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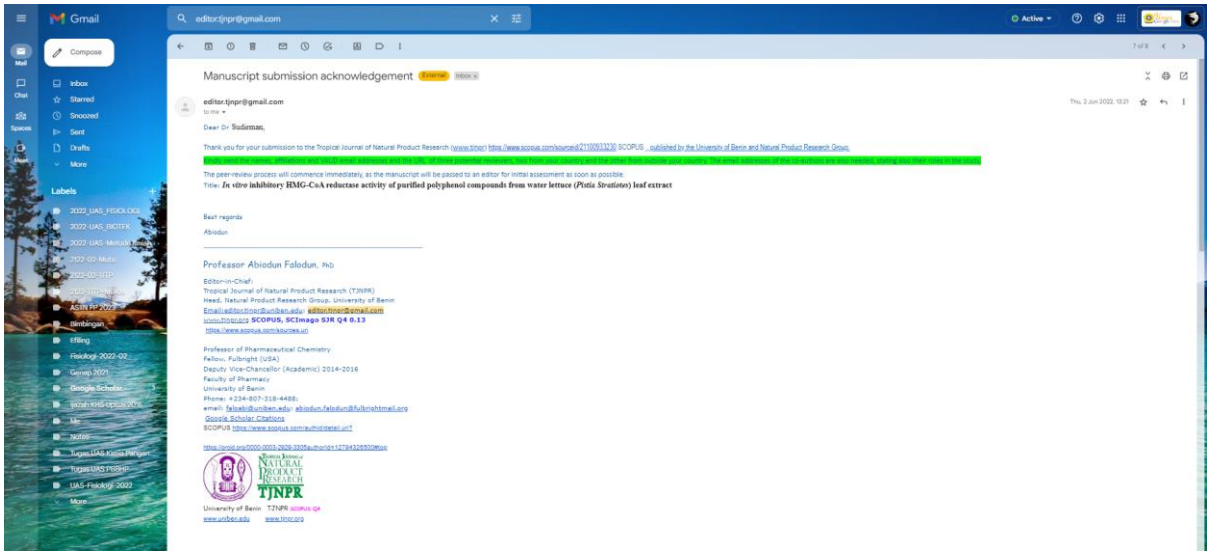

COVER LETTER

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[June 2nd, 2022]

Dear Prof. Abiodun Falodun,

We wish to submit a new manuscript entitled "*In vitro* inhibitory HMG-CoA reductase activity of purified polyphenol compounds from water lettuce (*Pistia Stratiotes*) leaf extract" for consideration by the **Tropical Journal of Natural Product Research**.

We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

In this paper, we report on anti-hypercholesterolemia of polyphenol purified extract from water lettuce (*Pistia stratiotes*). This is significant because this extract can inhibit HMG-CoA reductase. The paper should be of interest to readers in the areas of pharmaceutical and food supplement.

The results showed that the total polyphenols in each extract were 29.03 mg GAE/g dry sample (crude extract) and 65.63 mg GAE/g dry sample (purified extract). The total flavonoids were 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample (purified extract). The inhibitory activity of the HMG-CoA reductase enzyme showed that the purified extract could inhibit (34.74%) higher than the crude extract (2.61%). The purified extract of water lettuce (*Pistia stratiotes*) has higher levels of polyphenols and flavonoids and inhibits the HMG-CoA reductase enzyme more effectively than crude extract. Therefore, a purified extract of polyphenol compounds from water lettuce (Pistia stratiotes) has a potential alternative to develop as an anti-hypercholesterolemia agent.

We have no conflicts of interest to disclose.
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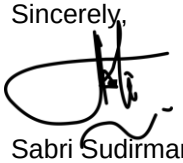

Sabri Sudirman

In vitro inhibitory HMG-CoA reductase activity of purified polyphenol compounds from water lettuce (*Pistia Stratiotes*) leaf extract

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Abstract

The 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase is an enzyme that plays a role in the synthesis of cholesterol. Synthetic anti-cholesterol drugs have side effects, so natural HMG-CoA reductase inhibitors such as from plant origin are needed. This study aimed to determine the inhibitory HMG-CoA reductase activity of polyphenol compounds from water lettuce (*Pistia stratiotes*) leaf extract. The research was conducted experimentally in a laboratory with a treatment consisting of two levels (crude and purified extracts) and repeated three times. Total polyphenols, flavonoids, and HMG-CoA reductase inhibitory activity assay were carried out by *in vitro* analysis. The results obtained were analyzed quantitatively, followed by using an independent sample t-test and presented in graphical form. The results showed that the total polyphenols in each extract were 29.03 mg GAE/g dry sample (crude extract) and 65.63 mg GAE/g dry sample (purified extract). The total flavonoids were 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample (purified extract). The inhibitory activity of the HMG-CoA reductase enzyme showed that the purified extract could inhibit (34.74%) higher than the crude extract (2.61%). The purified extract of water lettuce (*Pistia stratiotes*) has higher levels of polyphenols and flavonoids and inhibits the HMG-CoA reductase enzyme more effectively than crude extract.

