The Effectiveness of Melanin from Squid Ink (*Loligo* sp.) as Antibacterial Agent Against *Escherichia coli* and *Listeria monocytogenes*

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Abstract: Melanin from squid ink contain bioactive compounds that can be used as antibacterial agents. *E. coli* and *L. monocytogenes* are pathogenic bacteria that cause food damage and disease in human so a natural antibacterial compound is required to replace synthetic antibacterial agent for inhibiting the growth of pathogenic bacteria. Melanin was extracted from squid ink, then it was analyzed quantitatively and qualitatively using phytochemical, testing on antibacterial activity of melanin squid ink against *E. coli* and *L. monocytogenes*. The variables measured were yield from melanin, quantitative and qualitative phytochemical test, positive control test of Amoxicillin, negative control test by using HCl and aquabidest, and inhibitory zone melanin from squid ink extract using paper disc method with concentration of 4%; 8%; 16%; and 32% with three replications. The result showed that yield from melanin was 0.692%. Dominant substances found by quantitative phytochemical on melanin squid ink were alkaloids 2390.87±0.77ppm; flavonoids 365.11±0.84ppm; phenols 292.03±0.74ppm; and saponins 173.86±0.66ppm. Qualitative phytochemical test detected steroids and triterpenoids compounds. The inhibition zones from melanin against *L. monocytogenes* and *E. coli* were between 2.15–6.18 mm and 1.84–4.69 mm, respectively which is considered as concentration of weak to medium antibacterial activity. Best concentration of melanin from squid ink was 32% on diameter of inhibition zone.

1. Introduction

Squid, a family of Cephalopods release a colored ink secretion to defense against predators [1]. The general black ink composition is undoubtedly due to the presence of biological entities such as melanin, polysaccharides and some proteins [2]. Squid ink properties as antiretroviral, antitumor, antioxidant, and the ability to protect cells from damage due to chemotherapy and antibacterial potency of squid ink against pathogenic bacteria [3]. The squid ink can be used for therapeutic applications. Example antibacterial activity of the squid ink has already been reported against biofilm bacteria [4]. Melanin from squid ink contains active substances that are beneficial for human health [5]. The melanin pigment is manufactured in the mature cells of the ink gland a highly
specialized organ situated in the bottom of the ink sac and deputed to the continuous production of the ink. Within mature ink gland cells, melanogenesis depends on the activity of the copper-containing enzyme tyrosinase, which catalyses the committed step of the process, namely the oxidation of tyrosine to dopaquinone [6] [7]. Antibacterial activity of squid ink Sepioteuthis lessoniana and Sepia pharaonis have ability against S. aureus [8].

Safety of food is a scientific domain requiring advanced handling, preparation, and storage condition [9]. Pathogenic bacteria are often found in food stuffs, including fish and fishery products, such as E. coli and L. monocytogenes [10]. L. monocytogenes often contaminates food of animal origin and is pathogenic in both animals and humans [11]. A protein extracted from cuttlefish ink in very low concentration has been shown to inhibit the growth of S. aureus [12]. Antibacterial efficient of crude squid ink was against clinically important pathogenic strains such as E. coli [13]. The higher melanin were added, the larger area inhibition zone will formed. This was supported by [14], who showed that at volume of 20 μl crude extracts of squid (L. duvauceli) caused a 7 mm inhibitory zone against Vibrio fischeri. While a 100 μl volume inhibit zone of 17 mm against the strain of Candida albicans.

2. Research Methods
2.1. Sample extraction (fitrial and khotimah, 2017) [15]
Fresh squid was obtained from fishermen at Tambak Lorok, Semarang, Central Java. The squid were medium size, 17-20 cm in length. Fresh squid was separated from ink bag then the ink bag put into the sample bottle. The ink bag is solved with HCl using a stirrer in order for the ink to come out. Then, 50 ml of squid ink was mixed with 100 ml of HCl 0.5 M in a light-tight condition. The solution was stirred using a magnetic stirrer for 30 minutes, then stored for 24 hours at 10°C. The precipitate is separated from the supernatant by using a cold centrifuge (10,000 rpm at 5°C for 15 minutes). The precipitate was washed again with HCl 0.5 M solution 3 times, followed by aquadest, acetone, and aquadest. The next step is lyophilization for approximately 24 hours to separate the solvent and stored in the freezer prior to further testing.

