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Active Compounds on Squid (*Loligo* sp.) Ink Extract Powder as Immunostimulants Candidate to Against Shrimp Disease

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KEYWORDS

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Ink Extract Powder
Immunostimulant
FTIR
LC-MS

Abstract The business of developing shrimp farming cannot be separated from the existence of disease. In shrimp farming health management, disease prevention strategies can use various methods, one of which is immunostimulant. One alternative immunostimulant source that can be used to improve the body defense system in shrimp is squid (*Loligo* sp.) ink extract powder who has antibacterial ability. This study aims to analyze the presence of active compound in squid ink extract powder can be used as an immunostimulant candidate against shrimp disease. The method used in this research is descriptive explorative and experimental method. This research was conducted with several stages of squid ink extraction until it becomes powder. Identification of squid ink extract powder is using FTIR and LC-MS test. The results showed that the squid ink extract powder contained alkaloid and carboxylic acid from the FTIR test results. Based on LC-MS test results, it was found that squid ink extract powder contained betaine, cinnamic acid, and choline compounds with large amounts of content. Betaine, cinnamic acid, and choline have several biological activities as antibacterial, antioxidant, antiviral, antifungal, etc. so that it can be used as an immunostimulant against shrimp disease.

Introduction

National shrimp production volume in 2018 reaches 1.4 million tons of shrimp which includes white shrimp and tiger shrimp (Ministry of Marine Affairs and Fisheries, 2018). High production is the goal of intensive shrimp farming to meet market needs. One characteristic of intensive aquaculture is high stocking density. Stocking density of cultivated shrimp affects feed requirements, space and oxygen, which it will affect the quality of water maintenance, growth and survival rate of shrimp (Budiardi *et al.,* 2005).

The development of aquaculture systems from traditional to intensive in the majority of white shrimp ponds potentially to increase environmental pollution. Less optimal use of excessive feed will cause accumulation of organic matter. Pollution materials that are difficult to decomposed by microorganisms also cause accumulation and result in damage to the environment which will directly disrupt organism living in the environment. The above factors are the cause of the decline of the organisms body resistance to disease attacks because poor environmental quality, if this is left continuously then mass deaths will occur so that the population will decline (Kilawati & Maimunah, 2015). The business of developing shrimp farming cannot be separated from the existence of disease. Disease is a major obstacle in developing aquaculture businesses because it can cause relatively high mortality (Atmojo *et al.,* 2017).

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In shrimp farming health management, disease prevention strategies can be use various methods such as the use of chemicals and antibiotics, vaccinations, probiotic bacteria, SPF (Specific Pathogen Free) and SPR (Specific Pathogen Resistance), biosecurity production systems, and immunostimulant. The use of antibiotics has a negative impact like the accumulation of residues in fish tissue and the appearance of drug-resistance pathogens. Vaccination, although it is very effective but it requires time, effort, expensive costs, and the resulting specific protection. Probiotics are useful in controlling microbial infections through competition with harmful/pathogenic microorganisms, the production of inhibiting materials or by stimulating the shrimp immune system. SPR shrimp is only resistant to certain pathogens and with the presence of genetic mutations, SPR shrimp that are initially resistant become susceptibility to new pathogens. Shrimp resistance to pathogens also varies based on the life cycle of shrimp. Although biosecurity strategies such as reducing water changes, filtering, drying ponds, post larvae screening to limit the entry of pathogens in the aquaculture environment, and even combined with SPR shrimp significantly increase production, disease continues to occur in aquaculture. Balanced nutritional use is now being studied to improve the response to stress and infection of pathogens such as UFA supplementation, sterols and vitamins in feed. Another approach is the use of immunostimulants in preventing infectious diseases (Manoppo *et al*., 2011).

Immunostimulant sources for aquaculture can be produced chemically or biologically. These immunostimulatory ingredients can be grouped according to their functions and sources and consist of various groups like bacteria and bacterial products, yeast, carbohydrate complexes, nutritional factors, animal extracts, plant extracts, and synthetic drugs (Manoppo *et al.,* 2011). The giving of good immunostimulant must pay attention to the optimal dose and frequency of administration. High doses of immunostimulant can suppress the defense mechanism, while low doses are less effective in increasing the immune response. The frequency and administration of continuous immunostimulant is needed to provide more immune capabilities to achieve optimal protection (Febriani & Nuryati, 2013). One alternative immunostimulant source that can be used to improve the body defense system in shrimp is squid ink extract powder.

