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Hypoglycemic Effect of Antioxidant Astaxanthin from Vaname Shrimp Waste Extract Using Soybean Oil in Streptozotocin-Induced Mice (*Mus Musculus*)

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Abstract **Original Research Article**

Vaname shrimp (*Litopenaeus vannamei*) waste contains powerful biological compound called astaxanthin which has high antioxidant activity compared to other carotenoids pigment or α-tokopherol. In this study, the vaname shrimp waste were extracted using soybean oil and was used to treat streptozotocin-induced diabetic mice. The astaxanthin of vaname shrimp waste extract was counted using spectrophotemer and the antioxidant activity was determined by using DPPH method. The astaxanthin concentration was found highest in pigmented oil phase with the amount of 209.616 ppm and the IC50 value of the pigmented oil phase was 122.744 ppm. Hypoglycemic effect was evaluated in streptozotocin-induced mice which were devided into 5 groups and received different oral treatment in each group (glibenclamide in dose 5 mg/bw, aquadest 0.2 ml and astaxanthin in dose of 10, 15 and 20 mg/bw). The blood glucose levels were examined every 2 days for 14 days. The treatment of astaxanthin from vaname shrimp waste with dose 20 mg/bw showed remarkable fall on blood glucose in streptozotosin-induced diabetic mice, while a slight fall can be seen in dose 10 and 15 mg/kg bw. Therefore, these result present that vaname shrimp waste extract contained high astaxanthin and had high antioxidant activity which can be effectively used to reduce blood glucose in streptozotocin-induced diabetic mice.

Keywords: astaxanthin, hypoglycemic, soybean oil, streptozotocin, vaname shrimp waste.

1. INTRODUCTION

Indonesia is the largest archipelagic country. Shrimp is the main commodity export in the fisheries sector because of its economic and nutritional value. Central Bureau of Statistics Indonesia (BPS) (2020) stated that the shrimp export rate in Indonesia each year has increased by about 6%, where the exported shrimps are headless or skinless and about 60-70% of the shrimp weight becomes waste. Small quantity of the shrimp waste in Indonesia is mostly being used as animal feed, manure or shrimp paste. Thus, the large amount of shrimp waste leftover become problematic for the environment. Shrimp waste is a natural source which has rich carotenoids. Shrimp waste contains some major components such as 35-50% protein, 15-25% chitin, 10-15% minerals and carotenoid pigments (Sachindra *et al*., 2006). The optimal processing of shrimp waste would not only minimize the pollution problem but also increase the economy value and in addition would assist to separate precious components such as astaxanthin. Astaxanthin (3, 3-dihydroxy-β,β-carotene-4,4-dione) is red-orange carotenoid pigment, oxidized form of β-carotene being liable and widely distributed in crustacean waste such as crabs, lobster and shrimp (Silva *et al*., 2018). Astaxanthin is oil soluble pigment, with that being said, several attempts had been done to isolate astaxanthin from crustaceans waste using several animals oils such as menhaden, herring and salmon oil and vegetable oil such as sunflower oil, soybean oil, groundnut oil and palm oil (Chen and Meyers, 1982; Sachindra and Mahendrakar, 2005; Handayani 2008).

Antioxidant activity of astaxanthin is reported approximately 500 times higher than α-tocopherol and about 10 times higher compared to other carotenoids (zeaxanthin, lutein, canthaxanthin and β-caroteneand) (Naguib, 2000). Due to its high antioxidant activity, astaxanthin can be applied for various health sector such as enhance immune system, treat cardiovascular, hypertension, anti-cancer, anti-diabetes (Higuera *et al*., 2006). Lately, various studies have suggested that free radicals caused an oxidative stress which can trigger excess production of blood glucose in the body, resulting in various tissues and cells body damage. The antioxidant ability of astaxanthin to treat diabetes have been reported in numerous studies. Astaxanthin has

shown to improve the progression and acceleration of diabetic nephropathy in type 2 diabetic mice (Naito *et al*., 2004). Furthermore, astaxanthin is proven effectively in reducing oxidative stress in pancreatic cells caused by oxidative stress and in addition astaxanthin also able to lower blood sugar by improving insulin sensitivity and glucose intake (Feng, 2020; Uchiyama *et al*., 2002).