Keywords: Anti-hypercholesterolemia, HMG-CoA reductase, Polyphenol, Water lettuce.

Introduction

The lifestyle of today's society is experiencing significant changes. This change such as highest fast food consumption and this is also accompanied by a lack of physical activity including exercise. This condition causes a metabolic imbalance in the body that can cause the accumulation of fat and increase cholesterol. A previous study reported that cholesterol is a very important compound in human life. However, the cholesterol showed adverse effects if its quantity is too high, especially for low-density lipoprotein-cholesterol (LDL-C).[1] High cholesterol levels or hypercholesterolemia cause some diseases related to cardiovascular diseases (CVDs), such as atherosclerosis, stroke, and heart disease.[2]

Pharmacological management is a treatment for reducing cholesterol levels, such as the consumption of statin drugs to decrease cholesterol levels in the body by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase enzyme. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis.[3] However, the use of this drug has shown some adverse effects, such as headache, muscle pain, and digestive system problems.[4, 5] Therefore, research to find an alternative inhibitor of the HMG-CoA reductase is an emerging field, such as by using plant extract as either functional food or food supplement.

Water lettuce (*Pistia stratiotes*) is an aquatic plant that contains some bioactive compounds, such as polyphenols, flavonoids, and tannins.[6, 7] A previous study also reported polyphenol compounds show lowering cholesterol activity by inhibiting the HMG-CoA reductase enzyme.[8] Additionally, flavonoids also possess the potential anti-cholesterol activity and prevent CVDs.[9] Polyphenol extraction with organic solvent was obtained as a crude extract which is still composed of other non-polyphenol components, such as lipid, sugar, and organic acids. Thereby, a purification process is required to remove these compounds.[10] Based on this condition, we hypothesized that the polyphenol extract showed different activity

before and after purification. Therefore, this study aimed to investigate the polyphenol content from water lettuce (*Pistia stratiotes*) and its HMG-CoA reductase activity.

Materials and Methods

Sample preparation and extraction

The water lettuce (*Pistia stratiotes*) was harvested from Sukaraja Village, South Sumatra, Indonesia. The leaf was cleaned and dried by oven at 45ºC for 24 h. After the drying process, the sample was ground and kept for the extraction process. The polyphenol compound was extracted by the maceration method by using 70% ethanol as a solvent at room temperature for 3 h.[7] Briefly, 20 mg of sample and 200 mL of 70% ethanol were mixed in the Erlenmeyer flask, then stirred by using a magnetic stirrer. After 3 h, the filtrate and residue were separated by using a filter paper (Whatman 42). The filtrate was kept in a new collection tube, then the residue was extracted by a fresh solvent under the same condition as the first extraction and five extractions were performed in total. filtrate-mixed was then evaporated by using a vacuum rotary evaporator at 40ºC to obtain the concentrated extract. Half of the concentrated extract was dried by using a freeze dryer to obtain polyphenol extract in powder form (crude extract). On the other hand, one was purified by using a HyperSep Retain PEP cartridge to obtain purified extract.

Purification process

The purification process was performed by solid-phase extraction (SPE) and a HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described by a previous method.[11] Briefly, 2 mL of dH_2O and then 2 mL of methanol were rinsed for cartridge preconditioned. Then, 2 mL of crude extract was loaded into the cartridge. The sample was eluted by using 2 mL of n-hexane and then 2 mL of 1 N H_2SO_4. The cartridge

was washed with absolute methanol to obtain purified polyphenol extract in an aqueous form. Then, it was dried with a freeze dryer to obtain the powder form of purified extract.

Total polifenol and flavonoid analysis

The total polyphenol and flavonoid contents were analyzed according to Chandra *et al.*[12] Total polyphenol content was analyzed by using Folin-Ciocalteu's phenol reagent. Briefly, 50 mg dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL by dH_2O. The mixture then was incubated at room temperature and in dark conditions for 30 min. After that, the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Total flavonoid content was analyzed by using the aluminum chloride method. Briefly, 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min. After reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC) was expressed as mg quercetin equivalent (QE) per g of dry sample.