The chemical content in squid ink is still being investigated. Some studies say that the main content of squid ink is melanin. Ink content such as eumelanin consists of 5,6-dihydroxyindole (DHI), 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and 2-carboxyl indole (Nasution *et al*., 2017). Squid (*Loligo* sp.) ink extract is known to be used to treat diseases by bacteria. Nair *et al*. (2011) suggested that squid ink has antibacterial ability. According to Fadjar *et al*. (2016), squid ink raw extract can be used as bactericidal against *V. algynolyticus* at a dose of 265.5 mg/L, giving a highly significant effect to hematological profile and survival rate of tiger grouper juvenile. The potential of squid ink extracts as immunostimulant for aquatic organisms that is still little discussed.

Based on the above problems, it is necessary to further analyze the compound content of squid ink extract powder by FTIR and LC-MS tests. This is done to prove whether the compound in squid ink extract powder can be used as an immunostimulant candidate to against shrimp disease. Through the identification of compounds in squid ink extract powder, it is expected to provide information that squid ink extract powder can not only be used for treatment but also can be used as immunostimulant.

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Materials and methods

This research was conducted in July to August 2019 at the Aquaculture Laboratory of Disease and Health Fish Division and Laboratory of Fisheries and Marine Resources Exploration (Faculty of Fisheries and Marine Sciences, University of Brawijaya), Unit Operations Laboratory and Chemical Process Technology (Faculty of Engineering, University of Surabaya), Chemistry Laboratory (Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University of Malang), and Central Laboratory of Life Sciences (LSIH) University of Brawijaya. The equipment used in this study is set of rotary evaporator, shaker, spray dryer and glassware which includes glass beakers, measuring cups, measuring flasks, measuring pipettes, micropipette, watch glass, stirrers, porcelain dishes and mortars, refrigerator, tweezers, container, Erlenmeyer, scissors, Fourier Transform Infra-Red (FTIR), and Liquid Chromatography-Mass Spectrometer (LC-MS). The materials used in this study are squid (*Loligo* sp.) ink extract powder, methanol, aquades, label paper, and tissue.

Squid (Loligo sp.) Ink Extraction

The making of squid ink extract powder starts with taking ink from squid. The squid used came from Sendang Biru Beach, South Malang, East Java in a fresh condition. 1 kg of squid used to contain 40-50 tails. Taking ink begins with cutting the part of the mantle (the lower part of the squid body) vertically or longitudinally. The tool used to take squid ink bag is tweezers. Taking is done carefully to avoid tearing the ink bag. Then, the squid ink bag is placed in the container. The squid ink bag is cut using scissors and squeezed to take the black ink. 1 kg of squid produces 300 ml of ink. The squid ink that has been taken is placed in a sterile bottle and then put into refrigerator to prevent the ink from being damaged.

The extraction process begins with maceration. Maceration is an attempt to soak up squid ink with solvents. The comparison of mixing between squid ink and solvent is 1:3. The solvent used in this study is methanol because methanol is universal solvent. Taking squid ink and solvent is using measuring cup and then poured into Erlenmeyer and homogenized using a shaker for 1.5 hours. The next step is the Erlenmeyer mouth is closed using cotton and aluminum foil and then tied with rope. Furthermore, Erlenmeyer is stored in the refrigerator at 4°C for 7 days. After 7 days, evaporation was carried out using a rotary evaporator at 60 rpm for 4.5 hours (Girija *et al*., 2012). After the evaporation process, 16.65 gr of crude extract in the form of paste is obtained.

The next process is using a spray dryer. Spray drying is a drying process by changing the liquid to be dried into small pieces of fluid using an atomizer and drying it with hot air which is flowed into a drying chamber (Arwizet, 2009). Spray dryer has four stages of the process consisting of atomizing materials by spraying, contact between droplets with drying air, evaporation of water vapor, and finally the dry product from dry air. 16.65 gr of crude extract produces 94.74 gr of squid ink extract powder.