In this study we used soybean oil to extract astaxanthin due to its availability in Indonesia. Aside from that, soybean has approximately 85% of high fatty and long carbon chains which can affect in its hydrophobic ability (Winarsi, 2010). Aside from soybean oil has high availability in Indonesia. Taken together, we suppose that antioxidant activity of astaxanthin from vaname shrimp waste extract using soybean oil could reduce blood glucose level in diabetic mice. Therefore, the aim of this study was to evaluate the hypoglycemic effect of antioxidant astaxanthin from vaname shrimp waste extract using soybean oil in streptozotocin-induced mice and to determine important compounds from vaname shrimp waste extract.

2. MATERIALS AND METHOD

2.1 Preparation of Shrimp Waste

Litopenaeus vannamei shrimp waste was collected from a local fishery at Red-One Fisheries Jogja and transported to the laboratory under clean condition and repeatedly washed under flowing water, then dried. The dried shrimp waste were mashed and filtred using a 60-mesh siever.

2.2 Extraction and Determination of Astaxanthin Content

Astaxanthin in dried shrimp waste was extracted according Sachindra and Mahendrakar, (2005) using soybean oil with slight modification. Thirty grams of dried shrimp waste was homogenized with 60 ml of soybean oil (waste: oil ratio = 1:2) and heated in the waterbath at 60°C for 150 minutes. Thereafter, the extract was filtered using Whatman filter paper and centrifuged at 3000 × g for 10min. Afterwards, the volume of pigmented oil layer from the supernatant is presented as astaxanthin. The astaxanthin is calculated using spectrophotometer at 487 nm. Standard solution of astaxanthin (Sigma, USA) was made in 6 different concentrations (30, 25, 20, 15, 10 and 5 ppm) by dissolving 0.005 grams of astaxanthin 100 ml of soybean oil. The optical density was measured using a spectrophotometer at 487 nm. The absorbance in each concentration is noted and used to create a standard curve and to calculate the astaxanthin content.

2.3 Determination of Secondary Metabolites

Screening for secondary matabolites constituents in vaname shrimps waste extract was performed using chemical reagents for qualitative determinations. The constituents screened for phenolics, flavonoids and terpenoids. Flavonoid test was carried out using shinoda test according to Hanani (2017). Crude extract of vaname shrimp waste 1 mL was mixed with 2 mL HCl, Mg metal powder was added until completely dissolved. Positive reaction is shown by the presence of red, yellow or orange colors. Phenolic content was performed using ferric chloride method according to Banu dan Catherine (2015). Crude extract of vaname shrimp waste was taken 2 ml of, and then 3-4 drops of iron (III) chloride ($FeCl_3$) were added. A positive reaction is indicated by the presence of a dark green, blue or purple color. For the confirmation of terpenoids in crude extract of vaname shrimp waste, 0, 3 grams were added in test tube, then 2 ml of chloroform were poured and 3 ml of sulfuric acid were added. The reaction between sulphuric acid and chloroform formed two layers to the extract and gave a color change. Formation of reddish-brown color showed a positive result of terpenoids.

