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The Antibacterial Activity of Melanin in the Cuttlefish (*Sepia* sp.) Ink against *Aeromonas* sp.

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ABSTRACT

The marine environment consists of many organisms recognized to have bioactive compounds as a mechanism of self-defense or protection of eggs and embryos. One of them is cuttlefish (*Sepia* sp.). This study aimed to determine the potential and bactericidal action of the cuttlefish ink melanin against *Aeromonas* sp. Ink extraction and purification were carried out to obtain melanin mechanically using 0.5M HCl. The growth patterns of bacteria were studied by the Total Plate Count method, and the bactericidal mechanism of melanin was observed by Transmission Electron Microscopy (TEM). The results showed that cuttlefish ink melanin inhibited *Aeromonas* sp. as indicated by the shrinkage of cell size and irregular cell shape. The results of this study are important information for dealing with *Aeromonas* sp. attack in cultured fish.

INTRODUCTION

Cuttlefish is one of the fisheries commodities which utilization is still limited. So far, the use of cuttlefish has only been cooked with a mixture of spices, while the ink is thrown away or used as part of the cuttlefish processing in food. Several studies have shown that squid ink contains melanin, protein, fat and glycosaminoglycans **(Fiore *et al.*, 2004)**. Squid ink can act as a cell-protective drug in the treatment of cancer with chemotherapy by increasing the number of leukocytes and bone marrow cell nucleates, whose numbers are decreasing due to the use of drugs such as tumor cell killer **(Naraoka *et al.*, 2000)**. In addition, melanin from squid ink has anti-tumor activity that inhibits plasmin activity from increasing thromboxane, and meanwhile, it increases the body's resistance to kill cancer cells **(Zhong *et al.*, 2009)**. Melanin also contributes as an antioxidant **(Fahmy, 2013; Guo *et al.*, 2014)**, anti-radiation **(Pralea *et al.*, 2019; Solano, 2020)**. Moreover, it works as an antirotavirus **(Rajaganapathi *et al.*, 2000)** and antibacterial **(Aruldhason *et al.*, 2014; Zakaria *et al.*, 2019)**. In addition, melanin also can absorb metals, especially in the phenolic hydroxyl (OH), carboxyl (COOH) and amine (NH) functional groups of the melanin molecule **(Chen *et al.*, 2009)**.

Nair *et al*. (2011) stated that Sepia ink consists of melanin granules on thick colorless media. The pigment melanin is the main element in the ink glands that continuously produce ink. At the end of the ripening process, the ink gland cells store ink sacs that function as reservoirs. Each Sepia ink bag contains ± 1 g melanin **(Derby, 2014)**, and the amount of melanin is ± 15% of the total ink wet weight **(Wang *et al*., 2014)**. Melanin Sepia is formed by many aggregates. This aggregate is also formed by small round grains with different size distributions. Small grain diameter ranges from 100-200 nm **(Mbonyiryivuze *et al*., 2015)**.

The squid and/or cuttlefish ink had antibacterial activity **(Nair *et al*., 2011).** Melanin's action as an antibacterial has not been much revealed. Some researchers have tested antibacterial activity against only extracts from cuttlefish and/or squid ink. **Nithya *et al*. (2011)** examined the antibacterial activity of cuttlefish hexane extract (*Sepia pharaonis*) purified with diethyl ether. The results of this study indicate that the extract has inhibitory activity against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis, Klebsiella pneumoniae*, and *E. coli*. **Sari *et al.* (2019)** revealed that melanin from squid ink contains steroid and triterpenoids, which has a weak to medium antibacterial activity against *L. monocytogenes* and *E. coli*. The results of the current study tested the antibacterial activity by approximating the ability of the ink component (melanin) to chelate metals. The cytotoxic effect of melanin squid ink is thought to affect the growth of fish microbes, such as *Aeromona*s sp.

Aeromonas sp. is gram-negative, facultative anaerobic genus, which causes disease for both terrestrial and aquatic animals, as well as humans. *Aeromonas* sp. is considered an important bacterium among disease-causing agents in fish **(Praveen *et al*., 2016; Olga *et al*., 2020)**. It is necessary to study the antibacterial activity of cuttlefish melanin against *Aeromonas* sp. Therefore, this study aimed to determine the potential and bactericidal activity of the cuttlefish ink melanin against *Aeromonas* sp.