Fourier Transform Infrared (FTIR)

Fourier Transform Infrared (FTIR) analysis was performed on pellet samples made from KBr (99.99%) mixed with squid ink extract powder. Samples of squid ink extract powder for FTIR test of about 4 mg. This amount is mixed with about 1400 mg KBr. To ensure that the resulting pellets allow an accurate spectrum, the mixture is mixed using mortar and pestle. The obtained powder is put into a macro-micro KBr pellet and compressed into a pellet using a hydraulic press (Beckman 00-25 Glenrothes five Scotland). This pellet is ready for FTIR analysis. The FTIR spectrum was collected at 4 cm^{-1} resolutions in transmission

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mode (400-4000 cm^{-1}) using a Thermo Scientific Nicolet Is10 FTIR spectrophotometer (Mbonyiryivuze *et al*., 2015).

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS test is used to determine the content of compounds in a material. The first step of 20 mg of squid ink extract powder was oxidized in 4.0 mL K_2CO_3 1 mol/L with 0.5 mL of 30% H_2O_2 for 20 hours and to be an HPLC product at 25°C and at a flow rate of 7.0 mL/minute. The mobile phase is 0.4 mol/L. The next step is injection of 10 µL from the product at a concentration of 60-80 µM in a mixture of 75:25 methanol grade LC and water, then injected into the Agilent 1200 Series liquid chromatography system (HPLC; Agilent Technologies Inc.) and separated using the C18 Ascents Express column 5 cm x 2, 1 mm x 2.7 µm (Supelco Analytical). The HPLC is connected to the standard ESI interface to Agilent Technologies 6224 MS-TOF to obtain precise high-resolution mass measurements. LC-MS-TOF is operated at a flow rate of 0.3 mL/min using linear gradient of formic acid 0.3%, water 98%, and acetonitrile 2% (A) and formic acid 0.3%, acetonitrile acid 98%, and 2% water (B) as the mobile phase. The gradient program starts with 0% B at 0 minutes and increases to 55% B for the 13 minutes program. MS uses the electrospray ionization (ESI) source in negative mode. The desolvation temperature is set to 300°C using nitrogen as gas desolvation at 11 L/min at a nebulizer pressure of 227.5 kPa (Glass *et al*., 2012).

Results and discussion

Fourier Transform Infrared (FTIR)

Squid (*Loligo* sp.) ink extract powder identified the contents of its active compound using FTIR in transmission mode (400-4000 cm^{-1}). The results obtained can be seen in Figure 1.

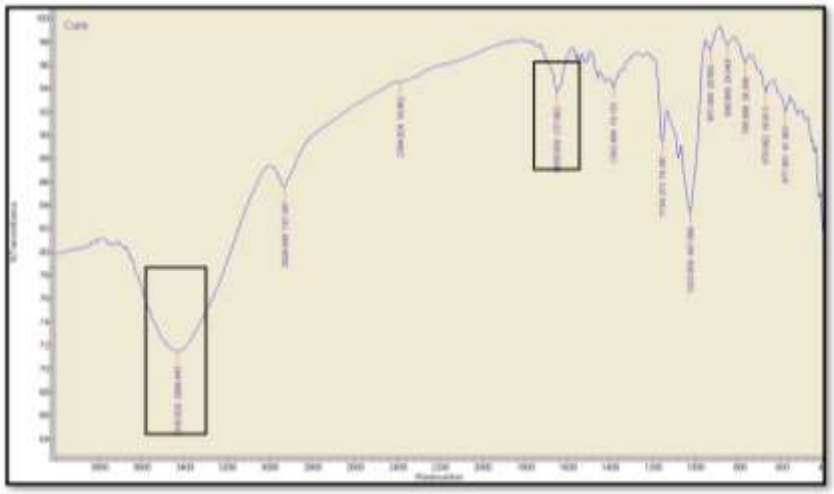

Figure 1. FTIR Test Results Squid (*Loligo* sp.) Ink Extract Powder

Based on the above results, it can be seen that the squid (*Loligo* sp.) ink extract powder contains compounds in absorbance of 3430.825 cm^{-1} at peak 1; 2928.548 cm^{-1} at peak 2; 2384.624 cm^{-1} at peak 3; 1650.099 cm^{-1} at peak 4; 1383.494 cm^{-1} at peak 5; 1154.373 cm^{-1} at peak 6; 1023.954 cm^{-1} at peak 7; 931.084 cm^{-1} at peak 8; 848.988 cm^{-1} at peak 9; 765.086 cm^{-1} at peak 10; 670.062 cm^{-1} at peak 11; 577.051 cm^{-1} at peak 12. The absorbance at:

