Antioxidant potential of squid ink (*Loligo duvauceli*)

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Abstract. Squid ink has secondary metabolites to avoid enemy attacks. Secondary metabolite compounds have been able to determine the presence of antioxidant activity. Maceration is a way to get extracts and secondary metabolites in a sample. This study aimed to identify secondary metabolites and determine antioxidant activity in *L. duvauceli* ink extract. The method in this research was descriptive. The treatments used were solvents with different polarity levels, n-hexane, ethyl acetate, and ethanol. The parameters carried out in this study were yield, qualitative and quantitative secondary metabolites compound testing, and antioxidant activity testing. The results showed that n-hexane extract showed the presence of saponin compounds, ethyl acetate extract contained steroid compounds, and ethanol extract contained alkaloids and phenolic compounds. Saponin content in n-hexane extract was 0.23%. The levels of alkaloids and phenolics in the ethanol extract were 0.02% and 0.72%, respectively. The results show that *L. duvauceli* ink has antioxidant activity obtained from the IC_{50} value, n-hexane extract was 128.35 ppm, ethyl acetate extract was 78.81 ppm, and ethanol extract was 75.68 ppm.

1 Introduction

Squid (*Loligo duvauceli*) is one of the molluscs from marine fisheries in Indonesia which is commonly consumed by the wider community. Squid has a fairly complete nutritional content such as protein, fat, vitamins, etc. According to [1] that the number of squid caught in all Indonesian waters for the benefit of commercial commodities, in 2018 was 216,977.64 tons.

Squid produces offal and ink sacs which are considered as by-products with low selling value, and can cause quite serious ecological problems if not used properly. Squid ink has many benefits for humans, and contains nutrients that are important for human consumption. Squid ink contains 89.15% water, 9.37% protein, 0.85% carbohydrates, 0.28% fat and 2.35% ash [2].

Squid ink can be applied to various pharmaceutical fields. Squid ink can be used as an antibacterial [3], antihypertensive [4], antiretroviral [5], [6] stated that squid ink has anti-inflammatory activity which inhibits gastric secretion. Research by [7] on squid ink powder extract showed antioxidant activity. Research by [8] on squid ink extract without melanin also showed antioxidant activity.

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Squid ink is thought to contain several secondary metabolites that lead to antioxidant activity. Each secondary metabolites compounds obtained is based on the polarit of solvents during extraction. So, it necessary to use different solvent to see the variuos compounds that extracted. Antioxidants are important components that play a role in the natural activities of living things, because they are antioxidant compounds needed by organisms to ward off free radicals and prevent damage caused by free radicals. Antioxidant compounds consist of several molecular structures that are able to release electrons to free radical compound molecules without being completely disturbed by their function and work [9].

The use of squid ink is quite rare, because some people think that squid ink (*Loligo duvauceli*) is a by-product that has no selling value. Previous studies on squid ink powder extract [7] and squid ink extract without melanin [8] have been carried out. Therefore researchers are interested in conducting research on antioxidant activity tests of fresh *L.duvauceli* ink extracted with different solvents to determine the presence of secondary metabolites and antioxidant activity present in fresh squid ink extract (*Loligo duvauceli*).

2 Materials and methods

2.1 Materials and Tools

The material used is squid ink which is obtained from squid purchased at one of the markets in Pekanbaru. The materials used for the extraction of squid ink were ethanol p.a (Merck), ethyl acetate p.a (Merck) and n-hexane p.a (Merck). The materials used for testing secondary metabolites were H_2SO_4 (Merck), chloroform (Merck), ammonia (Merck), Dragendorf reagent (Merck), Mayer's reagent (Merck), Wagner reagent (Merck), distilled water, 1% $FeCl_3$ (Merck), HCl 2N (Merck), Mg powder (Merck), concentrated HCl (Merck), glacial CH_3COOH (Merck) and concentrated acid H_2SO_4 (Merck), methanol (Merck), gallic acid (Merck), folin ciocalteau (Merck), Na_2CO_3 (Merck), aquabides, and caffeine standards. The materials used for testing antioxidant activity were DPPH (Sigma), vitamin C (Merck) and methanol p.a (Merck).

The tools used are brown bottles, beaker glass (Pyrex), aluminum foil, vortex, erlenmeyer (Pyrex), rotary vacuum evaporator (Cole Parmer), UV-Vis spectrophotometer (Optima), cuvettes (Hellma), analytical scales (Boeco Germany), test tubes (Pyrex), test tube racks, dropper pipettes, gloves, and masks.