MATERIALS AND METHODS

2.1 Materials

The material used was cuttlefish (ink) obtained from the Muara Kintap Fishing Port, Tanah Laut Regency, and South Kalimantan. Other ingredients used included 0.5M HCl (Merck), acetone (Merck), aquades, Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), Sterile-EO Syringe Filter (Sartorius Minisart pore size 0.20 μm), Microbact TM GNB12A/B/E, 24E Identification Kits (Oxoid) and 70% alcohol.

The tools used in this study were a refrigerated centrifuge (Labogene Scanspeed 1580R), Freeze dryer (Model Christ alpha 2-4 LD Plus), autoclave (Pressure Steam Sterilizer Electric Model No.25X-2), laminar flow (Biobase), incubator (Memmert), colony counter (Quebec), incubator shaker (Wisd), spectrophotometer (Genesys 10uv), SEM with Energy Dispersive X-ray (SEM-EDX) EDAX.SL, TEM JEOL, JEM1400, and beaker glass.

2.2 Methods

2.2.1 Preparation of melanin

Isolation and purification of cuttlefish ink melanin was performed according to the method of **Magarelli *et al*. (2010)** as follows: 50 g of cuttlefish ink was added to 100 ml

of HCl (0.5 M) in the dark. The solution was stirred for 30 minutes (in a magnetic stirrer) and then stored for 24 hours at 10°C. The pellet was separated from the supernatant by centrifugation (10000 rpm at 5°C for 15 minutes). The suspension was rinsed three times, each with 0.5M HCl, sterile water, acetone and finally with water, sequentially. Lyophilization was then carried out to remove the solvent using a freeze dryer. Melanin was obtained and then characterized using infrared (IR) spectroscopy and its morphology was identified by SEM, TEM, and the percentage of melanin elements using EDX (Energy Dispersive X-ray Spectroscopy).

2.2.2 Culture of the organism

The microbes were isolated from rotten meat and digestive fish (*Euthynnus affinis*). Isolates were grown on GSP agar medium (*Pseudomonas-Aeromonas*-Selective agar, Merck). Species were determined using the manual protocol of Microbact identification kit GNB 12A/B/E, 24E (Oxoid).

2.2.3. Analysis of *Aeromonas* cell leakage

The methods of cell leakage analysis were performed following those of **Bunduki *et al*. (1995)**. The analysis of *Aeromonas* cell leakage was carried out using a spectrophotometer at 280 and 260 nm. Evidently, the 280 nm wavelength is used to measure the nitrogen content of cell proteins, while the 260 nm wavelength is used to measure the nucleic nitrogen content in cells. 10 mL of *Aeromonas* cultures were centrifuged at 10,000 rpm for 10 minutes. The filtrate was removed and added to 5 mL of physiological salt solution (0.85% NaCl) to the cell sediment. The solution was then vortexed. Furthermore, melanin was added in a concentration of 0; 5; 10; 15; 20 mg/mL, and incubated at room temperature for 24 hours. The suspension was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was filtered with filter paper (0.20 µm sterile syringe filter) to separate cells. The analysis was carried out by observing the OD (Optical Density) of the liquid supernatant.

2.2.4 Analysis of melanin activity as antibacterial

Melanin activity was analyzed using the direct contact method between melanin and bacteria in liquid nutrient broth. The test tube consisted of 2,970 mL of sterile NB and 0.030 mL of bacterial suspension (3,000 mL of final solution). Powdered melanin was added to the test tube so that the melanin concentration in the tube was 0; 2; 6; 10 mg/mL. For the first series of test tubes (melanin concentration 0 mg/mL), 2,970 NB sterile + 0 mg melanin was used. A second series tube (melanin concentration 2 mg/mL), was prepared by adding 2.964 mL of sterile NB + 6 mg melanin, and so on.

The bacteria (10^8-10^9 CFU/mL) were freshened and incubated for 24 hours at 37 °C, then diluted ten times. The test tube was inoculated with 0.030 mL of bacterial suspension, vortexed for 1-2 minutes, and then incubated in a shaker incubator at 37°C for 24 hours. The Total Plate Count was used to count the bacteria by incubating the bacteria for 24 hours at 37°C; the number of colonies was counted. The total colony is the average count on the three Petri dishes that match a particular sample.