1. 3500-3370 cm^{-1} is a bond of N-H group (Centeno & Shamir, 2008)
2. 3100-2800 cm^{-1} is a bond of C-H group (Centeno & Shamir, 2008)

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3. 1650-1620 cm^{-1} is a C=C and C=N bond of aromatic group in addition to C=O double bond (COOH) of carboxylic group (Tarangini & Mishra, 2013)
4. 1400-1300 cm^{-1} can be due to OH bending of the phenolic and carboxylic groups (Mbonyiryivuze *et al*., 2015)
5. 1059-1041 cm^{-1} is the indication of CH in-plane/CH out of plane deformation (Centeno & Shamir, 2008)
6. 700 cm-1 ascribed to alkene C-H substitution (Tarangini & Mishra, 2013)

FTIR spectrum of squid (*Loligo* sp.) ink extract powder indicates that squid ink extract powder contains amine group. This is indicated by the absorption that height and width is 3430 cm^{-1} which part of N-H group that belongs to the amine group. Melorose *et al*. (2015) said that one of the compounds that include amine group is an alkaloid. According to Sari *et al*. (2019), alkaloids contain nitrogen as part of the cyclic system and contain varying substituents such as amine, amide, phenol and methoxy groups so that the alkaloids are semi polar and can act as antibacterial compounds. The mechanism of this compound in antibacterial activity is by destroying the cell metabolism so that bacterial growth can be inhibited. Andayani *et al.* (2018) state that bioactive alkaloids increased non-specific immune activity and can be used as immunostimulants mainly concerned with the role of disease prevention. Alkaloids increased the number of phagocytes activity of macrophages. Macrophages play a role in both innate and adaptive immune systems. In innate immunity, macrophages serve as phagocytic cells that eat the pathogens, produce proinflammatory cytokines and ROS production by the NADPH oxidase enzyme as well as RNS by nitric oxide (NO^-) synthase. ROS and RNS are also called as oxidative burst that was formed through oxidation process. ROS formed are superoxide anion (O_2^-), H_2O_2, HOCl, singlet oxygen (O^-) and hydroxyl radical (OH^-). HOCl is a powerful oxidant of H_2O_2 formed by the MPO enzyme.

Beside amine group, squid ink extract powder contains carboxylic group. This is indicated by the absorption that height and width is 1650 cm^{-1} which part of C=O double bond that belongs to the carboxylic group. According to Crocker (2012), one of the derivates from carboxylic acid is oleic acid. Oleic acid content in squid ink raw extract could kill the bacteria directly by stick in the bacterial membranes (e.g., carrageenan and lipopeptides) and proteic (lipoglycopeptides) or lipidic (glycodepsipeptides) cell wall, damaging the cell wall structure, break the cell wall and kill the bacteria (Fadjar et al 2016). Guzman (2014) states that cinnamic acids are a group of aromatic carboxylic acids. Several reviews and studies have appeared in the literature focusing on a particular medicinal application of cinnamic-related molecules, for example on anticancer, antituberculosis, antimalarial, antifungal, antimicrobial, antiatherogenic and antioxidant activities.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Based on the LC-MS test, it can be seen that some secondary metabolites contained in squid ink extract powder. The lists of dominant compounds identified were shown in Table 1.

Table 1. LC-MS Test Results Dominant Compounds of Squid (*Loligo* sp.) Ink Extract Powder

Compound	Formula	Molecular Weight (g/mol)	Retention Time (min)	Area	mzCloud
Betaine	$C_5H_{11}NO_2$	117.0787	0.925	270,127,830.54	98.8
Cinnamic Acid	$C_9H_8O_2$	148.0519	1.508	59,555,693.09	80.4
Choline	$C_5H_{14}NO$	103.0997	1.072	42,791,025.71	95.7

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Based on the above results, it is known that there are three dominant compounds of Squid (*Loligo* sp.) Ink Extract Powder including betaine, cinnamic acid, and choline. The dominant compound content of a material can be determined from the area. This is following the opinion of (Tuli & Ressom, 2009) who stated that relative abundance is determined by area in the LC-MS test. From the table above, it can be seen that betaine has the largest area of 270,127,830.54. The second compound that has the largest area is cinnamic acid with an area of 59,555,693.09. The third compound that has the largest area is choline with an area of 42,791,025.71. Single chromatogram based on molecular ion (m/z) of betaine, cinnamic acid, and choline respectively is shown in Figure 2, Figure 3, and Figure 4.