2.2. Phytochemical screening test
Analysis of Flavonoid Compounds refers to Suryanto [16]; Phenol Compounds; Saponin Compound; Alkaloid Compounds [17]; Steroid and Triterpenoid Compounds [18].

2.3. Antibacterial activity test
Antibacterial activity test was conducted according to [19]. Concentration of 1% melanin of cuttlefish ink (Sepia sp.) has inhibitory activity against E. coli. [15]. Previously, trial research was conducted at 2% melanin concentration of squid ink had no inhibitory activity against E. coli and L. monocytogenes so the concentrations was made two times and used in this study were 4%; 8%; 16%; 32%. A positive control concentration of Amoxicillin was used with concentration of 4%, 8%, 16% and 32%. The negative control used was HCl and Aquabidest. Whatman filter paper No. 41 impregnated by soaking the paper.
3. Results And Discussion

3.1. Quantitative and qualitative phytochemical test

The result of quantitative and qualitative phytochemical test of melanin powder from squid ink can be seen on Table 1.

| Parameter                  | Concentration (ppm) |
|----------------------------|---------------------|
| Alkaloids                  | 2390.7±0.77         |
| Flavonoids                 | 365.11±0.84         |
| Phenols                    | 292.03±0.74         |
| Saponins                   | 173.86±0.66         |
| Steroids/ Triterpenoids    | Detected            |

Note:
Data is the average of two replication ± SD

The purpose of the quantitative phytochemical test is to know the bioactive compounds contained in melanin from squid ink. Phytochemical test include phenol, flavonoids, saponin, alkaloids, steroids and triterpenoids.

The polysaccharides from squid ink have also been studied and are shown to have superior antioxidant, antitumor and anti-chemotherapeutic properties [2]. Other study results also stated that phytochemical test showed that squid ink contains triterpenoid group chemical compounds [20]. Squid ink contains alkaloid compounds and steroids [21]. Melanin in squid ink contains more alkaloid bioactive compounds. Alkaloid compounds are the dominant compounds compared to other bioactive compounds. 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 semipolar 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. Alkaloid can be detected because alkaloid is generally soluble in semi-polar solvent to nonpolar, while some groups of pseudoalkaloid and protoalkaloid dissolve in water as polar [21]. This indicates that the squid contains pseudoalkaloid and protoalkaloid groups. Protoalkaloid is a relatively simple amine. There is no heterocyclic ring, and it is obtained based on photosynthesis of basic amino acids. Pseudoalkaloid is not derived from amino acid precursors and usually these compounds are alkaline.

Taufiq et al. [22], also argues that alkaloids have antibacterial ability, a mechanism that is thought to interfere with peptidoglycan components in bacterial cells, so that the cell wall layer is not fully formed and causes cell death. Peptidoglycan itself is the substance that forms the cell wall of bacteria. Components of peptidoglycan include amino acids and sugars. Fahmy et al. [5], protein has made from amino acids in crude squid ink can significantly damage cell membrane integrity leading to increase leakage in microbial cell content. This is reinforced by Chambell [23], the alkaloid works by disrupting the peptidoglycan component and inhibiting the topoisomerase enzyme that has a very important role in the process of replicating, transcribing, and recommending DNA by cutting and connecting to a single strand or double strand. Flavonoids are polar compounds because they have a number of hydroxyl groups. Polar solvents such as HCl can be used to extract flavonoids from plant or animal tissues. Flavonoid is a polar compound because it has a number of hydroxyl groups [24].

Other bioactive compounds contained in melanin are saponins and steroids. Saponins are polar compounds which have a glycosome bond, and are easily soluble in a polar solvent. Saponins have a non-polar group of steroids and triterpenoids, but are more likely to be polar because of their glycosome bonds. 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 semipolar [1]. The presence of a steroid component is thought to be an adrenal hormone and sex hormone (progesterone, 17β-estradiol, testosterone, 4-androstene-dione and cortisol) eg steroids detected in Achatina fulica and snail.
(Pomacea canaliculata) which is a type of gastropod [25]. This steroid is thought to have a stamina enhancing (aprodiasiak) and anti-inflammatory effect.

Other bioactive compounds contained in melanin are phenols. The phenol compound is a polar compound, so the phenol compound will be more easily soluble in the polar solvent as well [26].