The percentage of bacterial inhibition was determined by a modification of the method of **Capasso *et al*. (1995)** with the following formula:

$$Bacterial\ inhibition = 100 - (N_t \times 100/N_o) \quad (1)$$

Where, Nt is the number of CFU bacteria/mL in the addition of melanin treatment, while No is the number of CFU bacteria/mL initial (initial inoculum). The Total Plate Count was used to count the bacteria by incubating the bacteria for 24 hours at 37°C, and then the number of colonies was counted. The total colony evaluation was carried out every 3 hours with repetitions.

2.2.5 Analysis of melanin activity to *Aeromonas* sp. growth

The inhibition of melanin on the growth of *Aeromonas* sp. was carried out in the same method as the melanin activity test, with a concentration of 10,000 mg/mL. This concentration is defined by the percentage of inhibition relative to the number of initial microbes close to 100%. Observations were carried out every three hours for 24 hours.

2.2.6. Preparation of *Aeromonas* sp. for TEM analysis

Sample preparation was done following the method of **Kim *et al*. (2007)** with some modifications. Bacteria were inoculated in NB medium, and 5,000 mg/mL and 10,000 mg/mL melanin were added, and then incubated for 24 hours at 37°C. Negative controls (without melanin) were prepared in NB medium. After incubation, the suspension was centrifuged at 1200 rpm for 10 minutes. The supernatant was washed with a phosphate buffer solution pH 7.2 and centrifuged at 2,600 rpm for 5 minutes (4°C). For the sample, cell pellets were fixed with 2% glutaraldehyde. *Aeromonas* sp. Cell images were observed by transmission electron microscopy (TEM, JEOL, and JEM 1400).

RESULTS

3.1 Isolation of *Aeromonas* sp.

The colony, presumably *Aeromonas* sp., is the yellow color on GSP agar, tested for biochemical properties using a microbact (oxoid) identification kit. The test results of the microbact kit are presented in Table (1). The identification results showed that *Aeromonas hydrophilla* similarity reached 94.20%.

Table 1: The results of the microbact kit

Microbact (oxoid) identification	**Result**
Oxidase	(+)
Motility	(+)
Nitrate	(+)
Lysine	(+)
Ornitine	(+)
H_2s	(+)
Glucose	(+)

Mannitol	(+)
Xylose	(+)
Onpg	(+)
Indole	(+)
Urease	(+)
Vp	(+)
Citrate	(+)
TDA	(-)
Gelatin	(-)
Malonate	(+)
Inositol	(-)
Sorbitol	(+)
Rhamnose	(+)
Sucrose	(+)
Lactose	(+)
Arabinose	(+)
Adonitol	(-)
Raffinose	(+)
Salicin	(-)
Arginine	(-)

3.2 Purification of melanin

Cuttlefish melanin is an aggregate composed of many spherical grains. Small spherical grains also make these aggregates with different size distributions. The small grain diameter ranged from 100-200 nm (Fig. 1 & 2). TEM micrograph shows spherical melanin granules (Fig. 3).