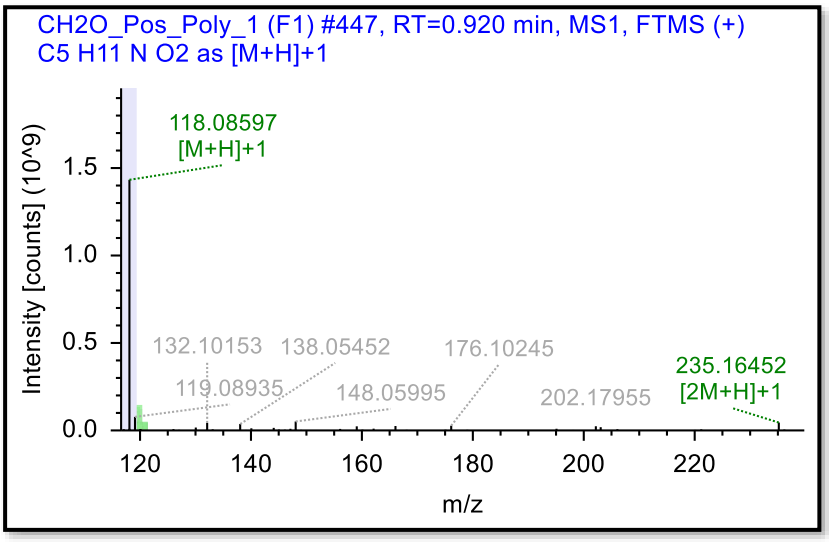

Figure 2. Single Chromatogram Based on Molecular Ion (m/z) of Betaine

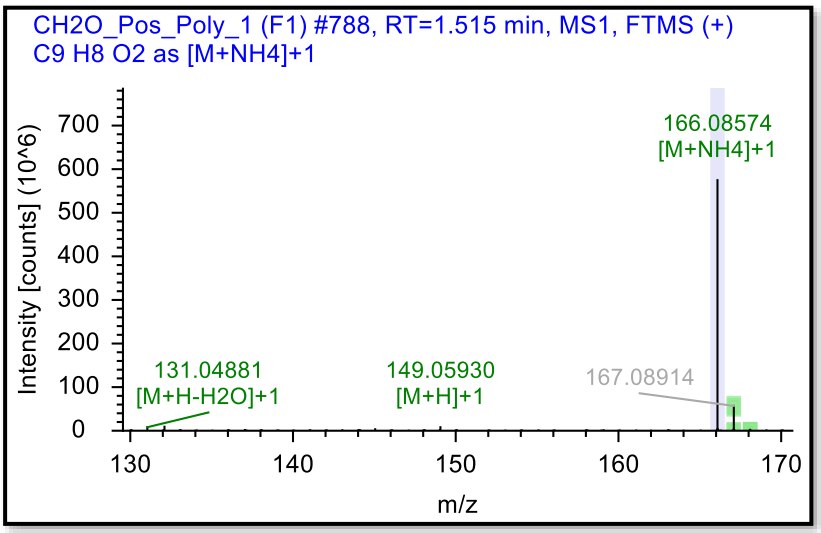

Figure 3. Single Chromatogram Based on Molecular Ion (m/z) of Cinnamic Acid

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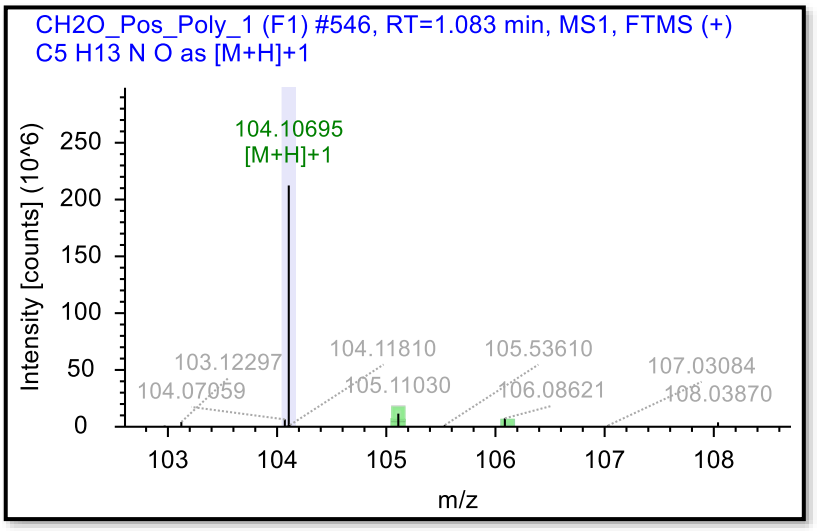

Figure 4. Single Chromatogram Based on Molecular Ion (m/z) of Choline

From Figure 2, it is known that betaine has molecular ion 118 m/z so it has a molecular weight 117 g/mol. Figure 3 showed that cinnamic acid has molecular ion 149 m/z so it has a molecular weight 148 g/mol. Figure 4 showed that choline has molecular ion 104 m/z so it has a molecular weight 103 g/mol. The value of molecular ion and molecular weight that are not much different or even the same. Committee (2015) who stated the for ion with either a +1 or -1 formal charge, as is found with most "small molecules", the weight of the molecule is the same or not much different as the m/z.

Betaine is one of alkaloid compounds (Preedy, 2015); (Mahibalan *et al.,* 2016). Betaine has antioxidant, antibacterial, and antifungal activity. Betaine acts by preventing bacterial reproduction and killing the infection causing bacteria (Mahibalan *et al.,* 2016). In line with that opinion, He *et al.* (2012) states that betaine plays an important role in protein and energy metabolism, and can significantly increase the colonial outgrowth of bacteria. Betaine could stimulate the total number of bacteria and growth-stimulate some bacteria species in tilapia intestine. The gut microbiota is known to be closely related to host health, nutrition absorption, pathogen resistance and enhancement of immune responses. From the research result of Adjoumani *et al*. (2017), it also notes that supplementation of betaine at 1.2% can improve growth performance and antioxidant capacity, as well as reduce fatty acid synthesis and enhance mitochondrial β-oxidation and lipid transportation in high-fat diet-fed blunt snout bream (*Megalobrama amblycephala*). Thus, effectively alleviating fat accumulation in the liver by changing lipid metabolism. Ali & Al-faragi (2017) also argue that betaine has nutritional function commonly used as feed additive in animal, poultry and aquatic nutrition. Betaine improve growth performance, health status, feed digestibility, palatability, flesh quality, and immune status of fish species. Dietary betaine supplementation improves growth performance and survival rate in common carp (*Cyprinus carpio* L.). Kumar *et al.* (2014) state that betaine was found to has immunostimulatory role in fish.

Cinnamic acid belongs to the group of carboxylic acid compounds. The research of Yılmaz & Ergün (2018) has result that dietary cinnamic acid improved the immunity by increasing granulocyte (%), phagocytic activity, phagocytic index, respiratory burst activity, potential killing activity in the blood of fish, and lysozyme, myeloperoxidase, total Ig and CH50 in

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the serum of rainbow trout (*Oncorhynchus mykiss*). High content of natural antioxidants in herbal medicine as cinnamic acid can obstruct reactive oxygen species (ROS) generation and scavenge free radicals from tissues (Hassaan *et al*., 2019). In line with that opinion, Enis Yonar *et al.* (2011) reported that cinnamic acid content in propolis has antioxidant activity. While cinnamic acid content in cinnamon extract has antiparasitic activity against *Dactylogyrus intermedius* (Monogenea) in goldfish (*Carassius auratus*) (Ji *et al*., 2012). Sova (2012) states that cinnamic acid has antioxidants and antibacterial activity by showed inhibitory activity against several Gram-positive and Gram-negative bacteria. Cinnamic acid also has antiviral activity against viruses belonging to different taxonomic groups, antifungal properties have also been observed. Syahidah *et al.* (2015) reported that several aquatic pathogens such as *Mycobacterium* sp., *Staphylococcus* sp., *Enterococcus* sp., *Pseudomonas* sp. and *Micrococcus* sp. could be effectively inhibited by cinnamon's extract which contain cinnamic acid, while essential oils from this herb also possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties.