HMG-CoA reductase inhibitory activity assay

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory activity was measured by using commercial kits from Sigma-Aldrich Co. (CS1090-1KT,

Merck) and performed according to the manufacture protocol. Briefly, 50 mg of each extract (crude and purified extract) was dissolved in Assay buffer. The solution was centrifuged at 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated according to these formulas:

$$\text{Enzyme activity (Units/mgP)} = \frac{(\Delta A340/\text{min}_{\text{sample}} - \Delta A340/\text{min}_{\text{blanko}}) \text{ x TV}}{12{,}44 \text{ x V x } 0{,}6 \text{ x LP}}$$

Where: TV = Total volume of the reaction in ml (1 ml for cuvettes and 0.2 ml for plates); V = volume of enzyme used in the assay (ml); LP = Light path in cm (1 for cuvettes).

$$\text{Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)} \text{ x } 100\%$$

Table 1. Reaction Mixed of HMG-CoA reductase inhibitory activity (for 1 mL of cuvette)

Sample	Assay buffer	Crude extract (CE	Purified extract (PE)	Pravastatin (Prava)	NADPH	HMG-CoA	HMG-CoA Reductase
Blanko	920 µL	-	-	-	20 µL	60 µL	-
HMG-CoA reduktase activity	915 µL	-	-	-	20 µL	60 µL	5 µL
CE inhibition	910 µL	5 µL	-	-	20 µL	60 µL	5 µL
PE inhibition	910 µL	-	5 µL	-	20 µL	60 µL	5 µL
Prava inhibition	910 µL	-	-	5 µL	20 µL	60 µL	5 µL

Results and Discussion

Total polifenol and flavonoid content

In the present study, we found that purified extract shows significantly higher total polyphenol and flavonoid contents when compared to crude extract as shown in **Figure 1**.

Total polyphenol contents (TPC) were about 29.03±4.15 mg GAE/g dry sample (crude extract) and 65.63±6.51 mg GAE/g dry sample (purified extract). Whereas, total flavonoids were about 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample (purified extract). The purified extract is majorly composed of polyphenol and flavonoid compounds due to unwanted compounds being removed during the purification process. A previous study reported that the quality or bioactivity of crude extract was increased after purification.[13] A solid-phase extraction (SPE) method was used for the purification process due to its rapid, simple, and economic.[14] The n-hexane was used to remove non-polar from the crude extract and a low concentrate of sulfuric acid was used to remove non-polyphenol polar compounds, such as sugar and organic acids.[11] A previous study reported that the total polyphenol of crude extract of *Inga edulis* increased from 496.5 mg GAE/g to 518.8 mg GAE/ after purification process.[15] Additionally, the luteolin (a flavonoid compound) increased from 0.68 mg/g to 3.52 mg/g and some flavonoids compounds of olive pomace also increased after purification process.[16]

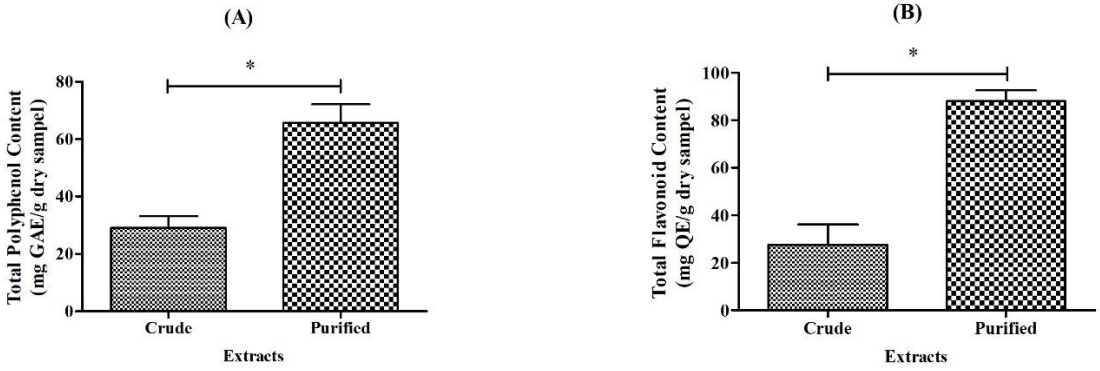

Figure 1: Total polyphenol **(A)** and flavonoid **(B)** content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (n=3). Statistically significance at *p<0.05 versus purified extract.

HMG-CoA reductase inhibitory activity

Figure 2 showed the representative reduction of absorbance at 340 nm during the measurement of the HMG-CoA reductase activity assay. After the calculation, we found that purified extract (34.74±5.40%) showed HMG-CoA reductase activity significantly higher than crude extract (2.61±0.28%) as shown in **Figure 3**.

