Antioxidant activity of biopigment fractions from golden apple snail eggs (*Pomacea canaliculata*)

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Abstract. Biopigment is a natural non-toxic, renewable and environmentally friendly dye. The golden apple snail eggs is known to contain carotenoid groups such as *astaxanthin* pigment. The purpose of this study was to determine the antioxidant activity of biopigment from acetone and methanol fractions with various in vitro test. Biopigment extracted with acetone had the highest antioxidant activity with IC₅₀ DPPH method of 130.52±3.07 μg/mL and ABTS of 125.89±1.70 μg/mL. The result of total antioxidant capacity with FRAP method of 184.11±3.92 μmol Fe²⁺/g extract and CUPRAC method of 29.17±3.58 μg/mL ascorbic acid/g extract. The second fraction from acetone solvent was the best fraction with IC₅₀ DPPH method of 92.08±2.85 μg/mL and ABTS of 144.80±3.33 μg/mL. The result of total antioxidant capacity with FRAP method of 259.11±3.93 μmol ascorbic acid g⁻¹ extract. Identification with LC-MS/MS on the second fraction found *astaxanthin* pigment and five other pigments.

Keywords: *astaxanthin*, carotenoid, LC-MS/MS, *Pomacea canaliculata*

1. Introduction

Golden Apple Snail (*P. canaliculata*) is one of the introduced animals from South America. The spread of golden apple snail in Indonesia occurred in 1981 as an animal in the aquarium. The introduction of the golden apple snail in Indonesia has caused various problems, one of which is to become pests in plants. The golden apple snail egg has pink characteristics and is clustered like a mulberry. Pink colour from golden apple snail eggs turned out to have a fairly high pigment content. Abdullah *et al* (2017) stated that the golden apple snail egg extract with acetone and methanol as solvents contained various kinds of pigment and non-pigment active compounds.

Biopigment or natural dyes do not have toxic effects, renewable, environmentally friendly and easily degraded. This source of natural dyes can be obtained from plants, animals, and microorganisms (Pujilestari 2015). The natural pigment from golden apple snail eggs was the carotenoid group. This carotenoid group was a chemical compound (yellow, orange and red). The carotenoid pigment content in golden snail eggs is 313.48±19.73 ppm (Ameliawati 2013). The carotenoid pigment has benefits as compounds that have antioxidant activity (Fasset and Coomers 2011). Antioxidants from natural ingredients are currently interesting to develop. Sources of antioxidants from natural ingredients are
safer to use to prevent various causes of chronic diseases in humans (Himaja et al. 2010). Identification of active compounds with LC-MS/MS shows that golden apple snail eggs contain active compounds of carotenoid pigments and non-pigment compounds. One of the potential pigment compounds is the astaxanthin compound (Abdullah et al. 2017). Astaxanthin compounds have biological activity as antioxidants that are beneficial to human and animal metabolism (Ambati et al. 2014).

Miki (1991) stated that the antioxidant properties of astaxanthin ten times better than β-carotene, zeaxanthin, lutein, and canthaxanthin compounds. These antioxidant compounds can provide protection against various kinds of diseases for humans and animals. Chien (1996) mentions the administration of astaxanthin in shrimp farming can increase shrimp survival to 77% compared to administration of beta-carotene which only increases 44% survival in shrimp. Astaxanthin can also inhibit the generation of Reactive Oxygen Species (ROS) in neuroblastoma cells in humans (Liu and Osawa 2007). Moreover, astaxanthin has important benefits for human health such as improving skin health, LDL cholesterol oxidation inhibitors, reducing eye fatigue, suppressing the development of obesity, atherosclerosis, diabetes, hypertension, and hyperlipidemia (Yamashita 2013). Astaxanthin can also overcome the problem of inflammatory disease, cardiovascular disease, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (Yang et al. 2013).

The identification of the active component in the golden apple snail eggs pigment extract with acetone solvent treatment contained alkaloids, flavonoids, steroids, and triterpenoids, while the treatment of methanol solvent contained alkaloids and saponins. Identification of active compounds with LC-MS/MS found that methanolic extract of 11 carotenoid pigments in the xanthophyll group and 2 carotenoid pigments in the carotene group in acetone extract there were 11 carotenoid pigments in the xanthophyll group. One of the pigment compounds found is the astaxanthin compound (Abdullah et al. 2017). Research on the natural pigment of golden apple snail eggs and their antioxidant activity has never been done. Therefore, the purpose of this study is to determine the antioxidant activity of golden apple snail eggs extract and determine the best natural pigment fraction between acetone and methanol solvents to be used as antioxidants by various in-vitro tests.