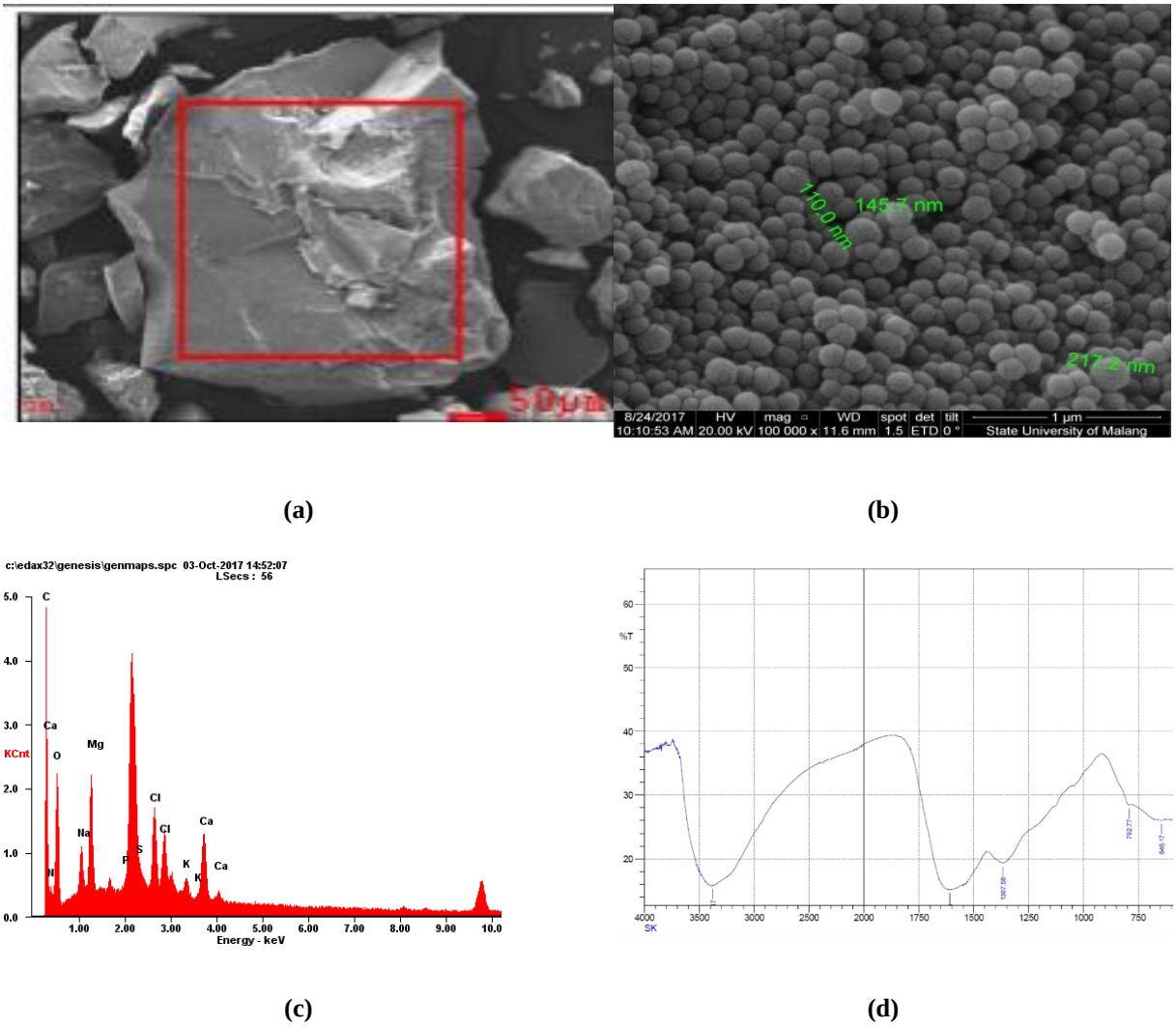
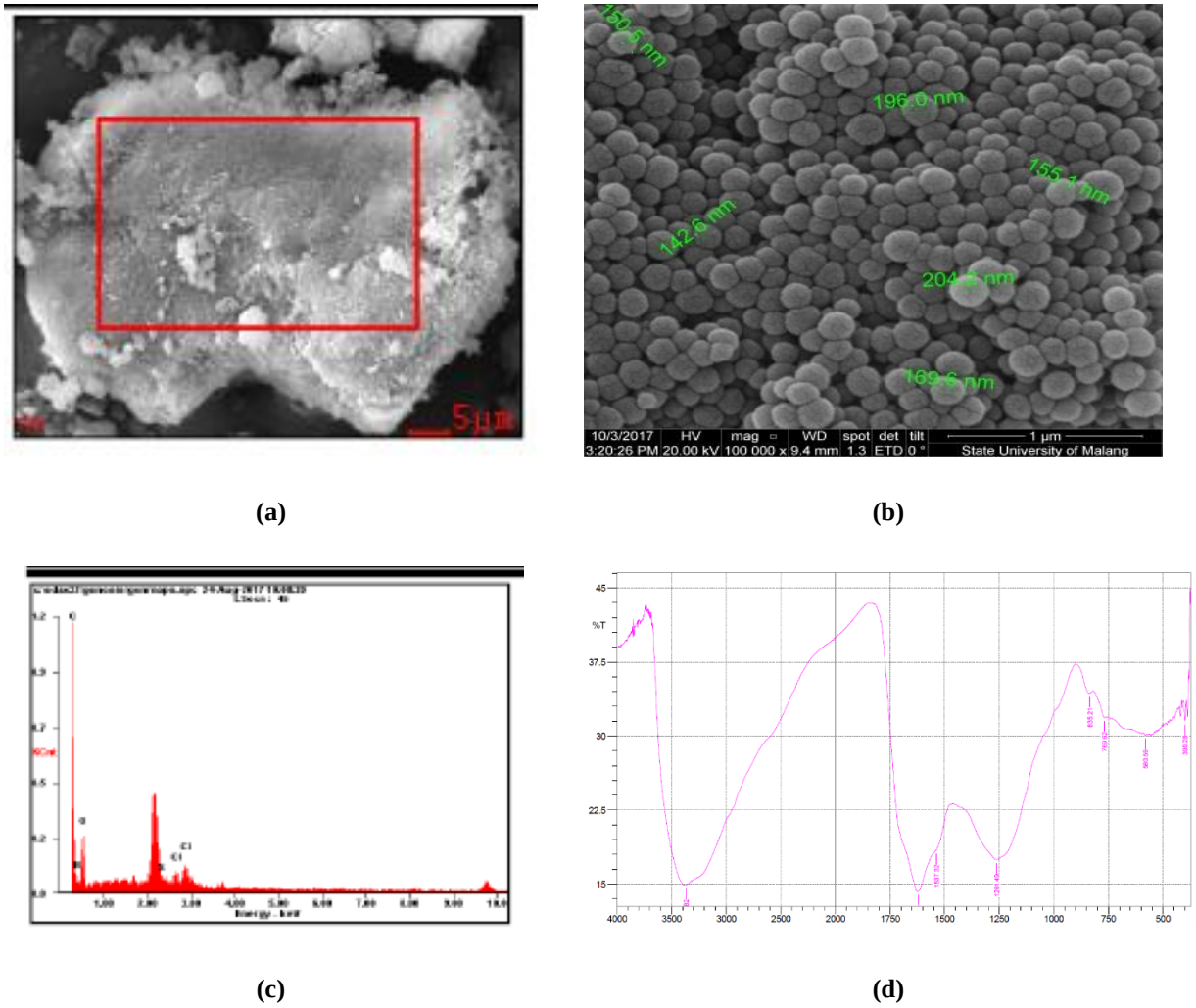


