International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Extraction of Melanin from Squid Ink And It's Activity as Food Bio Preservative

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ABSTRACT

Squid ink is known to contain melanin, a natural pigment with potential biopreservative properties. This study aimed to extract melanin from squid ink and evaluate its biopreservative activity. Squid ink was collected and processed to isolate melanin using a series of extraction techniques. The extracted melanin was then tested for its antimicrobial properties against common foodborne pathogens.The extracted melanin exhibited significant biopreservative activity, showing inhibitory effects against various bacteria and fungi. These findings suggest that squid ink melanin could be utilized as a natural preservative in food products. In conclusion, the extraction of melanin from squid ink proved successful, demonstrating promising biopreservative potential. Further research is warranted to explore its application in food preservation on an industrial scale.

Keywords: Squid ink, Extraction of melanin, Antibacterial activity, Natural preservative.

INTRODUCTION

Healthy eating trend and increased health consciousness among the people of this century has generated a need for the development of novel bioactive compounds. In recent years, there is a great interest in finding new and safe bioactive compounds from natural sources for their use in food and medicinal materials to replace the synthetic compounds.

Facing an alarm of antimicrobial resistance globally, the importance of identifying antimicrobial agents from natural sources is considered as the need of the hour. One of the most serious dangers to world health and food security is antibiotic resistance. Though it occurs naturally, the overuse of antibiotics in humans and animals is hastening the process (Allsop, 1998). Due to the advent of new infectious diseases and the re-emergence of already vanished diseases, the necessity to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action is become a never-ending process. The growing demand for novel antibacterial agents, which are capable to control the emerging diseases and resistant strains of bacteria, is motivating the researchers to explore the biologically diverse and dynamic ocean for novel bioactive substances.

The un-explored and untapped marine ecosystems are thought to be a wealthy resource of many organisms, with tremendous potential to generate fascinatingly new bioactive substances (Hong et al., 2009). Marine environment has been focused for the invention, isolation and characterization of novel compounds with versatile medicinal, industrial, or agricultural uses (Ajila et al., 2007). Marine environment comprises complex ecosystems and a lot of creatures which are well-known to possess bioactive compounds as a standard way of selfdefense or to guard the eggs and embryos (Nair et al., 2011). Recently many bioactive

compounds has been isolated, characterized and purified from a variety of marine species, primarily from marine bacteria, cyanobacteria and invertebrates like sponges, tunicates and molluscs (Donia and Hammen, 2003).

Cephalopods, the successful predators, can use a variety of substances to subdue their prey, becoming an interesting font of bioactive compounds. The high species diversity and substantial resources make these animals a reliable source of raw materials for large-scale commercial production.

Cephalopoda is a class coming under the phylum mollusca and it comprises a diverse group of Octopuses, cuttlefish, squids, and nautiluses (Voss, 2013). Cephalopods are solely marine invertebrates and are scattered all around the world, from the intertidal regions to deep ocean (Boyle and Rodhouse, 2008). They are not only having an imperative place in balancing the marine ecosystems, but also they are performing as valuable fishery resources too. Cephalopods can be easily distinguished by their unique morphological and biological characters. Octopuses possess muscular arms which are capable of performing a variety of elusive tasks. The sensitive chemoreceptors present in their suckers can detect even the diminutive changes or differences in their immediate surroundings. With the distinctive dual mode of locomotion squid and cuttlefish can be identified easily. Pulsed jetting drives bursts of speed, and fin flapping allows high-precision manoeuvring.

Cephalopods are abundant and represent one of the ocean's greatest potential food resources. The mantle, limbs, and portion of the intestines, such as the liver, have all been utilised for food. Cephalopods have been prepared using a variety of culinary techniques, including boiling and steaming, frying, grilling, marinating, smoking, drying, and fermenting, in addition to being consumed fresh and raw. Cephalopods are generally good providers of proteins, minerals, omega-3 fatty acids, and micronutrients, and they have a low fat content (Mouritsen and Styrbaek, 2018).

The cephalopod are unique for their defenses, from their fast getting escape travels to changes in coloration that can be disruptive, cryptic, or startling, to toxin venom, to arm autotomy and inking (Hanlon and Messenger, 1996; Norman, 2000). Inking is a self-protective action that helps cephalopods to escape from predators by producing a visual mimic or visual smokescreen, according to observational evidence (pseudomorph). Another suggested character of cephalopod ink is to interfere with predators' chemical sensors; either as a deterrent or as a distracting food mimic (phagomimic). The cephalopods are able to produce and release ink even at their small and young stages (Boletzky, 2017).