2.4 DPPH Free Radical Scavenging Activity Assay

Antioxidant activity of vaname shrimps waste extract was performed using DPPH scavenging ability with three replications. The test was conducted in a 96-well microplate according to Nasution and Bella (2019) with slight modification. The antioxidant activity test was done using 6 series of concentrations with graded dilutions. 100 µL stock solution of extract in 6 series concentration (1000, 500, 250, 125, 62,5, 31,25 ppm) were added from well A- F then 50 µL methanol were added to well B-H. Methanol in well H is used as blank. Then 80 µL of DPPH with concentration 40 ppm were added to each well A-G. Absorbanced was read at 517 nm after 30 minutes incubation at room temperature in dark room. The scavenging ability (%) was calculated as follows:

$$\% \text{ inhibition} = \frac{absorbance\ of\ standard - absorbance\ of\ extract}{absorbance\ of\ standard} \times 100\%$$

Concentration of sample resulting in 50% inhibition on DPPH known as IC_{50} were calculated using the standard curve correlation between % inhibition and sample concentration to determine the antioxidant activity of vaname shrimp waste extract.

2.5 Antidiabetes Activity Assay

Male mice (*Mus musculus*) weighing between 20-29 grams were used in this experiment. The mice were adapted 3 days earlier and given commercial pellet and water ad libitum. The experimental protocol was approved by the Research Ethics Committee of Medical School of Duta Wacana Christian University number 1323/C.16/FK/2021. The mice were fasted 16 hours

earlier and made diabetic by injecting streptozotosin dissolved in normal saline solution (0.9% NaCl) at a dose of 40 mg/kg BW intraperitoneally (Wang, 2012). After 72 hours blood glucose of mice were tested and diabetic mice were used for the experiment when the blood glucose was greater than 120 mg/dl. Streptoztosin-induced mice were divided into 5 groups of 4 animals in each group. Group 1 as positive control received 0,2 ml of streptozotosin, group 2 as negative control received 0,2 ml aquadest, group 3 to group 5 were given astaxanthin extract from vaname shrimp waste at different dose (10, 15 and 20 mg/kg BW). This treatment was given for 14 days everyday orally everyday and the blood glucose was measured every 2 days.

3. RESULT AND DISCUSSION

3.1 Astaxanthin Concentration of Vaname Shrimp Waste Extract Using Soybean Oil

Since astaxanthin is oil soluble pigment, various vegetables and animals oil have been used to extract this pigment from crustaceans waste (Chen and Meyers, 1984; Sachindra and Mahendrakar, 2005; Handayani *et al.*, 2008). The use of soybean oil for astaxanthin discovery from crustaceans waste have been done severally (Meyers and Chen, 1984; Sachindra and Mahendrakar, 2005). Result showed that the used of soybean oil for recovery of astaxanthin from crustaceans waste indicated higher result of astaxanthin concentration compared than using herring, menhaden and salmon oil (Chen and Meyers, 1984), meanwhile the concentration of astaxanthin using soybean oil according Sachindra and Mahendrakar (2005) gave lower result of astaxanthin concentration than sunflower oil. However in this present study the astaxanthin concentration is higher compare than previous studies have done.

The solvent extracted carotenoid in this study was found in the form of a red-orange pigmented oil and red-brownish paste. The highest astaxanthin concentration (209.616 ppm) from vaname shrimp waste extract using soybean oil was obtained in a red-orange pigmented oil phase and the lowest followed by red-brownish bottom paste phase (35.720 ppm) (Table 1). In this present study, it was observed that pigmented oil phase gives higher astaxanthin concentration compare to bottom paste phase. This may be due to the reason that the free form of astaxanthin in vaname shrimp waste has been bound to fatty acids of soybean oil, causing the increase rate of mass transfer of astaxanthin from solid to liquid phase. As a result, the pigmented oil obtained higher astaxanthin concentration than paste phase. It is supported by Higuera-Ciapara *et al.* (2006), stated that astaxanthin in crustaceans waste is commonly found in free form or in association with other compounds and readily dissolved in oils.