Fig. 1: Scanning electron micrograph (SEM) of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin before being purified

The FTIR spectrum showed that melanin had a strong Infrared absorption spectrum at 3300, 1620, and 1260 cm-1. It detected phenolic hydroxyl (OH), carboxyl (COOH), and amine (NH) groups (Fig. 1d). The difference between before and after purification was mainly seen in the intensity of each functional group. Cuttlefish ink contained the most significant components, specifically C (50.22%), O (29.18%), and N (7.99%), as well as a small portion of Mg, Na, S, K, Ca, P and Cl. After the purification stage, several components of melanin were released, such as Ca, Na, Mg, P, and K (Table 2).



(a) **(b)**

(c) **(d)**

Fig. 2: Scanning electron micrograph of cuttlefish ink melanin (a) and (b) aggregates melanin, (c) EDX pattern of melanin and (d) The FT-IR spectra of melanin after being purified

Table 2: Elemental percentages of melanin by EDX (Energy Dispersive X-ray Spectroscopy)

	C (%)	N (%)	O (%)	Na (%)	Mg (%)	P (%)	S (%)	Cl (%)	K (%)	Ca (%)
Before purified	50.22	7.99	29.18	2.38	3.98	0.83	0.98	1.99	0.52	1.93
After purified	62.76	16.02	20.28	-	-	-	0.49	0.45	-	-