In processing industries most probably these ink sacs are discarded as waste along with the viscera of cephalopods. The improper disposal of these can cause serious ecological problems and environmental pollution (Hossain et al., 2018). However, this ink can be a hopeful source for many bioactive compounds with an immense range of chemical structures and functions that can lead to a jump in the manufacturing of nutraceuticals, functional foods and food additives. But the presence of black coloured pigment melanin limits the utilization of ink extract as antioxidants. Hence, the removal of this black pigment before use can widen its application in food industry.

Among the squids,the Indian squid (L. duvauceli Orbigny, 1835) is the dominant species,catching about 97% from Indian waters.(Thomas and Kizhakudan 2006) 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

Diagnostic features:

Broad mantle with wide fins, which are nearly as long as the mantle. Moderately long tentacular clubs with protective membranes (but not reaching the base). A total of 8 suckers, arranged in transverse rows with 5 or 6 middle ones are fairly enlarged. Left-arm IV is hectocotylized: 12 normal quadriserial rows and 10 rows with ventral suckers. Have a vivid transverse tiger-stripe pattern (especially in males) on the dorsal mantle and head; a narrow, light, interrupted line along the bases of fins

Systematic position

Kingdom: Animalia
Phylum: Mollusca
Class: Cephalopoda
Subclass: Coleoidea
Order: Myopsida
Suborder: Decapodiformes
Family: Loliginidae
Genus: Loligo
Species: *Loligo duvacelii*

AIM AND OBJECTIVE

Evaluate the antimicrobial activity of melanin from squid ink against the most common food borne pathogens.

To extract melanin from selected cephalopods landed at Thoothukudi Coast.

Evaluation of Antibacterial assay of melanin from Squid ink by well and disc diffusion

To investigate the functional characteristics of melanin from selected cephalopod species

MATERIALS AND METHOD

SAMPLE COLLECTION

Squid were collected from the Thoothukudi Harbour fish market and were stored at 4°C .The sample were selected based on size and physical appearance and kept in a large container with ice cubes to maintain freshness of squid.Then posteroventrally dissected and ink glands were manually removed from the viscera of squid .The ink were separated from the squid and collected in the sample bottle

COLLECTION OF FOOD BORNE PATHOGENS

The food borne pathogens such as E.coli., S.aureus.,Vibrio sp., Salmonella were collected from KMCH coimbatore and fisheres college,Thoothukudi.

SPECIES IDENTIFICATION

The three collected cephalopod species (Cuttlefish, squid and octopus) were identified up to the species level based on the key morphological characteristics described by FAO Species Catalogue Vol. 3, Cephalopods of The World, An Annotated and Illustrated Catalogue of Species of Interest to Fisheries (Roper et al., 1984).

INK EXTRACTION

Ink sacs were carefully separated and hygienically taken out from cephalopods. Using ethanol, the outside area of the ink sacs was sterilized and was blotted with sterile cotton. With the help of sterile scissors, the ink duct was cut opened and the crude ink was contained in sterile sample containers by gently squeezing the ink sacs

SEPARATION OF MELANIN FROM SQUID INK

The ink were separated from the squid and collected in the sample bottle. About 50 ml of squid ink were mixed with 100 ml of HCl 0.5 M.The solution was stirred using a magnetic stirrer for 30mins ,then stored for 24 hours at 10°C. Then the precipitate is separated from the supernatant by using a cold centrifuge process at 6000 RPM.

ANTIBACTERIAL ASSAY

PREPARATION OF BROTH CULTURE

All the bacterial culture was cultured in differntial media and grown overnight at 37º c for 24 hours, it was used in antibacterial assay.

ANTIBACTERIAL ACTIVITY USING DISC DIFFUSION METHOD

The ability of melanin from the ink of cephalopods to inhibit the growth of bacteria was estimated by the disk diffusion method given by Bauer et al., (1966). The sterile cotton swab was dipped into the inoculums tube and excess fluid was removed by rotating the swab against the side of the tube (above the inoculums level) using a firm pressure. The dried surface of the Muller Hinton Agar plates (5 to 6mm depth) was seeded with individual test organism by streaking the swab three times over the entire surface of the agar plate. In order to ensure the uniform distribution of inoculums, the plate was rotated approximately at an angle of 60 degrees each time. The plate was rimed with cotton swab to pick up the excess fluid and the inoculum was allowed to dry for 3 to 5 min. Then the sterile antimicrobial disc (Hi-media) was impregnated with 50µl of melanin at three different concentrations (5, 10 and 20%) and placed on the surface of the inoculated agar plate with the help of a sterile forceps. 30 µg chloramphenicol disc was used as the positive control. The plates were incubated at 37°C for 24 h and the diameter of zone of growth inhibition was measured in millimeters.