Table-1: Concentrations of astaxanthin from vaname shrimp waste extract

Replications	Astaxanthin concentration pigmented oil phase (ppm)	Astaxanthin concentration bottom paste phase (ppm)
1	209.984	32,267
2	209.590	37,464
3	209.275	37,070
Average	209,616	35,720

In this research we assume that temperature and extraction time had influence on astaxanthin concentration. The influence of some other factors such as body part and shrimp species is also suspected to have a little effect on astaxanthin concentration in this study. The stability of astaxanthin is known at 50°C to 70°C. Therefore, we presume that the high astaxanthin concentration in this study is obtained by optimizing the extraction process, which was done at 60°C for 150 minutes. We also suspected that the enhancement amount of astaxanthin release in soybean oil in this study is caused by the sterification between hydroxyl groups in astaxanthin and fatty acid in soybean oil that arises during the extraction. Sachindra and Mahendrakar (2005) stated that carotenoid degrades at high temperature, consequently increasing temperature above 70°C or heating time above 150 min can result in decrease in astaxanthin concentration. Handayani *et al.* (2008) said that at the right temperature and maximum time of extraction, the hydroxyl groups in astaxanthin became more reactive and the reaction became faster and resulted in high yield of astaxanthin.

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3.2 Secondary Metabolites in Vaname Shrimp Waste Extract Using Soybean Oil

Investigations on the secondary metabolite of vaname shrimp extract using soybean oil were revealed the presence of flavonoid, phenolic and terpenoid in the pigmented oil phase extract of vaname shrimp waste, while the bottom paste phase only contains flavonoids and terpenoids (table 2). Astaxanthin is one of carotenoid pigments that belong to major group of terpenoids. The presence of terpenoid showed by 2 layers of brownish-red color in both phase showed that both phase contained astaxanthin. The orange color appeared in the flavonoid test indicated a reaction between HCl and Mg powder which produces a reddish orange flavilium salt complex compound (Marliana dan Suyono, 2006). Phenolic compounds indicate the presence of antioxidants in the sample; this is because phenolic plays a major role in preventing oxidation and inhibits free radical.

Table-2: Secondary metabolit content from vaname shrimp waste extract

Extract Phase	Secondary Metabolites Test	Result
Bottom paste phase	Phenolic	-
	Flavonoids	+
	Terpenoids	+
Pigmented oil phase	Phenolic	+
	Flavonoid	+
	Terpenoids	+

3.3 Antioxidant Activity of Vaname Shrimp Waste Extract Using Soybean Oil

Antioxidant activity of vaname shrimp waste extract were evaluated using DPPH scavenging activity and the highest percent inhibition was obtained by 1000 ppm pigmented oil phase (92.652%) whereas 1000 ppm bottom paste phase resulted in lower percent inhibition (90.878%). In this study, it was observed that increasing the sample concentration would increase the percentage of inhibition (figure 1 and figure 2). This indicated that the higher sample concentration, the more antioxidant compounds contained in the sample that were able to inhibit DPPH free radicals.