Note: - = not detected

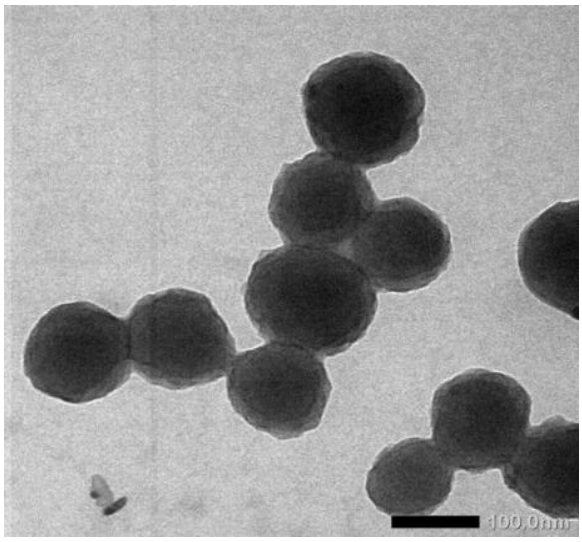


Fig. 3: Granules melanin with TEM

3.3 Evaluation of antibacterial activity

Melanin activity from *Sepia* sp. to *Aeromonas* sp. growth is shown in Table (3). The concentration that causes mortality is close to 100% and then the action is observed on the growth of *Aeromonas*. After 6 hours of incubation, 10 mg/ml of melanin caused a decrease in the number of live colonies more than 1 log. The decline continued till it reached 2 log10 after 21 hours of incubation. Melanin causes an extension of the adaptation phase and a decrease in the number of living colonies. After 24 hours of incubation, no more live colonies were found (Fig. 4). Fig.(4) shows the total bacteria measured every 3 hours. The existing method does not explain in detail, especially the method of measurement. It indicates that in addition to prolonging the adaptation phase, melanin also accelerates the period of bacterial cell death.

Table 3: The growth of *Aeromonas* sp. in Nutrient Broth containing melanin

Bacteria test	Melanin concentration after purification (mg/ml)	The number of bacteria (CFU/ml)		(%) inhibition relative to the initial microbial number [100-(Ntx100/No)]
		Incubation 0 hour (No)	Incubation 24 hours (Nt)	
Aeromonas sp.	2	$8.55x10^5$	$1.59x10^9$	0
	6	$8.55x10^5$	$3.6x10^5$	57.89
	10	$8.55x10^5$	$3.0x10^0$	99.99

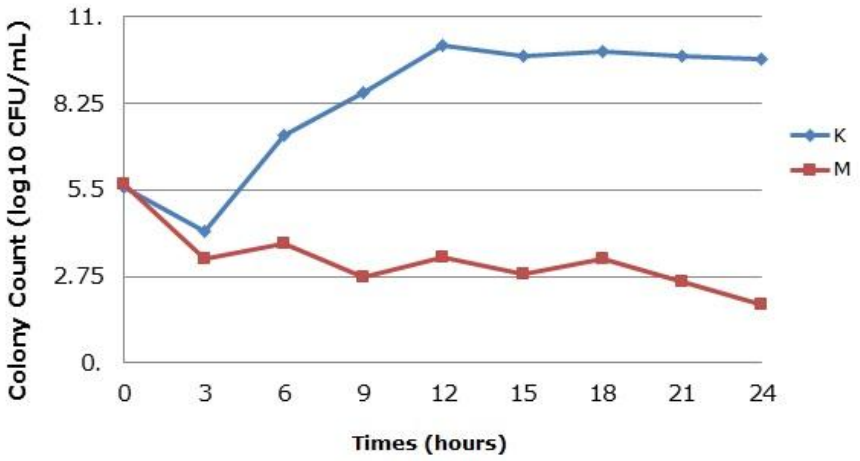
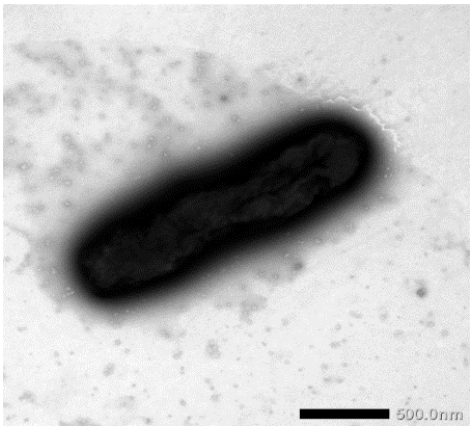
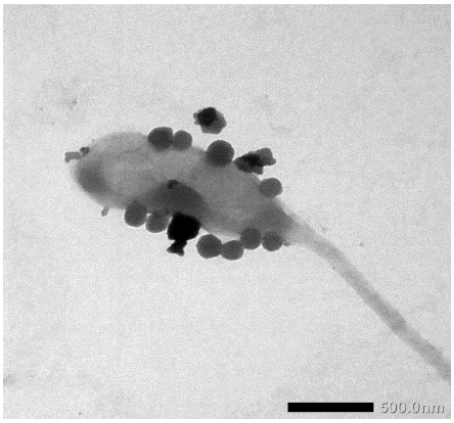