BIO PRESERVATIVE

Potensial Test For Squid Ink Substance Preservatives On Making Meatballs . There are three diferrent treatments: using the appropriate concentration of squid ink with an optimal concentration as an antibacterial test, formalin as a positive control, and untreated as a negative control. After that, the durability time of meatballs preserved by squid ink were observed and compared with the results from the positive and negative control, respectively**.**

Organoleptic test Organoleptic tests were conducted to determine the flavor and appearance with a rating between 1 to 10. Respondents were asked to try test samples. One sample contains the addition of squid ink and another sample does not contain the addition of squid ink. Then the results were written on the provided accreditation forms**.**

RESULT AND DISCUSSION

SAMPLE COLLECTION

PLATE 1: SQUID

SAMPLE EXTRACTION

PLATE 2: SQUID INKBAG

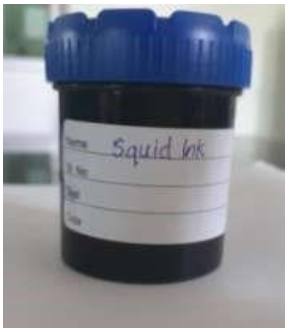


PLATE 3: SQUID INK

PLATE 4: MELANIN OF SQUID

ANTIBACTERIAL ACTIVITY OF MELANIN

Antibacterial activity of melanin was tested at three different concentrations such as 5, 10 and 20% against eight pathogenic bacterial strains and the results observed are given in Table and Plate. Melanin from all

the cephalopods showed antibacterial activity against the tested organisms and it was concentration dependent i.e. the activity was high at higher concentration and low at lower concentration