3.2. Antibacterial Activity Test of Melanin Squid Ink Against E. coli and L. monocytogenes

The result of inhibitory zone in various concentration to E. coli and L. monocytogenes can be seen on Table 2.

### Table 2. The Result of Inhibitory Zone In Various Concentration to E. coli and L. monocytogenes

| No. | Extract Concentration (%) | Area of Inhibitory Zone (mm) |
|-----|----------------------------|-------------------------------|
|     |                            | E. coli                       | L. monocytogenes              |
| 1.  | 4                          | 1.84 ± 0.14<sup>a</sup>       | 2.15 ± 0.13<sup>a</sup>       |
| 2.  | 8                          | 2.74 ± 0.32<sup>b</sup>       | 2.56 ± 0.24<sup>a</sup>       |
| 3.  | 16                         | 3.66 ± 0.4<sup>c</sup>        | 4.58 ± 0.14<sup>b</sup>       |
| 4.  | 32                         | 4.69 ± 0.3<sup>d</sup>        | 6.18 ± 0.3<sup>c</sup>        |

Note:
- Data has been reduced the diameter of disc paper by 6 mm
- Data obtained from 2 different diameter measurement points, then averaged 3 reps ± SD
- Data followed by different notation show significant difference (P<0.05)

Some studies reported that the ink of cephalopods exhibit antimicrobial activity [14] [27]. Crude extracts of the Loligo duvauceli is reported to have antifungal and antibacterial activity against Candida albicans, Lactobacillus acidophilus, Escherichia coli, Klebsiella pneumonia, and Staphylococcus aureus. These findings corroborate the results of the present study.

The mechanism of antibacterial compounds is divided into two, namely bacteriostatic growth inhibitory or bactericidal, killing the bacteria. Based on the inhibit zone formed, melanin from squid ink only weak to moderate bacteriostatic broad spectrum against gram positive and negative test bacteria. The bioactive compounds that are most likely to be instrumental in inhibiting bacterial growth are alkaloids and flavonoids because of their high content in melanin from squid ink. Alkaloid compounds as antibacterial compounds have a working mechanism disrupting the components of peptidoglycan in bacterial cells. The higher levels of bioactive compounds are increasingly bactericidal, whereas lower levels are usually only bacteriostatic. [28]

In present study the concentration 32% melanin from squid ink developed 6.18 mm against L. monocytogenes. Similarly, antibacterial activity of squid ink Sepioteuthis lessoniana and Sepia pharaonis have ability against gram-negative bacterial as S. aureus. [31].

Based on the results, melanin from squid ink is able to inhibit the growth of gram-positive bacteria better than gram-negative bacteria. Very similar to the present study, the gram-negative bacteria like E. coli were also inhibited, but the zones were comparatively smaller [30]. Another study also reported similar antibacterial effect of cuttlefish ink against S. aureus. [12].

This occurs due to differences in the arrangement of the cell wall. Gram-positive cell wall bacteria is single-layered with 1-4% lipid content, whereas in gram-negative bacteria it has 3 layers (lipopolys, outer membranes of phospholipids and lipopolysaccharides) and lipid content of 11-22%. The outer membrane of the phospholipid causes an antibacterial chemical component to penetrate gram negative bacterial cell wall. The L. monocytogenes bacterial cell wall is more easily penetrated than the cell wall of E. coli bacteria [11]. This is in accordance with research conducted by Jawetz et al. [27], the test results show the diameter of inhibition zone in gram-positive bacteria in general tend to be larger than gram-negative bacteria. This suggests that gram-positive bacteria are more susceptible to antibacterial compounds than gram-negative bacteria.
The difference in cell wall structure determines the penetration, bonding and activity of antibacterial compounds. Gram-positive bacteria have cell wall structures with more peptidoglycan, less lipids and cells containing polysaccharides (teicocic acid). This acid is a water-soluble polymer, which acts as a positive ion transport to get out or enter. Adding water-soluble properties shows that gram-positive cell wall is more polar [29]. While the flavonoid compound in melanin is a polar part so it is easier to penetrate the polar layer of peptidoglycan than nonpolar lipid layer. Thus causing inhibitory activity in gram-positive bacteria is greater than gram negative.