Fig. 4: *Aeromonas* sp. growth curves (K) without melanin, and (M) with melanin

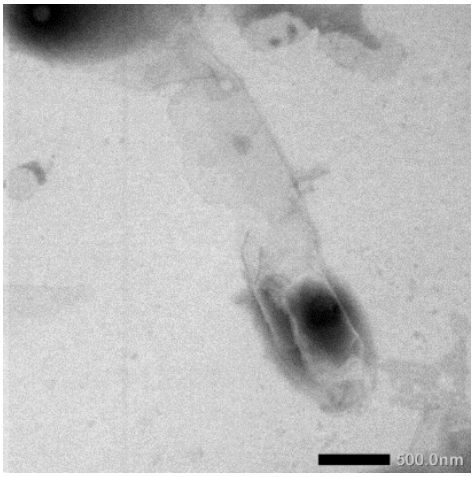
Fig. (5b) shows the TEM of *Aeromonas* sp. without adding melanin (a) or incubated with 5 mg/mL melanin and with 10 mg/ml melanin (arrows describe how melanin granules penetrate cells). Melanin causes increased cell wall permeability and leads melanin molecules to penetrate the cells due to cell walls and damages of membranes. This concentration of 5 mg/mL melanin caused the cell size to be small and irregular.



(a) Untreated *Aeromonas*



(b) *Aeromonas* treated with 5 mg/mL melanin



(c) *Aeromonas* treated with 10 mg/mL melanin

Fig. 5: TEM image of *Aeromonas* sp. morphology (a) before, (b) and (c) after treated with melanin

Table (4) shows the relationship between melanin concentrations and the cell-free supernatant of *Aeromonas* cells. Higher OD values at 260 nm and 280 nm indicated more significant cell leakage due to melanin. The higher the melanin concentration is, the greater the rate of cell leakage.

Table 4: OD values of *Aeromonas*-free supernatant after incubated with melanin from *Sepia* sp.

Melanin concentration (mg/ml)	**Optical Density**	
	260 nm	**280 nm**
0	0.038	0.037
5	0.179	0.198
10	0.297	0.321
15	0.359	0.395
20	0.451	0.499

DISCUSSION

Melanin is a tyrosinase that has been identified in squid ink **(Derby, 2014)**. Squid ink consists of a suspension of eumelanin granules in a thick colorless medium. Eumelanin is heterogeneous, generally, an insoluble polymer that develops through the enzymatic oxidation of the amino acid tyrosine. The production of eumelanin in pigment cells takes place in special organelles called melanosomes. Eumelanin consists of about 20% 5, 6-dihidroxyindol (DHI) and 5,6-dihydroxyindol-2-carboxylic acid (DHICA)

(Magarelli *et al*., 2010). It was reported that melanin inhibits bacterial proliferation **(Mackintosh, 2001)**.

Natural melanin is classified as a pigment molecule capable of absorbing metals at high concentrations. The ability to bind eumelanin to the metal side is an important parameter for understanding melanin metal complexes **(Lei *et al*., 2008; Chen *et al*., 2009).** Phenolic hydroxyl (OH), carboxyl (COOH), and amine groups (NH) act as functional groups that may be responsible for metal binding in melanin **(Chen *et al*., 2009)**. **Zerrad *et al*. (2014**) showed that there is a tenuous vibration of NH and OH groups at a wavelength of 3365-2880 cm^{-1}. The band centered at 1637 cm^{-1} is associated with the vibration of the aromatic ring of the C = C boundary and/or the aromatic conjugated C = O group. The FTIR spectrum of the melanin pigment, centered at 1261 cm^{-1} corresponds to the bending of the carboxylic ion group (COOH). In comparison, other small peaks can be ascribed to the substitution of C-H alkenes in melanin pigments **(Sajjan *et al*., 2010; Surwase *et al*., 2012)**.