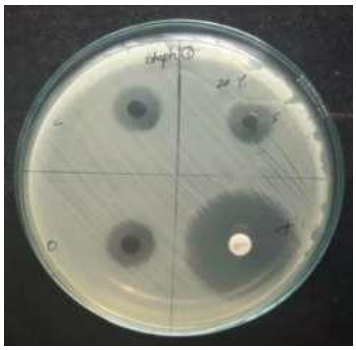


PLATE 5: *S.aureus*

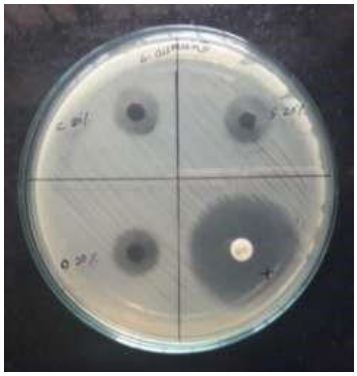


PLATE 6: *Salmonella*

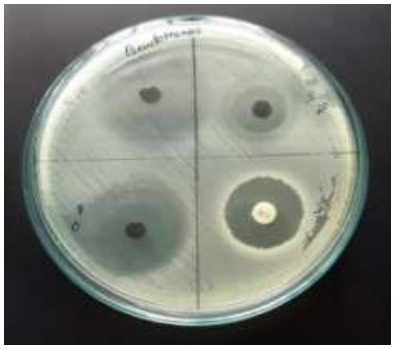


PLATE 7: *E.coli*

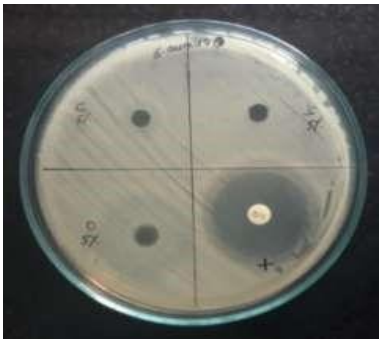


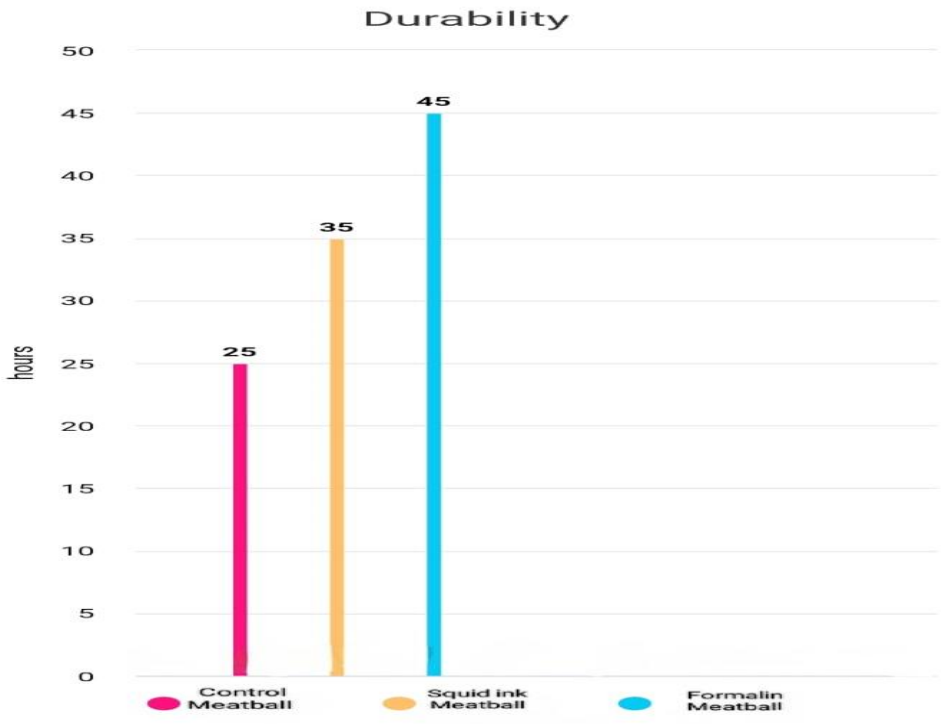
PLATE 8: *vibrio*

Melanin from the ink showed the highest inhibitory activity of 24 mm against *Salmonella* and 21 *Vibrio* a concentration of 20%. At the same time,lower concentrations of melanin from squid ink showed inhibitory activity of 8 mm and 7 mm against *Staphylococcus aureus* and *Salmonella sp.,*respectively. And *E.coli* also the highest inhibitory activity of 20 mm against the concentration of 20%.

BIO PRESERVATIVE

Organisms	**5%**	**10%**	**20%**	**PC**
S.aureus	8mm	11mm	15mm	32mm
Salmonella	7mm	9mm	24mm	32mm
E.coli	9mm	14mm	20mm	34mm
Vibrio	9mm	16mm	21mm	32mm

Squid ink with a concentration was mixed with meatballs to determine its potential as a natural preservative. The potential of squid ink as a preservative was determined based on the length of durability time of the food. The food material were mixed with squid ink can last for 30 hours without additional preservatives while only survive for 20-25 hours. Addition of preservative formaldehyde can survive for 48-72 hours. Based on this, squid ink has good potential as a natural preservative, but its ability can not exceed the ability of formaldehyde . Comparison of the appearance of meatballs with the addition of borax , without the addition of formaldehyde squid ink and the addition of squid ink



CONCLUSION

The present study investigated the antibacterial property in melanin from selected cephalopods landed in Thoothukudi coast. The cephalopods such as *loligo duvacelii* were selected for this study. The ink was then extracted and melanin ink were separated out from the crude ink by centrifugation at 6000 rpm for 15mins. at 4°C.The ability of melanin from cephalopods to inhibit the growth of bacteria was estimated by the disk diffusion method. The test was conducted against common food brone pathogens such as Gram positive strains : *Staphylococcus aureus* and Gram negative strains : *Escherichia coli*, *Salmonella typhimurium* , *Vibrio vulnificus* . Melanin has showed the highest inhibitory activity of 24 mm against *Salmonella typhimurium* In Japan, squid ink is used as traditional foods. Based on previous research, squid ink is believed to exhibit antiseptic effect on meat product . It has been shown that product treated with the ink had an extended shelf life. The potential of squid ink as a preservative has not been able to exceed the ability of formalin so a further research is needed to determine the content contained in squid ink that has potential for a safe use of natural preservatives.