Cell walls in gram-negative bacteria are more complex than gram-positive bacteria. E. coli is a gram negative bacteria that is resistant to some antibacterial compounds [27]. This is caused by three layers of cell wall in these bacteria, so some compounds are not able to damage the tissue from E. coli bacterial cell wall. The outer walls of E. coli bacteria are of high permeability, so that the active substances in the simplicia extract can’t enter the bacterial cells resulting in no growth inhibition.[30]. Mode of action in delivering antibacterial, antifungal and antibiofilm efficacy may conceivably due to the high level of protein content [9].

Gram-positive cell wall contains peptidoglycan and also teikoic acid and teikuronic acid. The teikoc acid (TA) is a polymer of phosphate ribitol or glycerol phosphate. Each monomer of the theopholic acid is linked by a phosphodiester bond. The acetic acid acts as one of the gram-positive cell wall organisers alongside the peptidoglycan chain. Theoric acid is another polymer of carbohydrates found in each bacterium is a cyclohexic acid bound to cyelonate and the two acids can be separated from the peptidoglycan by hydrolysis. Theic acid and theic acid are bound covalently to peptidoglycan. Therefore, the gram-positive cell wall of bacteria is partially polysaccharide, whereas in gram negative bacterial cell wall there is little peptidoglycan between the external membrane and inside cell wall membrane. Gram positive bacteria experienced the process of cell denaturation in advance compared to gram-negative bacteria.

3.3. Negative control and positive control test
3.3.1. Negative control test
Based on the negative control test, the Aquabidest and HCl solvent has no inhibition zone. This proves that the Aquabidest and HCl solvent is not bactericidal or bacteriostatic to the test bacteria, so it is certain that the resulting inhibitory zone results are not affected by the solvent used. The negative controls showed significant differences with various concentrations of the extract [31]. Negative controls show no inhibition zone. This indicates that the negative controls used do not affect the antibacterial test.

3.3.2. Positive control test
This test aims to compare the diameter of the inhibitory zone formed from melanin squid ink and Amoxicillin. Amoxicillin concentrations were used according to the concentrations used in melanin 4%, 8%, 16% and 32%. The result of inhibitory zone positive control test of amoxicillin against E. coli and L. monocytogenes for 48 hours can be seen on Table 3.
Table 3. The Result of Inhibitory Zone Positive Control Test of Amoxicillin Against E. coli and L. monocytogenes for 48 hours

| No. | Concentration (%) | E. coli    | L. monocytogenes |
|-----|------------------|------------|------------------|
| 1   | 4                | 15.71±0.11 | 17.13±0.1^a      |
| 2   | 8                | 18.7±0.28  | 20.26±0.12^b     |
| 3   | 16               | 22.78±0.08 | 24.53±0.09^c     |
| 4   | 32               | 24.6±0.14  | 25.39±0.13^d     |

Note:
- Data has been reduced the diameter of disc paper by 6 mm
- Data obtained from 2 different diameter measurement points, then averaged 3 reps ± SD
- Data followed by different notation show significant difference (P <0.05)

Based on the inhibitory zone diameter results, it is known that Amoxicillin has strong antibacterial activity. Amoxicillin is a penicillin derivative that has a broad spectrum that can inhibit the growth of gram-positive and gram-negative bacteria. The mechanism of action of Amoxicillin (penicillins) is by preventing peptidoglycan crosslinking in the final stages of cell wall synthesis, by inhibiting penicillin binding proteins [14]. This protein is an enzyme in the plasma membrane of bacterial cells normally involved in the addition of crosslinked amino acids with bacterial cell wall peptidoglycan, and block the activity of transpeptidase enzymes so that the bacterial cell wall becomes brittle and susceptible to lysis. Research argues that amoxicillin may inhibit S. aureus bacteria at a concentration of 32 mg/L with an inhibitory zone of 9.9 mm [32].

The difference in the mechanism working of Amoxicillin and melanin from squid ink is that Amoxicillin works to inhibit bacterial cell wall synthesis, while melanin from squid ink works by disrupting the peptidoglycan component in bacterial cells, so that the cell wall layer is not completely formed. Amoxicillin is one of the antibiotics that is effective against both gram positive and negative bacteria.

4. Conclusion
The conclusions obtained from this research, the most effective concentrations of melanin from squid ink as antibacterial agent against E. coli and L. monocytogenes was 32 %.

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