2. Materials and methods

2.1. Materials
The main materials used in this study were golden apple snail eggs (P. canaliculata) obtained from rice fields in Situ Gede Village, West Bogor, Bogor City and Cultivation Experiment Ponds, Department of Aquaculture, Fisheries and Marine Sciences, IPB University. Golden apple snail eggs were prepared by cleaning up the dirt that sticking using flowing water. Golden apple snail eggs chopped and dried for 24 hours at 50°C. The dried golden apple snail eggs mashed using a mortar, thus the fine powder form obtained. The materials used for extraction were distilled water, acetone (Merck, Germany) and methanol (Merck, Germany), and dimethyl sulfoxide (DMSO) 10% (Merck, Germany). The material used for testing the antioxidant activity of ascorbic acid (Merck, Germany), DPPH (2,2-diphenyl-1-picrylhydrazul) (Sigma-Aldrich), FRAP reagent (mixture of 300 mM acetate buffer solution, TPTZ solution in 40 Mm HCl, FeCl3,6H2O 20mM), FeSO4·7H2O, ethanol 99.9%, CuCl2·2H2O 0.01 M, ethanolic neocuproine 0.0075 M, ammonium acetate buffer 1 M (pH 7) and ABTS solution.

The equipment used in this study included analytic scales (Sartorius TE64, Germany), oven (Yamato DV-41), Vortex tool, water-bath, orbital shaker (Wisd SHO-1D), UV-Vis spectrophotometer, UV light, Chromatography column, Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS): Ultra-high Performance Liquid Chromatography (UPLC) (Acquity ACCQ-Tag Ultra C18 Waters), Mass spectrometry (MS) (Xevo G2-S QTof), column acquity UPLC (HSS C18 1.8 μm (2.1 x 150 mm).

2.2. Methods
2.2.1. Pigment extraction and evaporation. Golden apple snail egg powder weighed as much as 70 g then immersed with DMSO 10% 1:2 (w/v) solution while stirring with a spatula for 10 minutes. The sample is then filtered and the filtrate is taken. The DMSO filtrate was added with acetone and methanol solvents each with a ratio of 1:8 (w/v), after which pigment was extracted (modified Seely et al 1972). The extraction method used was maceration with the orbital shaker at 30°C for 50 minutes, 150 rpm. The extraction was then evaporated at 30°C using a vacuum rotary evaporator so that the concentrated pigment extract in the form of an orange paste which was dissolved and weighed was obtained.

2.2.2. Bio-pigment fractionation. The sample extract was fractionated to separate the components by column chromatography method. Column chromatography is one of the purification techniques to isolate the desired compound in a mixture. Column chromatography used a stationary phase (solid adsorbent) as silica gel and the mobile phase (liquid adsorbent) as eluent. Separation by column chromatography is carried out vertically where the stationary phase and mobile phase are inserted from the top of the column and move down through the column.

3. Results and discussion

3.1. Characteristics of natural pigment extract of golden apple snail (P. canaliculata)

Water content testing was carried out on golden apple snail powder with the obtained value of 0.56%. The percentage of water content golden apple snail powder indicates that the water content is relatively low. Salim et al (2016) explained that the permissible threshold for water content of Simplicia extract is below 10%. The results of the percentage of water content which is relatively low in the study because the sample has been given oven drying treatment and smashed into powder form so that the water content in the sample becomes lower. Extraction in the study used samples in the form of dry powder. This aims to increase the surface area so that the penetration power of the solvent into the sample cell is better (Walker 2006). Extraction begins with a pre-treatment of 10% DMSO immersion which has the purpose of denaturing proteins, penetrating cell walls and entering into cell cavities containing active compounds, so that there is a difference in concentration between the solution of active compounds in cells with outside cells, concentrated solutions in cells can be pushed out (Seely et al 1972). There were two solvent treatments used in extraction, namely acetone and methanol with ratio sample:solvent (1:8). Comparison of samples with optimal extraction solvents will produce good yields (Voight 1994). The results of the characteristics crude bio-pigment extract of golden apple snail egg with the treatment of two different solvents can be seen in table 1.