There are differences in the components after purification of the commercial melanin *Sepia* in the study of **Mbonyiryivuze *et al*. (2015)** which contains the main ingredients, including C, O, Na and Cl, as well as minor compounds, such as Mg, Ca, K, S and N. Melanin has the ability to bind various metal ions, such as Ca, Fe, Cu, Zn, and Mg **(Zou *et al*., 2015)**. Mg (Magnesium) is one of the bacterial metals that play a role in cell wall orders **(Matthews *et al*., 1979)**. The antibacterial activity of melanin is thought to be by storing metal ions needed by bacteria for cell wall ordering **(Aisiah *et al*., 2020)**. The present results coincide with those of **Sari *et al*. (2019)** who noted the ability of melanin in the bacterial cell wall ordering event. Moreover, C and N elements increase after purification, presumably because after purification, the release of ions turns C and N elements to be more concentrated. **Poernomo *et al*. (2020)** stated that carbon and nitrogen play a role in strengthening the antibacterial activity of a material.

Bacterial cell walls contain many types of cations, including Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+}. Those ions are responsible of various bacterial activities, including enzyme work, metabolism regulation and maintaining the integrity of the outer layer as well. Mg^{2+} and Ca^{2+} ions, in particular, play an important role in protecting the stability of the external structure **(Ferrero *et al*., 2007; Peshenko & Dizhoor, 2007; Chen *et al*., 2009)**. The outermost layer of the outer membrane in gram-negative bacteria is lipopolysaccharide (LPS). These molecules are individually negatively charged. The different cations help stabilize and maintain the integrity of the outer membrane by binding to adjacent LPS molecules. These divalent cations function as bridges that bind negatively charged lipid molecules **(Raetz *et al*., 2007)**. The outer membrane of bacterial cells serves as a barrier to the entry of unnecessary compounds into cells (such as bacteriocins, enzymes, and hydrophobic compounds). If the cation can be adsorbed by the melanin functional group **(Hong and Simon, 2007)**, the metabolic system where the bacterial cells will be disrupted so that the growth of bacterial cells is also disturbed. Fig.(5) shows the

disruption of the bacterial cell wall due to melanin's activity, which disrupts the metabolic system of the bacterial cell wall.

The outer membrane of bacterial cells is protected by Ca^{2+} and Mg^{2+} ions against Polymyxin B, which can interact with divalent cations and replace them from their binding sites on lipopolysaccharides (LPS) **(Sahalan *et al*., 2013)**. This condition causes disorganization of the outer membrane of gram-negative bacteria. The LPS components detached from the surface of the bacteria, causing membrane leakage and ultimately cell death. Ca^{2+} proved to be more effective in protecting bacterial cells than Mg^{2+}. This can be seen in Table (3) and Fig. (4), where melanin has the property of chelating metal ions so that the formation of bacterial cell walls is not formed. The formation of a bacterial cell wall requires logomes such as Ca and Mg

The binding of melanin functional groups, such as phenolic hydroxyl (OH), carboxyl (COOH), and amino (NH) groups **(Chen *et al*., 2009)** to Ca^{2+} and Mg^{2+} ions in the outer membrane of *Aeromonas*. *Aeromonas* cells leakage could be seen from the production of bacterial cell-free supernatant after the cells were incubated for 24 hours with melanin ink (Table 3). This can lead to irregular whole formation in the outer membrane and alter membrane permeability due to the gradual release of LPS molecules and membrane proteins. The results of this study are supported by previous research, which shows an increase in the value of optical density (wavelengths of 260 nm and 280 nm), to the administration of melanin extract on the growth of *E. coli* **(Fitrial & Khotimah, 2017)**. The increase in optical density is closely related to the presence of bacteria, presumably due to the effect of melanin which inhibits bacterial growth by damaging cell walls **(Zhang *et al*., 2015)**. It was suggested that a similar mechanism causes degradation of the *Aeromonas* sp. membrane structure during the addition of melanin. Hence, extensive investigations aiming at a better understanding of the interactions between melanin and bacterial components should shed light on how this melanin material acts as a bacteriocidal material.

CONCLUSION

Melanin from *Sepia* sp. ink has inhibitory activity against the growth of *Aeromonas* sp., and causes reduced cell size and irregular cell shape. This is important information to overcome *Aeromonas* sp. attack on cultured fish.

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