REFERENCE

1. Affandi, R.I., Fadjar, M. and Ekawati, A.W., 2019. Active compounds on Squid (*Loligo sp*.) ink extract powder as immunostimulant candidate to against shrimp disease. *Research Journal of Life Science*, *6*(3), pp.150-161.
2. Agustini, T.W., Wijayanti, I., Amalia, U. and Benjakul, S., 2018. Effect of melanin free ink extracted from squid (*Loligo sp*.) on proximate and sensory characteristics of soft-bone milkfish (Chanos chanos) During Storage. In *IOP Conference Series: Earth and Environmental Science*, 116(1), pp. 012031.
3. Ajila, C.M., Naidu, K.A., Bhat, S.G. and Rao, U.P., 2007. Bioactive compounds and antioxidant potential of mango peel extract. *Food chemistry*, *105*(3), pp.982-988.

4. Alghazeer, R., Saeed, S. and Howell, N.K., 2008. Aldehyde formation in frozen mackerel (*Scomber scombrus*) in the presence and absence of instant green tea. *Food Chemistry*, *108*(3), pp.801-810.
5. Allsop, A.E., 1998. New antibiotic discovery, novel screens, novel targets and impact of microbial genomics. *Current opinion in microbiology*, *1*(5), pp.530-534.
6. Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disc method. *American Journal of clinical pathology*, *45*(4), pp.493-496.
7. Boyle, P. and Rodhouse, P., 2008. *Cephalopods: ecology and fisheries*. John Wiley & Sons.
8. Bush, S.L. and Robison, B.H., 2007. Ink utilization by mesopelagic squid. *Marine Biology*, *152*(3), pp.485-494.
9. Centeno, S.A. and Shamir, J., 2008. Surface enhanced Raman scattering (SERS) and FTIR characterization of the sepia melanin pigment used in works of art. *Journal of Molecular Structure*, *873*(1-3), pp.149-159.
10. Chacko, D. and Patterson, J., 2005. Effect of pharaoh's cuttlefish, Sepia pharaonis ink against bacterial pathogens. *Indian Journal of Microbiology*, *45*(3), pp.223.
11. Derby, C.D., 2014. Cephalopod ink: production, chemistry, functions and applications. *Marine drugs*, *12*(5), pp.2700-2730.
12. Diaz, J.M.J. and Thilaga, R.D., 2014. Screening of antimicrobial activities in the ink of cephalopods against human pathogens. *World Journal of Pharmacy and Pharmaceutical Sciences*, *5*(6), pp.2359-2367.
13. Fitrial, Y. and Khotimah, I.K., 2017. Antibacterial Activity of Melanin from Cuttlefish and Squid Ink. *Jurnal Pengolahan Hasil Perikanan Indonesia*, *20*(2), pp.266-274.
14. Ganesan, P., Brita Nicy, A., Kanaga, V. and Velayutham, P., 2017. Proximate analysis of cuttlefish ink procured from Thoothukudi coast: A comparative study. *International Journal of Fisheries and Aquatic Studies*, *5*(3), pp.253255.
15. Girija, A.S., Suba, K.P., Hariprasad, G. and Raghuraman, R., 2014. A novel study on the antibacterial effect of the crude squid ink extracts from the Indian squid against four bacterial pathogens isolated from carious dentine. *International Journal of Current Microbiology and Applied Sciences*, *3*(4), pp.904-909.
16. Girija, S., Priyadharshini, J.V. and Pandi, S.K., 2011. Isolation and characterization of LOLDUVIN-S: A novel antimicrobial protein from the Ink of Indian Squid *Loligo duvauceli*. *International Journal of Current Research*, *7*, pp.4-14.
17. Hanlon, R.T. and Messenger, J.B., 1996. Cephalopod behaviour. 1st ed. United Kingdom: Cambridge University Press.
18. Hossain, M.P., Rabeta, and Azan, T.H. 2018. Medicinal and therapeutic properties of cephalopod ink: a short review. *Journal of Food Science, 3*, pp.188-198.
19. Ismail, M. and Riad, R., 2018. Research Article Screening the Antimicrobial Activity of Different *Sepia officinalis* (Cephalopoda: Sepioidea) Parts Collected from Alexandria Mediterranean Waters, Egypt Against Some Human Pathogens. *Singapore Journal of Scientific Research*, 8(1), pp.1-7.
20. Jeyasanta, I. and Patterson, J., 2020. Research Article Bioactive Properties of Ink Gland Extract from Squid Loligo duvauceli. *Ecologia*, 10, pp.9-19.
21. Kumar, P., Kannan, M., ArunPrasanna, V., Vaseeharan, B. and Vijayakumar, S., 2018. Proteomics analysis of crude squid ink isolated from *Sepia esculenta* for their antimicrobial, antibiofilm and cytotoxic properties. *Microbial pathogenesis*, *116*, pp.345-350.