| Solvent Type | Yield(%) | Yield(%)* | Colour | Colour* | Form | Form* |
|--------------|----------|-----------|--------|---------|------|-------|
| Acetone      | 3.25±0.59| 33.80     | Orange | Orange  | Paste| Concentrate |
| Methanol     | 7.39±1.81| 36.10     | Brown orange | Orange | Paste| Concentrate |

Note : *Abdullah et al (2017)

The results showed that the highest yield was found in methanol (polar) solvent treatment. This is thought to be related to the characteristic of golden apple snail eggs tends to dissolve into polar solvents as well. The colour of the extracts in acetone solvents was orange while methanol solvents were brown-orange. The characteristic form of the extract-treated two solvents in the same form. Abdullah et al (2017) stated that the yield of golden apple snail egg’s natural pigment extract with methanol treatment has a value of 36.10% higher than acetone solvent with a value of only 33.80% with the characteristic of snail egg pigment extract in orange in the form of concentrate.

The difference of the extract’s characteristic from the research with the literature is due to the difference of the ratio 10% DMSO to the pre-extraction treatment. Abdullah et al (2017) states that 10% DMSO
used is 1:4 while this research uses 1:2. This indicates that a smaller ratio of 10% DMSO can produce fewer extracts and more extracted paste forms. The results also showed that the maceration extraction treatment with methanol solvent could produce higher yields of than acetone solvents.

3.2. Active components bio-pigment extract of golden apple snail (\textit{P. canaliculata})

Qualitative phytochemical test results showed that crude golden apple snail acetone solvent treatment had active compounds namely alkaloids, flavonoids, triterpenoids, and saponins. The treatment of methanol solvents has active compounds namely alkaloids, flavonoids, steroids, triterpenoids, phenols, tannins, and saponins. This qualitative result shows that crude bio-pigment extract with methanol solvent has more active compounds than crude bio-pigment extract with acetone solvent.

Abdullah \textit{et al} (2017) explained that crude snail natural pigment extract with acetone solvents had active compounds namely alkaloids, flavonoids, steroids, triterpenoids, and saponins while methanol solvents were alkaloids and saponins. The difference in the results of this test is allegedly due to the influence of the pre-extraction treatment using 10% DMSO. Abdullah \textit{et al} (2017) stated that the 10% DMSO used was 1:4 while this study used 1:2. This indicates that the snail egg natural pigment extract is better using a DMSO 10% 1:2 ratio to obtain more active compounds in the methanol solvent treatment.

3.3. Pigment component of crude extract golden apple snail in qualitative

Natural pigment’s components of crude extract golden apple snail with TLC showed that there were two spots in each treatment. The spot was identified as a xanthophyll, carotenoid pigment group with Rf value for acetone treatment 0.10 cm (spot 1) and 0.34 cm (spot 2) and methanol treatment 0.13 cm (spot 1) and 0.40 cm (spot 2). Britton \textit{et al} (1995) said the value of carotene Rf was 0.88 cm and xanthophyll was 0.10-0.30 cm in acetone: n-hexane (5:95). Heriyanto and Limantara (2006) stated the range of carotene Rf (orange) values was 0.87-0.93 cm while xanthophyll (yellow) was 0.26-0.34 cm and (orange) was 0.17-0.23.

3.4. Characteristics of the fractions golden apple snail egg

Fractionation of crude extract golden apple snail resulted in different amounts of colour fraction between acetone and methanol solvents. The number of colour fractions obtained in this study were five fractions for acetone samples. Parameters of the fraction characteristics observed were yield and colour. The characteristic results of the acetone treatment extraction fraction can be seen in table 2.

| Acetone Fraction | Yield (%) | Colour     |
|------------------|-----------|------------|
| 1                | 29        | Dark Orange|
| 2                | 10.25     | Light Orange|
| 3                | 30.71     | Light Yellow|
| 4                | 14.03     | Transparent|
| 5                | 16.01     | Light Orange|