Choline belongs to the group of alkaloid compounds. Khosravi *et al*. (2015) reported that choline concentration of 230 mg/kg diet largely meets parrot fish (*Oplegnathus fasciatus*) requirement for growth and normal lipid metabolism. The fish seems to synthesize enough choline for its normal growth and metabolism. Choline is an essential component of cell structure and plays important role in cell maintenance and certain metabolic functions. Therefore, deficiency of these cofactors can negatively affect the immune function and disease resistance. Baldissera *et al*. (2019) states that dietary vegetable choline (VC) supplementation can increase the growth performance of fingerlings Nile tilapia. At 60 days of experiment, the VC dose near 800 mg/kg of feed should be recommended for improving the fish performance. Moreover, 800 and 1200 mg VC/kg of feed were able to improve the hepatic energy metabolism and antioxidant/oxidant status which can be pathways involved in the increase of growth performance of Nile tilapia. The research of Wu *et al*. (2013) has resulted that dietary choline could enhance the disease resistance of juvenile Jian carp by promoting the immune responses and improving the balance of intestinal microflora. Dietary choline enhanced the immunity of juvenile Jian carp through improvement of non-specific and specific immune response and regulation of inflammation. This result is in agreement with Wu *et al*. (2014) who demonstrated that diet supplemented with 310, 607, 896, 1167, and 1820 mg vegetable choline (VC)/kg of feed decreased lipid peroxidation and protein carbonylation in spleen and head kidney of Jian carp, contributing to the improvement of immune and anti-inflammatory responses. Zhao *et al.* (2016) reported that the fish gills are not only responsible for gas and electrolyte exchange and excretion, but also been proposed as an immune organ. Dietary choline supplementation significantly improved the healthy statues of fish gill. Optimal dietary choline supplementation improved the antibacterial properties in the gills of fish. Optimal dietary choline supplementation decreased inflammation through decreasing pro-inflammatory cytokines and increasing anti-inflammatory cytokines. Optimal dietary choline supplementation improved the barrier function of fish gill. Optimal dietary choline improved the integrity of fish gills by decreasing the oxidative damage, which might be partially related to increases of glutathione content and Cu/Zn-SOD, CAT, GPx, GST, and GR activities.

From the results above, we know that squid ink extract powder has several biological activities. For many years the content of squid

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ink continues to be studied. The present study showed that squid ink extract powder contained alkaloid and carboxylic acid from FTIR test results. Further testing using LC-MS known that squid ink extract powder contains many active compounds. The dominant compounds of squid ink extract powder are betaine (alkaloid), cinnamic acid (carboxylic acid), and choline (alkaloid). Betaine has the largest area of 270,127,830.54. Cinnamic acid with an area of 59,555,693.09 and choline with an area of 42,791,025.71. Betaine, cinnamic acid, and choline content in squid ink extract powder have several biological activities as antibacterial, antioxidant, antiviral, antifungal, etc. From that information it can be said that squid ink extract powder can be used as immunostimulant in aquaculture, particularly shrimp disease.

Conclusions and Suggestion

The results of this study concluded that from the analysis of the compound content of squid ink extract powder by FTIR and LC-MS tests it was found that squid ink extract powder contained several compounds. The squid ink extract powder contained alkaloid and carboxylic acid from the FTIR test results. Based on LC-MS test results, it was found that squid ink extract powder contained betaine, cinnamic acid, and choline compounds with large amounts of content. Betaine and choline are alkaloid compounds, while cinnamic acid is a carboxylic acid. Betaine, cinnamic acid, and choline have several biological activities as antibacterial, antioxidant, antiviral, antifungal, etc. Therefore, the squid ink extract powder can be used as immunostimulants against shrimp disease. Based on the research that has been done, it can be suggested that further research needs to be conducted on the application of squid ink extract powder to shrimp disease at the field scale.

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