2.2 Methods

2.2.1 Sample extraction procedure

Squids that have just been obtained from market are dissected and ink sac is manually removed from innards. Ink is removed from the ink sac, then placed in a clean plastic container and frozen prior to testing. Extraction was carried out based on research by [7] which has been modified. Fresh squid ink ± 200 g mixed with each of 3 different solvents based on their polarity, namely 1000 mL (1:5) n-hexane p.a, ethyl acetate p.a, and ethanol p.a in a glass bottle. The glass bottle is wrapped in aluminum foil to prevent direct light from penetrating the extract.

Each of these mixtures was then macerated for 72 hours. Furthermore, the extracted solution is evaporated or concentrated with a rotary vacuum evaporator at 40ºC, evaporation is carried out to separate the filtrate from the solvent. Furthermore, the extraction results were analyzed for yield, secondary metabolite compounds qualitatively and quantitatively, and their antioxidant activity.

2.2.2. Qualitative analysis of secondary metabolites

Analysis of secondary metabolites is divided into two tests, namely qualitatively and quantitatively. Qualitative testing is observed from the color produced during testing, which determines the presence of certain secondary metabolites in the sample extract. Qualitative testing of secondary metabolites compounds consisted of alkaloid, phenolic, saponin, flavonoid, and steroid/terpenoid tests [10].

2.2.3. Quantitative analysis of secondary metabolites

Quantitative analysis of secondary metabolites was carried out to find out how many levels of a compound contained in an extract. The results of qualitative analysis of positive secondary metabolites were then tested for their assay. The compounds tested were total alkaloids [11], total phenols [12] (Singleton dan Rossi 1965), and saponin levels [13].

2.2.4. Analysis of antioxidant activity with the DPPH method

Analysis was carried out in several stages which were modified from the [14] method. DPPH reagent was weighed as much ± 6 mg and mixed with 60 mL methanol and obtained DPPH solution with a concentration of 100 ppm. The control solution was prepared by mixing 0.5 mL of 100 ppm DPPH solution with 4.5 mL of methanol. Then incubated in a dark place for 30 minutes, the absorption was measured at a wavelength of 517 nm. Then ± 30 mg of *L. duvauceli* extract was mixed with 30 mL of methanol solvent, and 1 mg/mL of sample solution concentration was obtained. Methanol was added for dilution to obtain solution concentrations of 1000, 500, 250, 125 and 62.5 ppm. Antioxidant analysis was determined by taking 4.5 mL of each concentration solution using a micro pipette and then putting it into a 25 mL Erlenmeyer, then given 0.5 mL of 100 ppm DPPH reagent solution. The solution was homogenized and incubated in the dark for 30 minutes. The wavelength of 517 nm is used to measure absorbance.

Vitamin C 1 mg/ml (1000 ppm) was made as a positive control in the same way as the sample treatment. The results obtained are calculated and expressed as a percentage value (%) of the free radical scavenging activity of the DPPH reagent by entering the value of the equation:

$$\%\ \text{inhibition} = \frac{(\text{control absorbance-sample absorbance})}{\text{control absorbance}} \times 100\% \qquad (1)$$

The parameter in measuring of a sample is IC_{50}, which is the number of compounds needed to reduce DPPH free radicals by 50%. The IC_{50} value of each sample concentration is obtained from the linear regression equation, namely y = a + bx where the sample concentration (ppm) is the abscissa (x-axis) and the value of % inhibition is the coordinate (y-axis).

The IC_{50} value is calculated by entering the equation:

$$IC_{50} = \frac{50\text{-}a}{b} \qquad (2)$$

where:

a = intercept; b = slope

3 Result and Discussion

3.1. Yield of Loligo duvauceli Ink Extract

Yield is result of comparison value between weight of *L. duvauceli* ink extract produced and weight of ink before it is macerated. The average yield values of extracts from squid ink (*Loligo duvauceli*) are shown in Table 1.

Table 1. Yield of fresh Loligo duvauceli ink extract

Solvents	Yield (%)
n-Hexane	1,69 ± 0,12
Ethyl Acetate	2,26 ± 0,01
Ethanol	2,77 ± 0,12

The yield of ethanol extract showed the highest value because ethanol solvent can attract more bioactive compounds than other sailors. The lowest yield value is shown in the n-hexane extract with the value (1.69%) of the non-polar n-hexane solvent which can only attract bioactive compounds in small amounts as seen in the yield value. The yield value produced in the ethyl acetate extract is (2.26%), ethyl acetate solvent is a semi-polar solvent, therefore it can only extract compounds with moderate polarity [15].

Extracts in polar solvents yielded the highest values, whereas n-hexane extracts (non-polar) yielded small extracts. The yield value obtained can indicate the amount of bioactive content contained in a sample [16]. Solvents with a polar type are able to extract compounds from polar to semi-polar properties [17].