Acetone fraction obtained as many as five different fractions. The highest percentage of yield was found in acetone fraction 3 and the lowest percentage of yield was found in acetone fraction 2. The colour of each fraction varied from clear, faded orange, concentrated orange and clear yellow. The existence of these colour differences can be analyzed that the acetone fraction of golden snail egg bio-pigment extract contains carotenoid pigments in the xanthophyll (yellow) and carotene (orange) groups. Heriyanto and Limantara (2006) say that carotenoids can be divided into two main groups, namely polar carotenoids (xanthophyll) and non-polar carotenoids (carotenes). The carotene group is orange while the xanthophyll is yellow. The amount of methanol extract fraction obtained in this study were six colour fractions. Parameters of the fraction characteristics observed were yield and colour. The characteristics of the methanol extract fraction can be seen in table 3.
Table 3. Characteristic of methanol fraction golden apple snail egg’s natural pigment.

| Methanol Fraction | Yield Percentage (%) | Colour               |
|-------------------|----------------------|----------------------|
| 1                 | 20.93                | Transparent          |
| 2                 | 3.32                 | Transparent Yellow Green |
| 3                 | 7.54                 | Dark Orange          |
| 4                 | 45.48                | Light Orange Yellow  |
| 5                 | 14.79                | Light Orange         |
| 6                 | 7.94                 | Transparent Yellow   |

Methanol fraction obtained as many as six different fractions. The highest percentage of yield was found in the methanol fraction 4 and the lowest percentage of yield was found in the methanol fraction 2. The colour of each fraction varied from clear, rather yellowish to clear, faded orange, concentrated orange and clear to slightly green-yellow. These colour differences can be analyzed that the methanol fraction of golden snail pigment extract contains carotenoid pigments in the xanthophyll (yellow) and carotene (orange) groups. Heriyanto and Limantara (2006) say that carotenoids can be divided into two main groups, namely polar carotenoids (xanthophylls) and non-polar carotenoids (carotenes). The carotene group is orange while the xanthophyll is yellow.

This study shows that acetone fraction has a smaller amount of colour fraction but the resulting colour is better than the methanol fraction which has a greater number of colour fractions. The different colours of acetone and methanol fraction are influenced by the solvent's ability to dissolve the compounds present in the sample. Acetone solvent is one solvent that has good solubility in dissolving carotenoid pigment compounds (Britton et al 1995). Methanol solvents can dissolve compounds that are universal (polar and non-polar) contained in the sample (Salamah and Widyasaari 2015).

3.5. Qualitative pigment components

Analysis with Thin Layer Chromatography (TLC) is one way to find out what compound components are found in the fraction. Test results of TLC of acetone fraction and methanol of crude extract golden apple snail showed that there were several spots on each fraction result. The spot in the acetone fraction was identified as a group of carotenoid pigments in the xanthophyll and carotene groups. Rf value data on spot acetone fraction can be seen in table 4. The spot in the methanol fraction was identified as a group of carotenoid pigments in the xanthophyll, carotene and two fractions no spots were found. Rf value data on spot acetone fraction can be seen in table 4.

Table 4. Rf value of acetone and methanol fraction golden apple snail egg’s natural pigment.

| Acetone fraction | Rf value (cm) | Methanol fraction | Rf value (cm) |
|------------------|--------------|------------------|--------------|
| 1                | 0.15         | 1                | 0.60, 0.79, 0.90 |
| 2                | 0.11         | 2                | 0.90         |
| 3                | 0.11         | 3                | 0.87         |
| 4                | 0.88         | 4                | 0.42         |
| 5                | 0.87         | 5                | -            |
| -                | -            | 6                | -            |

The results of this study indicate that pure astaxanthin pigment is thought to be in acetone fraction 1, 2, 3 and methanol fraction 4 because the Rf value of each fraction belongs to the xanthophyll carotenoid pigment. Ciapara et al (2006) explained that the astaxanthin pigment is a type of xanthophyll carotenoid found in microorganisms and marine animals. Britton et al (1995) stated that the value of carotene Rf was 0.88 and xanthophyll was 0.10-0.30 in acetone: n-hexane (5:95). Golkhoo et al (2007) also stated
that standard astaxanthin has an Rf value of 0.27 cm. Another study stated that free astaxanthin has an Rf value of 0.33 cm (Lorenz 1998).

3.6. Antioxidant activity of golden apple snail egg (P. canaliculata) DPPH and ABTS methods

The DPPH method is used in studying antioxidant activity in biological compounds and their ability to reduce DPPH free radical activity. This activity is measured by decreasing the absorbance value of the sample (Vasic et al 2012). The DPPH reaction takes place through a mechanism of hydrogen donation where DPPH compounds are captured by antioxidant compounds which release hydrogen radicals to form reduced DPPH-H.

