid *Loligo duvauceli*", Ecologia., 10 (1), 9-19.

[14] Kumar, V., Muzaddadi, U.A., Mann, S., Balakrishan, R., Bembem, K., and Kalnar, Y., 2018, "Utilization of fish processing waste: A waste to wealth approach", Emerging Post Harvest Engineering and Technological Interventions for Enhancing Farmer Income., 127-131.

[15] Lowry, O.H., Rosebrough, N.J., Far, A.L., and Randall, R.J., 1951, "Protein measurement with the folin phenol reagent", Journal of biological chemistry., 193 (1), 256-275.

[16] Luo, C., Wu, Y., Sun, H., Jian, X., and Chen, X., 2016, "Optimization of enzymatic hydrolysis of collagen-rich waste liquid from squid tentacle peeling for preparing antioxidant peptides using response surface methodology and their antioxidant activities", Food Science China., 37, 176-182.

[17] Liang, T., Lo, B., and Wang, S., 2015, "Chitinolytic bacteria-assisted conversion of squid pen and its effect on dyes and pigments adsorption", Marine Drugs., 13, 4576-4593.

[18] Laokuldilok, T., Potivas, T., Kanha, N., Surawang, S., Seesuriyachan. P., and Wangtueai, S., 2017, "Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzymes treatments", Food Bioscience., 18, 28-33.

[19] Moovendhan, M., Shanmugam, A., and Shanmugam, V., 2019, "Nutritional composition of liver (Digestive gland) from thondi squid (*Sepioteuthis lessoniana*)", Indian Journal of Geo Marine Science, 48 (09), 1398-1403.

[20] Nirmale, V., Nayak, B.B., Kannappan, S., and Basu, S., 2002, "Antibacterial effect of the Indian squid, *Loligo duvauceli* (d' Orbigny) ink", Journal of the Indian Fisheries Association,

[21] Nair, J., Pillai, D., Joseph, S.M., Gomathi, P., Senan, V.P., and Sherief, P.M., 2011, "Cephalopod research and bioactive substance Indian", Journal of Geo-Marine Sciences., 40 (1), 13-27.

[22] Nisha, N., and Suja, S., 2018, "Phyto chemical evalution and antioxidant activity of methanol extract of *Loligo duvauceli* ink", J. Pharmacogon Phytochem., 7, 1764-1767.

[23] Remyakumari, K.R., Ginson, J., Ajeeshkumar, K.K., Vishnu, K.V., Asha, K.K., and Suseela, M., 2018, "Biochemical profile and nutritional quality of Indian squid, *Uroteuthis duvauceli"*, International Journal of Fisheries and Aquatic Studies., 6 (3), 187-192.

[24] Shavandi, A., Hu, Z., The, S., Zhao, J., Carne, A., Beknit, A., and Beknit, A.E., 2017, "Antioxidant and functional properties of protein hydrolysates obtained from squid pen chitosan extraction effluent", Food Chemistry., 227, 194-201

[25] Sheu., Che, T.Y., and Chou, C, C., 1990, "Antimicrobial activity of squid ink", J. Chin. Agric. Chem. Soc., 28, 59-68.

[26] Sukmark, T., Rachtanapun, P., and Rachtanapun, C., 2011, "Antimicrobial activity of oligomer and polymer chitosan from different sources against foodborne pathogenic bacteria", Kasetsart., 45, 636-643.

[27] Singh, A., Benjakul, S., Olatunde, O., and Yesilsu, A., 2020, "The combined effect of squid pen chitooligosaccharide and high voltage cold atmospheric plasma on the quality of Asian sea bass slices inoculated with *Pseudomonas aeruginosa*", Turkish Journal of Fisheries and Aquatic Sciences., 21 (1), 41-50.

[28] Singh, A., Benjakul, S., and Prodpran, T., 2019, "Chitooligosaccharides from squid pen prepared using different enzymes characteristics and the effect on quality of surmi gel during refrigerated storage", Food Production, Processing and Nutrition., 1 (5), 1-10.

[29] Vazquez, J.A., Martin, A., Duran, A.I., Nogueria, M., Fernandez-Compas, A., and Perez-Martin, R., 2019, "Production of valuable compounds and bioactive metabolites from by-products of fish discards using chemical processing, enzymatic hydrolysis, and bacterial fermentation", Marine Drugs., 17 (3), 139.

[30] Yusharani, M.S., Stenley., Harmami., Ulfin, I., Suprapto, S., and Mah, T.L., 2019, "Synthesis of water soluble chitosan from squid pens waste for capsule shell material", Tech Science Press, 7, 7.

[31] Zaharah, F., and Rabeta, M.S., 2018, "Antioxidant and antimicrobial activities of squid ink powder", Food Research., 2 (1), 82-88.