(a) (b) (c)

Fig 1. *L. duvauceli* ink extract (a) n-hexane extract, (b) Ethyl Acetate extract, and (c) ethanol extract

The results of the crude extract for each solvent are shown in **Fig 1.** The n-hexane solvent showed less extract yield than the other solvent extracts, showing a cloudy white color. *Loligo duvauceli* ink in fresh form during maceration, with the presence of water content and ink precipitate mixed with n-hexane solvent which is non-polar, resulting in a cloudy white extract.

The semi-polar ethyl acetate solvent produces a yellow extract, with less extract yield than ethanol solvent. [18] explain that the extraction results increase with increasing polarity of the solvent used with the addition of organic solvent and swater which can facilitate a compound that dissolves in non-polar and semi-polar solvents.

The results of the ethanol extract from *Loligo duvauceli* ink show a higher value than other solvents, with a black extract color. The yield of an extract is greatly influenced by the polarity of the solvent used [19]. The difference in the average amount of extract yield can

be influenced by other factors such as the length of extraction time, the type of solvent used, the ratio of the number of samples to the amount of solvent used, and the conditions and time of storage, [20].

3.2. Qualitative of Secondary Metabolites

Identification of secondary metabolites by the method of analysis of secondary metabolites was carried out to qualitatively determine the content of secondary metabolites in an extract of *Loligo duvauceli*. The test results obtained are in the form of colors that are adjusted to the color standards in each reagent. The test results for secondary metabolites in the n-hexane, ethyl acetate, and ethanol extracts are presented in Table 2.

Table 2. Results of Qualitative of Secondary Metabolites

Compounds	**Extract**			**Reagent**	**Standard Color**
	n-Hexane	**Ethyl Acetate**	**Ethanol**		
Alkaloid	+	+	++	Mayer,	Orange percipitate
	-	-	-	Dragendroff,	
	-	-	-	Wagner	
Phenolic	-	-	+++	FeCl3 3%	Black
Saponin	++	-	-	H20, HCl	Bubble
Flavonoid	-	-	-	Sianidin Test	Red or orange
Steroid	-	+++	-	Libermen – Burchard	Blue

N.b: +++ (strong), ++ (moderate), + (weak), - (not detectioned)

The results of [21] on ethanol extract of *Loligo duvauceli* ink powder showed detection of phenolic compounds, flavonoids, steroids, terpenoids, alkaloids, and saponins. These results show differences in the results of research on the bioactive compounds of fresh *Loligo duvauceli* ink extract, namely the absence of detectable saponins, steroids, flavonoids and terpenoids.

The ink released by squid is not liked by predators, especially fish because it is alkaloid. Alkaloids are natural chemical compounds that contain a basic nitrogen atom. Alkaloids were reported to be therapeutically and biologically active (eg, atropine, quinine and morphine) and have many medical applications [22]. Several studies have shown that alkaloids have many types of biological activities, such as antimicrobial, antioxidant, anti-cancer, anti-inflammatory, and anti-viral activities [23],[24]. Several alkaloid compounds are indicated to have benefits in the field of medicine [25].

Other bioactive compounds that were detected qualitatively were steroids in the ethyl acetate extract which showed a blue color in the test results. Steroids/triterpenoids were only detected in non-polar and semi-polar solvents, but were not detected in polar solvents, namely ethanol. Steroid compounds are thought to have the effect of increasing body stamina (aphrodisiac) and anti-inflammatory [26]. Steroids isolated from marine animals and plants have high medicinal value [27].

Phenolic bioactive compounds were also detected in the ethanol extract of *L. duvauceli* ink by showing a dark black color. The black color results from the reaction between Fe^{3+} and the phenolic compounds contained in the *Loligo duvauceli* ink extract. Phenolic compounds have polar properties, so they can be identified in polar solvents such as ethanol [28].

The presence of phenolic in ethanol extract is suspected as a determinant of antioxidant activity in extract. [29] stated that content of phenolic compounds in a material can reduce free radicals by binding to Fe ions and counteracting enzymatic systems that play a role in

formation of free radicals such as cyclooxygenase, mono-oxygenase or xanthine oxidase. Phenolic compounds are one of the main compounds that support antioxidant activity in a sample. According to [30], phenol content has a positive relationship with antioxidant activity as a free radical inhibitor.

3.3. Quantitative Secondary Metabolites

The test results on the secondary metabolites of *Loligo duvauceli* ink extract qualitatively showed the presence of alkaloids, phenolic, steroids, and saponins compounds. The dominant secondary metabolites were then tested quantitatively and the results of the analysis of secondary metabolites are presented in Table 3.