The ABTS method is an antioxidant test method with its mechanism of action to react between oxidizing agents (potassium persulfate) and ABTS salts. Radical reduction of ABTS solution which is greenish-blue because of the administration of hydrogen antioxidant which is measured at a wavelength of 734 nm. This method is very flexible because it can be used at different pH levels. ABTS solutions can also dissolve in water and organic solvents on different media. The advantage of this method is that the sample reaction is very fast with the ABTS reagent because it can be stable within 30 minutes (Shalaby and Shanab 2013). Antioxidant testing using the DPPH and ABTS method showed that the difference in the solvent polarity of the extract did not affect the IC\textsubscript{50} value of the crude golden apple snail egg pigment extract. The smaller the IC\textsubscript{50} value the higher the antioxidant activity (Molyneux 2004). The IC\textsubscript{50} test results on crude pigment extracts using DPPH and ABTS methods can be seen in Table 5.

| Sample            | IC\textsubscript{50} (μg/mL) | DPPH       | ABTS       |
|-------------------|-------------------------------|------------|------------|
| Acetone extract   | 130.52±3.07                   | 125.89±1.70|            |
| Methanol extract  | 133.76±0.18                   | 169.45±0.91|            |
| Ascorbic acid     | 1.41±0.02                     | 3.89±0.11  |            |

The results showed that the IC\textsubscript{50} value of golden apple snail egg extract was higher than IC\textsubscript{50} ascorbic acid. Ascorbic acid has an IC\textsubscript{50} value which is included in the category of very strong antioxidants in the DPPH and ABTS methods. The acetone extract has an IC\textsubscript{50} value which is included in the moderate antioxidant category in the DPPH and ABTS method. The methanol extract has an IC\textsubscript{50} value which is included in the moderate antioxidant category in the DPPH method and is weak in the ABTS method. This shows that the acetone extract has better antioxidant activity in the DPPH and ABTS method while the methanol extract has better antioxidant activity on the DPPH method. Molyneux (2004) explains that antioxidants are very strong if the IC\textsubscript{50} value is less than 50 ppm, strong if the IC\textsubscript{50} value is between 50-100 ppm, medium if the IC\textsubscript{50} value ranges from 100-150 ppm and weak if the IC\textsubscript{50} value ranges from 150-200 ppm.

The IC\textsubscript{50} value with the DPPH method in the fraction shows that the difference in solvent fraction polarity has an effect (P <0.05) on the IC\textsubscript{50} value of the golden apple snail egg fraction. Fraction acetone 2 and acetone 1 have a significant effect on the IC\textsubscript{50} value. Fraction acetone 3 and methanol 4 have no significant effect on the IC\textsubscript{50} value. IC\textsubscript{50} value DPPH method acetone fraction 2> acetone fraction 1> methanol fraction 4> acetone fraction 3. The IC\textsubscript{50} value with the ABTS method in the fraction showed that the difference in fraction solvent polarity had an effect (P <0.05) on the IC\textsubscript{50} value of the golden apple snail egg fraction. Fraction acetone 2 and methanol 4 have a significant effect on the IC\textsubscript{50} value. Fraction acetone 1 and acetone 3 have no significant effect on the IC\textsubscript{50} value. IC\textsubscript{50} value of the ABTS method of acetone fraction 2> acetone fraction 1> acetone fraction 3> methanol fraction 4. IC\textsubscript{50} value from the antioxidant activity test on the fraction of mas snail egg pigment using ABTS method can be seen in Table 6.
The results showed that the fraction acetone 1, acetone 3 and methanol 4 with the ABTS method had a lower IC₅₀ value compared to the DPPH method while the acetone fraction 2 with DPPH method had a lower IC₅₀ value than the ABTS method. This shows that the acetone fraction 1, acetone 3 and methanol 4 have better antioxidant activity in the ABTS method while the acetone fraction 2 eggs of golden snail have better antioxidant activity on the DPPH method. IC₅₀ value of Fraction acetone 2 was lower than crude golden apple snail egg’s natural pigment and other fractions. The acetone 2 fraction has a characteristic faded orange colour and the yield is the least compared to the other fractions. This shows that the results of fractionation with acetone (semi-polar) solvents have antioxidant activity that is better than crude extracts of golden apple snail egg’s natural pigment. Gori et al (2016) stated that the results of fractionation of C. incanus leaf extract with ethyl acetate (semi-polar) solvents had a lower IC₅₀ value compared to crude’s natural pigment extract of Cistus incanus leaf with methanol (polar) solvent and fractionated with water (polar).