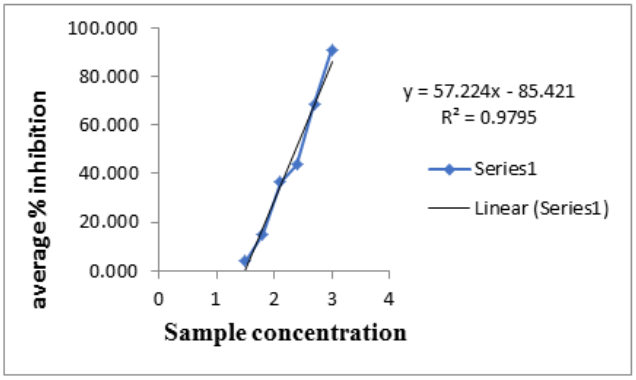

Fig-1: Bottom Paste Phase Linear Regression Curve of Vaname Shrimp Waste Extract

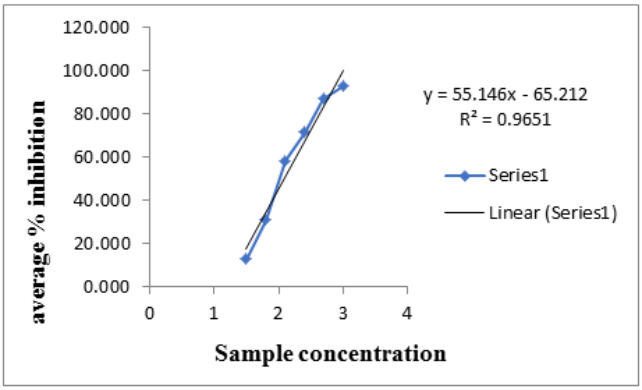

Fig-2: Pigmented Oil Phase Linear Regression Curve of Vaname Shrimp Waste Extract

Table-3: IC_{50} Value of Vaname Shrimp Waste Extract Using Soybean Oil

Test Sample Type	IC_{50} Value
Bottom Paste Phase of Vaname Shrimp Waste Extract	232.274 ppm
Pigmented Oil Phase of Vaname Shrimp Waste Extract	122.744 ppm

Inhibition concentration (IC_{50}) values of astaxanthin from vaname shrimp waste extract is presented in table 3. Inhibition concentration (IC_{50}) values of astaxanthin from vaname shrimp waste extract is presented in table 3. The IC_{50} values of the of the astaxanthin from vaname shrimp waste extract in bottom paste phase and pigmented oil phase were 122.744 ppm and 232.274 ppm, respectively. As per the definition, IC_{50} and antioxidant activity have an inverse correlation, means that the lower the IC_{50} values, the higher the antioxidant activity of the extract (*Prasad et al.*, 2009). It shows that bottom paste phase of vaname

shrimp waste extract possess low antioxidant activity while pigmented oil phase of vaname shrimp waste extract possess medium antioxidant activity. However, antioxidant activity in this present study is lower compare to the previous study Li *et al.* (2016) which recorded IC_{50} value of 2.39 from high pressure extraction (HPE) of astaxanthin from shrimp (*Penaeus vanname* Boone) waste.

3.4 Hypoglycemic Effect of Extract Astaxanthin From Vaname Shrimp Waste

The hypoglycemic effect of astaxanthin from vaname shrimp waste was observed after 14 days of oral administration in 5 groups of animal. It showed that blood glucose in 5 groups of animals was increased in the first day, which indicated the induction of streptozotosin succeeded. Statistical analysis of Duncan Multiple Range Test (table 4) showed no significant difference ($p<0.05$) between the positive control group of glibenclamide, 10 mg/kg BW and 20 mg/kg BW of vaname shrimp waste extract, but there was a significant difference ($P>0.05$) between the positive control group of glibenclamide with 20 mg/kg BW of vaname shrimp waste extract and the negative control of aquadest.

Table-4: Statistical analysis of blood glucose reduction in streptozotosin-induced diabetic mice

Treatment	Blood glucose (mg/dl)
Positive control	112.88[a]
Negative control	146.00[c]
Vaname shrimp waste extract 10 mg/kg BW	129.28[ab]
Vaname shrimp waste extract 15 mg/kg BW	135.31[bc]
Vaname shrimp waste extract 20 mg/kg BW	122.34[a]

As shown in table 5, blood glucose of all groups were significantly increased after 3 days of streptoztosin induction and then after 14 days of treatment, blood glucose reached to normal except for negative control aquadest group. As shown in table 5 and 6, the dose of 10 and 15 mg/kg bw of the astaxanthin slightly lowered the blood glucose of diabetic mice after 14 days, but did not reach significant level. The dose of 10 and 15 mg/kg bw of the astaxanthin from vaname shrimp waste slightly lowered the blood glucose of diabetic mice after 14 days, but did not reach significant level while the best treatment was obtained by using glibenclamide 5 mg/kg bw and followed by 20 mg/kg bw of astaxanthin from vaname shrimp waste extract with reducation 73.34% and 62.31%, respectively (table 6). The blood glucose reduction was also analyzed using reduction chart as shown in figure 3, the values of AUC from basal level showed a hyperglycemic condition in all group of animals treatment and it can be seen that values of AUC after administration of glibenclamide (5 mg/kg bw) and astaxanthin (20 mg/kg bw) were significantly smaller compared with to the negative control aquadest group and astaxanthin (10 and 15 mg/kg bw) ($P>0.05$). Therefore, the results obtained indicate that 20 mg/kg bw of astaxanthin from vaname shrimp waste had hypoglycemic effect in streptozotocin-induced diabetic mice.