Table 3. Result of Quantitative Secondary Metabolites from extracts

Extracts	Parameter	Average (%)
n-Hexane	Saponin	0,23±0,01
Ethanol	Alkaloid	0,02±0,01
	Phenolic	0,72±0,07

The n-hexane extract of *Loligo duvauceli* ink produces saponins. Saponin compound content is equal to 0.23%. The ethanol extract showed the presence of 2 types of bioactive compounds, namely alkaloids and phenolics, which were 0.02% and 0.72%, respectively. Active compounds are able to counteract activities that adversely affect human health during metabolism [31].

The secondary metabolites detected in the ethanol extract of *L. duvauceli* ink are alkaloids with an alkaloid content of 0.02%. These results showed that alkaloid content in ethanol extract of fresh *Loligo duvauceli* ink was only detected in small amounts. Several factors that allow the low levels of alkaloid compounds in this fresh ink extract are the extraction process and the time used. According to [32] states that the polar compounds present in marine animals, especially invertebrates, are dominated by alkaloids, saponins, steroids and proteins.

Alkaloids are known to exert antioxidant activity through scavenging or chelating processes [33]. According to [34] in the presence of bioactive alkaloid compounds which are antioxidants, it is suspected that extracts are able to reduce the work of cancer-causing free radicals because these compounds can donate one or more electrons to free radicals so that they can counteract the activity of these free radicals.

The levels of phenolic compounds obtained were quite high compared to the other two compounds identified in *Loligo duvauceli* ink extract. The results of [28] showed that the highest bioactive compound in the methanol extract of *Loligo duvauceli* ink was phenolic, with a total phenolic content of 2.65 mg. Phenolic compounds are a group of compounds that have potential as antioxidants [35]. Phenolic acids are major group of primary antioxidants [36].

Saponin compounds in *Loligo duvauceli* ink extract showed levels of 0.23%. Saponins are commonly used for the benefit of humans because saponins have broad activities such as antibacterial, antifungal [37], ability to lower cholesterol in the blood [38] and inhibit tumor cell growth. In addition, according to [39]saponins can be used as drugs, hyperglycemia, antioxidants, anticancer, hypercholesterolemia and anti-inflammation.

3.4. Antioxidant Activity of *Loligo duvauceli* Extract

The results of analysis of antioxidant activity in *Loligo duvauceli* extract showed results of percent inhibition as shown in Figure 2. The results showed that higher concentration of extract, higher percent inhibition obtained. This high percentage of inhibition indicates strong antioxidant activity at a sample concentration of 1000 ppm.

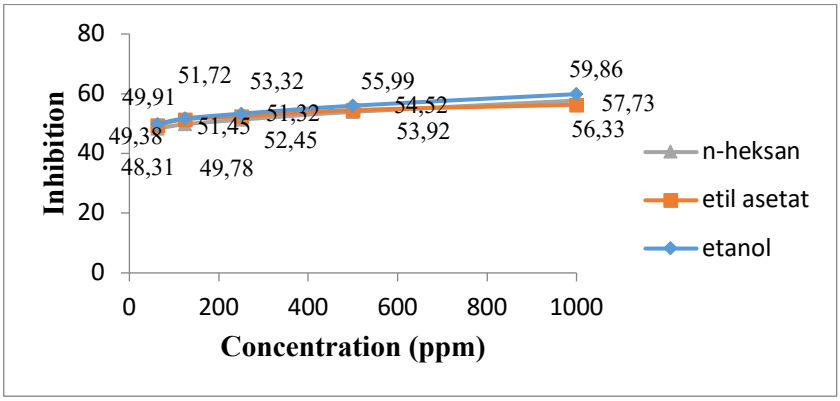

Fig 2. The effect of concentration on the antioxidant activity of *L.duvauceli* extract (n-hexane, ethyl acetate, and ethanol)

The results of analysis of the antioxidant activity of *Loligo duvauceli* extract using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method were then calculated by calculating the IC_{50} value, if the IC_{50}value was low, the antioxidant activity was stronger. IC_{50}calculation results are shown in Table 4.

Table 4. Result of Antioxidant Activity of *Loligo duvauceli* Extract

Solvents	**IC_{50}(ppm)**
n-Hexane	128,35±3,71
Ethyl Acetate	78,81±2,69
Ethanol	75,68±3,89

The ability of these extracts to ward off or reduce DPPH radicals can generally be seen from the change in the intensity of purple color of DPPH solution to a yellow color during testing. This is also in line with the theory put forward by [40] that the stable DPPH free radical compound is purple in color, and when it reacts with antioxidant compounds when incubated for 30 minutes in a sample it will form a yellowish color.