3.7. FRAP method
The FRAP method is one of the antioxidant test methods with a working mechanism to reduce iron (III) -tripiridyl-triazine compounds to iron (II) -tripiridil-triazine at pH 3.6 (Chanda and Dave 2009). FRAP reagents are blue and can be absorbed at a wavelength of 593 nm. The advantage of this FRAP method is that the method is cheap, the reagents are easy to prepare and quite simple and fast (Benzie and Strain 1996). FRAP testing results of crude golden apple snail natural pigment’s extract showed that the difference in the polarity of the solvent had a significant effect (P <0.05) on antioxidant capacity (reduction power). Acetone extract has a higher antioxidant capacity (reduction power) with a value of 184.1±3.92 μmol Fe²⁺/g extract compared to methanol extract with a value of 78.55±3.93 μmol Fe²⁺/g extract. Britton et al (1995) stated that acetone solvents have good solubility in carotenoid compounds so they can extract more compounds. More non-polar solvent and longer extraction time can increase total carotenoids of the sample (Wahyuni and Widjanarko 2015).

The FRAP test results in the fraction showed that the difference in fraction solvent polarity had an effect (P <0.05) on antioxidant capacity (reduction power). Acetone 1 and acetone 3 fraction gave no significant effect on antioxidant capacity (reduction power) of the golden apple snail egg fraction. Acetone 2 and methanol 4 fraction gave a significant effect on antioxidant capacity (reduction power). The antioxidant capacity of acetone 1 fraction was 134.1±3.92 μmol Fe²⁺/g extract, acetone 2 fraction was 259.1±3.93 μmol Fe²⁺/g extract, acetone 3 fraction was 128.55±3.93 μmol Fe²⁺/g extract and methanol 4 fraction of 56.33±3.93 μmol Fe²⁺/g extract. Antioxidant capacity (reduction power) of acetone fraction 2 > acetone fraction 1 > acetone fraction 3 > methanol fraction 4. Britton et al (1995) stated that acetone solvents have good solubility in carotenoid compounds so they can extract more compounds. More non-polar solvent and longer extraction time can increase total carotenoids of the sample (Wahyuni and Widjanarko 2015).

### Table 6. IC₅₀ value crude extract of golden apple snail egg fraction.

| Sample                  | DPPH (μg/mL)   | ABTS (μg/mL)   |
|-------------------------|----------------|----------------|
| Acetone Fraction 1      | 162.97±2.57ᵃ   | 156.18±3.90ᵃ   |
| Acetone Fraction 2      | 92.08±2.85ᵇ    | 144.80±3.33ᵇ   |
| Acetone Fraction 3      | 173.05±2.50ᶜ   | 159.37±1.33ᵃ   |
| Methanol Fraction 4     | 172.85±1.97ᶜ   | 171.95±2.22ᶜ   |
| Ascorbic Acid           | 1.41±0.02      | 3.89±0.11      |

Note: The numbers on the results followed by different letters superscripts (a, b, c) show significantly different results (p <0.05)
Antioxidant capacity (reduction power) in FRAP testing is also influenced by the presence of flavonoids in samples that can help the process of reducing metal ions such as iron (Benavente et al. 1997). This is related to the results of the golden apple snail egg phytochemical testing which proves that golden apple snail eggs have an active compound of flavonoids. Components that have reduced power indicate that the component can be an electron donor and can reduce fat oxidation in the peroxidation process (Yen and Chen 1995). The more concentration of Fe$^{3+}$-TPTZ reduced by the sample to Fe$^{2+}$-TPTZ, the greater antioxidant activity of the sample (Istiningrum 2013).