Table-5: Effect of astaxanthin on blood glucose in streptozotosin-induced diabetic mice

Group	Dose	Blood glucose (mg/dl)	
		Before	After
Positive control (glibenclamide)	5 mg/kg BW	171.5	46
Negative control (aquadest)	0.2 ml	151.5	130.5
Diabetic + astaxanthin	10 mg/kg BW	162.5	67.75
Diabetic + astaxanthin	15 mg/kg BW	168.5	96
Diabetic + astaxanthin	20 mg/kg BW	180.75	67

Table-6: Blood glucose percentage reduction in streptozotosin-induced diabetic mice

Replications	Blood glucose percentage reduction (%)				
	Control +	Control -	Dose 10 mg/kg BW	Dose 15 mg/kg BW	Dose 20 mg/kg BW
1	75,12%	24.62%	37.68%	61.81%	54.70%
2	71,52%	13.87%	59.40%	57.86%	59.07%
3	78,17%	-2.34%	69.94%	-7.74%	59.15%
4	68,54%	13.70%	62.77%	60.10%	76.335%
Average	73.34%	12.46%	57.45%	43.01%	62.31%

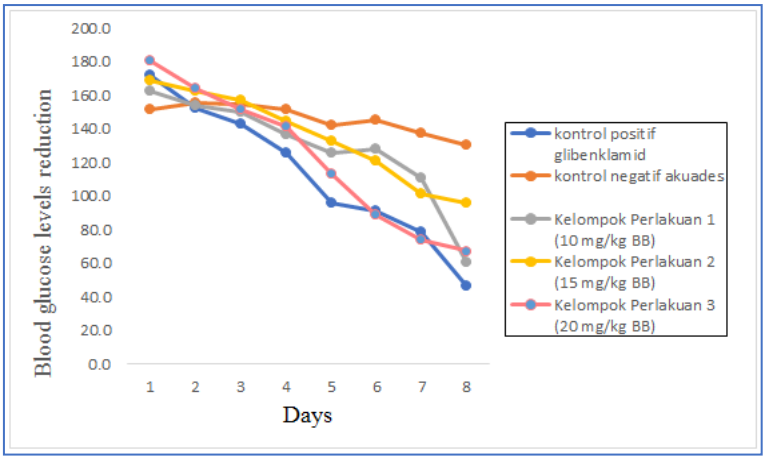

Fig-3: Blood glucose levels reduction after 14 days of treatment

In this study, our result suggest that 20 mg/kg bw of astaxanthin from vaname shrimp waste extract using soybean oil produces a significant hypogyclemic effect in streptozotocin-induced diabetic compared to 10 mg/kg bw and 15 mg/kg bw.

4. CONCLUSION

In this study, we conclude that the pigmented oil phase of vaname shrimp waste extract using soybean oil contained important secondary metabolit such as flavonoid, phenolic and terpenoid and indicate medium antioxidant activity (122.744 ppm) but high astaxanthin content (209,616 ppm). As further matter, 20 mg/kg bw astaxanthin from vaname shrimp waste showed a significant hypogyclemic effect in streptozotocin-induced diabetic mice with the highest percentage reduction of 62.31%. Further research needs to be done by extending the preclinical test to fully understand the mechanism of astaxanthin to reduce blood glucose in streptozotocin-induced diabetic mice, also histopathology test is needed to understand the effect of astaxanthin from vaname shrimp waste extract in vital organs.

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