IC_{50} value (Inhibition Concentration), is a parameter used in antioxidant analysis, namely the concentration of the sample extract required to scavenge 50% of DPPH free radicals. The IC_{50} value was obtained from a linear regression equation which states the relationship between the concentration of the test extract and the percent inhibition [41].

According to [42] that free radical scavenging activity is determined from the IC_{50} value. The smaller the IC_{50} value means the stronger the antioxidant activity of an extract. Specifically, an extract is said to have very strong antioxidant compounds if the IC_{50} value is less than 50 ppm (IC_{50} <50 ppm), strong (50<IC_{50} <100 ppm), moderate (100<IC_{50} <150 ppm), weak (150 ppm<IC_{50} <200 ppm), and very weak IC_{50} > 200 ppm). Based on this, the antioxidant activity of the ethanol extract and ethyl acetate extract of *L.duvauceli* ink had

strong antioxidant activity, while the antioxidant activity of the n-hexane extract showed moderate antioxidant activity.

Ethanol extract has the smallest IC_{50} value but provides the highest effectiveness in reducing DPPH radicals. The strong antioxidant activity of the ethanol extract is due to the presence of certain secondary metabolic compounds contained therein. In the ethanol extract, the high effectiveness of antioxidant activity is thought to be due to the presence of alkaloids and phenolic compounds.

The high level of total phenol in the ethanol extract is in line with the strong antioxidant activity in the ethanol extract. The strong scavenging ability is related to the hydroxyl groups present in phenolic compounds [43]. The strong antioxidant activity of the ethanol extract of *Loligo duvauceli* ink has a positive correlation to the total phenol content. This can be seen from the identification of phenolic compounds and reinforced by the relatively high yield of total phenols. [44] stated that phenolic as a secondary metabolite has potential as an antioxidant, due to the presence of compounds with hydroxyl groups in phenolic compounds.

According to the research results of [7] using squid ink powder (*Loligo* sp.), the percent inhibition value shows a fairly high number in the ethanol extract compared to the n-hexane extract, namely ethanol (67.57%), and n-hexane (2.10%) with a greater percentage of ethanol inhibition than the n-hexane extract, it is known that the ethanol extract has high antioxidant activity as well. The study showed higher antioxidant activity of *Loligo* sp. powder extract. than fresh *L.duvauceli* extract which showed the highest inhibition percentage, namely 59.86% in ethanol extract.

The another study from [8] showed radical scavenging activity of *Loligo formosana* ink extract free of melanin content or without melanin content (MFI) was 179.6 ± 2.1%. [8] stated that squid ink has a black pigment containing melanin, the presence of melanin may limit the application of bioactive compounds as antioxidants, as evidenced by the test results, the high percentage of inhibition indicates high antioxidant activity in the *Loligo formosana* ink extract without melanin, compared to fresh *L.duvauceli* ink extract (Table 4.).

According to research by [45] *Loligo duvauceli* ink extract without melanin content (MFI), showed an antioxidant activity of 63.21% at a concentration of 50 g/ml. The research by [46] showed ethanol extract of *Loligo vulgaris* ink free of melanin (MFI) showed an antioxidant activity of 62.7% at a concentration of 50 g/ml. The high percentage of inhibition in both extracts indicated high antioxidant activity in both *L.duvauceli* and *L.vulgaris* ink extracts without melanin compared to fresh *L.duvauceli* ink extract.

Research [47] regarding the antioxidant activity of cuttlefish ink (*Sepia officinalis*), showed antioxidant activity results of 88.32% to 50.36% in the concentration range of 10 to 80 mg/ml. Melanin-free ink (MFI) from *Sepia prebahari* cuttlefish also has antioxidant activity [48]. This shows that inks from various marine mollusk animals have antioxidant activity, both in the presence of melanin content and without melanin content.

The presence of antioxidant activity in *Loligo duvauceli* ink extract is due to the presence of secondary metabolics contained in *Loligo duvauceli* ink such as alkaloids, steroids, saponins and phenolics. In addition, according to [49] Squid ink polysaccharides also have antioxidant abilities from the ability to capture samples with DPPH radicals.

4. Conclusions

The secondary metabolics contained in each extract show that ethanol solvent (polar) attracts more bioactive compounds. Such as, phenolic, alkaloid, saponin, ans steroid. The antioxidant

activity of *Loligo duvauceli* ink extract shows that the ethanol extract has a stronger antioxidant activity than other extracts, which is equal to 75.68 ppm.

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