3.8. CUPRAC method
The CUPRAC method is an antioxidant test method with its mechanism of action copper (II)-neocuproine (Nc) as a CUPRAC reagent that effectively oxidizes antioxidant samples and is reduced to bis-neocuproine-copper (I). The advantages of the CUPRAC method are faster colour development, stable and inexpensive reagents needed, and simpler methods (Apak et al. 2005). The results of the CUPRAC testing of crude golden apple snail egg bio-pigment extract showed that the difference in the polarity of the given solvent had no effect ($P > 0.05$) on the antioxidant capacity (reduction power). Acetone extract has a higher antioxidant capacity (reduction power) than methanol extract. Antioxidant capacity of acetone crude extract is 29.17±3.58 μg/mL. Acetone acid/g extract and methanol crude extract is 28.45±2.88 μg/mL. Ascorbic acid/g extract. Britton et al. (1995) stated that acetone solvents have good solubility in carotenoid compounds so they can extract more compounds. Non-polar solvent and longer extraction time can increase total carotenoids of the sample (Wahyuni and Widjanarko 2015).

Antioxidant capacity (reduction power) in CUPRAC testing is influenced by the presence of flavonoids in samples that can help reduce the metal ion such as copper (Benavente et al. 1997). This is related to the results of the golden apple snail egg phytochemical testing which proves that golden snail eggs have an active compound of flavonoids. Components that have reduced power indicate that the component can be an electron donor and can reduce fat oxidation in the peroxidation process (Yen and Chen 1995).

3.9 Active compound biopigment fraction of golden apple snail egg in semi qualitative
Structural identification and characterization of a sample can be done using LC-MS/MS (Mayumi et al. 2006). Liquid Chromatograph tandem Mass Spectrometry (LC-MS/MS) is a LC technique that has a mass spectrometer detector. The final result of this identification is to know the name of the compound based on the molecular formula obtained through LC-MS/MS. The compounds that are the main focus of this analysis are pigment compounds. The results of the identification of LC-MS/MS pigment compounds in the second acetone fraction can be seen in table 7.

| Number | Retention time | Molecule formula | Molecule mass (m/z) | Molecule mass* (m/z) | Compound name | Percent identity (%) |
|--------|----------------|------------------|--------------------|----------------------|---------------|---------------------|
| 1      | 5.03           | C_{42}H_{50}O_{2} | 597.4656           | 596.94               | Spirilloxanthin | 9.74                |
| 2      | 9.02           | C_{40}H_{52}O_{2} | 597.3969           | 596.85               | Meso-Astaxanthin| 54.09               |
| 3      | 9.02           | C_{40}H_{52}O_{2} | 597.3901           | 564.85               | Cantaxanthin    | 5.07                |
| 4      | 9.89           | C_{40}H_{52}O_{2} | 569.4321           | 568.86               | Lutein         | 10.20               |
| 5      | 9.89           | C_{40}H_{52}O_{2} | 569.4321           | 568.89               | Zeaxanthin      | 10.20               |
| 6      | 12.01          | C_{40}H_{52}O_{2} | 597.3963           | 596.84               | Astaxanthin     | 78.17               |

Note: *online source database (www.chemspider.com)
The compounds that are the main focus of this analysis are astaxanthin pigment compounds. The identification results of LC-MS/MS in the second acetone fraction showed the presence of astaxanthin pigment compounds. The astaxanthin compound was found at a retention time of 12.01. The readable molecular mass is 597.40. The retention time for astaxanthin compounds in this study was lower than in previous studies. The results of identification of LC-MS/MS crude bio-pigment extract of golden apple snail egg showed that the astaxanthin compound was found at a retention time of 13.46 with a molecular mass of 597.40 (Abdullah et al 2017).

The difference in the retention time indicates that the form of sample used affects the results of the LC-MS/MS chromatogram. The lower retention time indicates that the sample matrix is clean and not too much impurity (Pan et al 2012). Validation of the molecular mass of the astaxanthin compound found compared to the molecular mass of Chemspider (www.chemspider.com). The difference in molecular mass is due to analysis with LC-MS/MS with ionization techniques usually producing molecular ions ([M + H]+ or [M + H]−). The ion charge produced cannot be ascertained because it depends on several factors such as analyte chemical properties, ESI voltage polarity, matrix properties, and solvent composition (Halket 2005).

4. Conclusion

To conclude, the acetone fraction number 2 had higher antioxidant activity compared to other fractions with IC50 values of the DPPH method of 92.08±2.85 μg/mL, the ABTS method of 144.80±3.33 μg/mL and FRAP antioxidant capacity was 259.11±3.93 μmol ascorbic acid/g extract. Semi-qualitative identification using LC-MS/MS found the acetone fraction number 2 contained astaxanthin pigment and five other pigments.

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