22. Marquinet, J.I.A., Lopez Moro Maria Del Pilar, 2001. *Process for producing a food colorant, colorant thus obtained and uses thereof*. U.S. Patent 6,329,010.
23. Nadarajah, S.K., Vijayaraj, R. and Mani, J., 2017. Therapeutic significance of Loligo vulgaris (Lamarck, 1798) ink extract: A biomedical approach. *Pharmacognosy research*, *9*(Suppl 1), p.S105.
24. Nair, J.R., Pillai, D., Joseph, S.M., Gomathi, P., Senan, P.V. and Sherief, P.M., 2011. Cephalopod research and bioactive substances.
25. Ngoile, M.A.K., 1987. *Fishery biology of the squid Loligo forbesi Steenstrup (Cephalopoda: Loliginidae) in Scottish waters*. University of Aberdeen (United Kingdom).
26. Nicomrat, D. and Tharajak, J., 2015. Antimicrobial Effect of Squid Ink on Common Microbial Causing Biofilm Attaching to Silicone. In *Applied Mechanics and Materials* (Vol. 804, pp. 191-194). Trans Tech Publications Ltd.
27. 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*, *29*, pp.65-69.
28. Nithya, M., Ambikapathy, V. and Panneerselvam, A., 2011. Effect of pharaoh's cuttlefish ink against bacterial pathogens. *Asian Journal of Plant Science & Research*, *1*(4), pp.49-55.
29. Ramasamy, P., Subhapradha, N., Srinivasan, A., Shanmugam, V., Krishnamoorthy, J. and Shanmugam, A., 2011. In vitro evaluation of antimicrobial activity of methanolic extract from selected species of Cephalopods on clinical isolates. *African Journal of Microbiology Research*, *5*(23), pp.3884-3889.
30. Sari, R.C., Wijayanti, I. and Agustini, T.W., 2019, March. The effectiveness of melanin from squid ink (Loligo sp.) as antibacterial agent against Escherichia coli and Listeria monocytogenes. In *IOP Conference Series:Earth and Environmental Science*, 246(1), pp. 012022.
31. Thiansilakul, Y., Benjakul, S. and Shahidi, F., 2007. Antioxidative activity of protein hydrolysate from round scad muscle using alcalase and flavourzyme. *Journal of Food Biochemistry*, *31*(2), pp.266-287.
32. Vate, N.K. and Benjakul, S., 2013. Antioxidative activity of melanin-free ink from splendid squid (Loligo formosana). *International Aquatic Research*, *5*(1), pp.1-12.
33. Vate, N.K., Benjakul, S. and Agustini, T.W., 2015. Application of melanin‐free ink as a new antioxidative gel enhancer in sardine surimi gel. *Journal of the Science of Food and Agriculture*, *95*(11), pp.2201-2207.
34. Vennila, R., Kanchana, S., Arumugam, M. and Balasubramanian, T., 2010. Investigation of antimicrobial and plasma coagulation property of some molluscan ink extracts: Gastropods and cephalopods. *African Journal of Biochemistry Research*, *5*(1), pp.14-21.
35. Voss, G.L., 1963. Cephalopods of the Philippine Islands. *Bulletin of the United States National Museum*. 234, pp.1-180
36. Williams, L.W., 1909. *The anatomy of the common squid, Loligo pealii, Lesueur*. Library and printing-office late EJ Brill.
37. Xiao'e, C., 2000. Study on Nutritional Composition of Sepialla maindroni Ink [J]. *Journal of Zhejiang Ocean University*, *4*.
38. Yang, Y.T., Zhu, J.F., Liao, G., Xu, H.J. and Yu, B., 2018. The development of biologically important spirooxindoles as new antimicrobial agents. *Current medicinal chemistry*, *25*(19), pp.2233-2244.
39. Zakaria, F., Fadjar, M. and Yanuhar, U., 2019. Cuttlefish (Sepia sp.) ink extract as antibacterial activity against Aeromonas hydrophila. *International journal of Scientific and Technology research*, 8(11), pp. 1175-77.

40. Zheng, G.L., Zhang, X.Y., Zhou, Y.G., Meng, Q.C. and Gong, W.G., 2002. Comparison between partial components and trace elements in sepia and squid ink. *Chinese Journal of Marine Drugs*, *21*(3), pp.12-14.