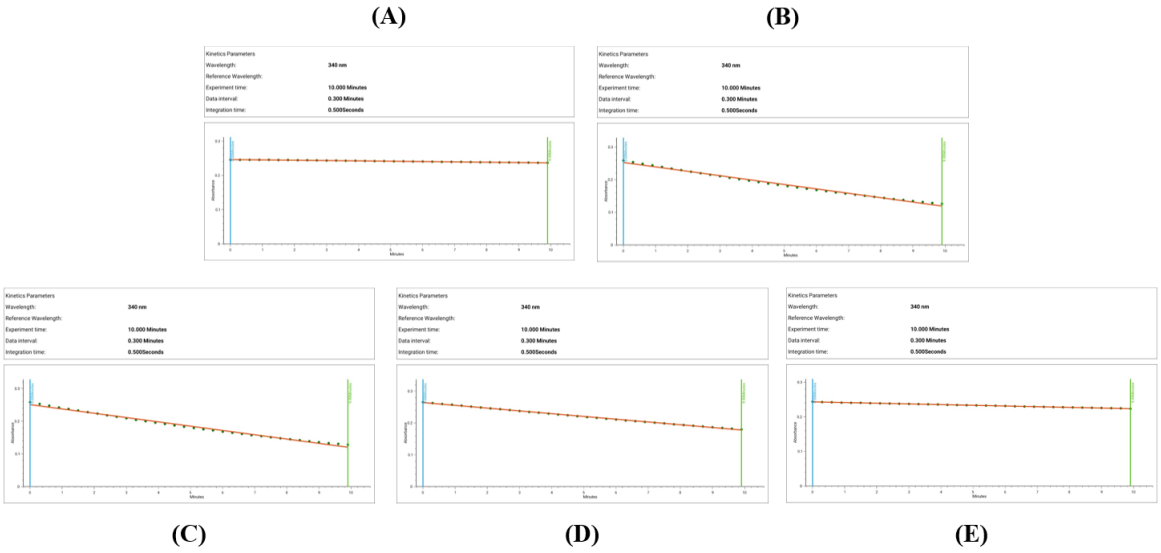

Figure 2: Representative of HMG-CoA reductase activity and inhibitory activity of sample: (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D) Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were measured at 340 nm for 10 min by a spectrophotometer.

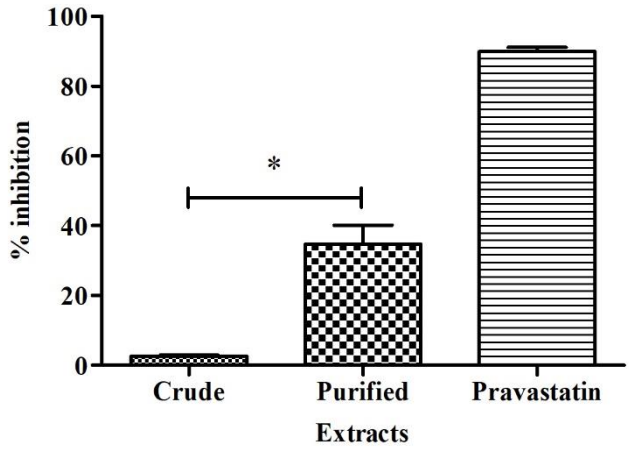

Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean ± SD (n=3). Statistically significance at *p<0.05 versus purified extract.

High inhibition of purified extract to HMG-CoA reductase due to this extract composed of high concentrate polyphenol and flavonoid compounds. A previous study reported that polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to inhibit HMG-CoA reductase activity.[17] A previous reference also reported that isoflavon (a flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during cholesterol synthesis.[18] Chen *et al*. also reported that catechin (a polyphenol compound) successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.[19] Additionally, Islam *et al*. reported that polyphenol and flavonoid compounds can block the electron transfer on the substrate HMG-CoA and bind on HMG-CoA reductase on the $NADP^+$ binding site.[8] The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis. This enzyme catalyzed HMG-CoA to Coenzyme A and mevalonate. The mevalonate then was converted to cholesterol by the mevalonate pathway.[3] Therefore, inhibited HMG-CoA reductase is an effective way to reduce cholesterol level in human and animal experiments.[20]

Conclusion

The purified extract showed a high polyphenol and flavonoid contents when compared to before the purification process (crude extract). The 3-hydroxy-3-methylglutaryl Coenzyme-A reductase inhibitory activity of the purified extract is also high when compared to crude extract. Therefore, a purified extract of polyphenol compounds from water lettuce (*Pistia stratiotes*) has a potential alternative to develop as an anti-hypercholesterolemia agent.

DECLARATIONS

Acknowledgment

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Conflict of interest

No conflict of interest associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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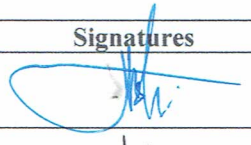
1	Sabri Sudirman		2-06-2022
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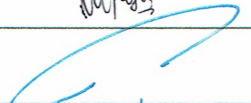
3	Herpandi		2-06-2022
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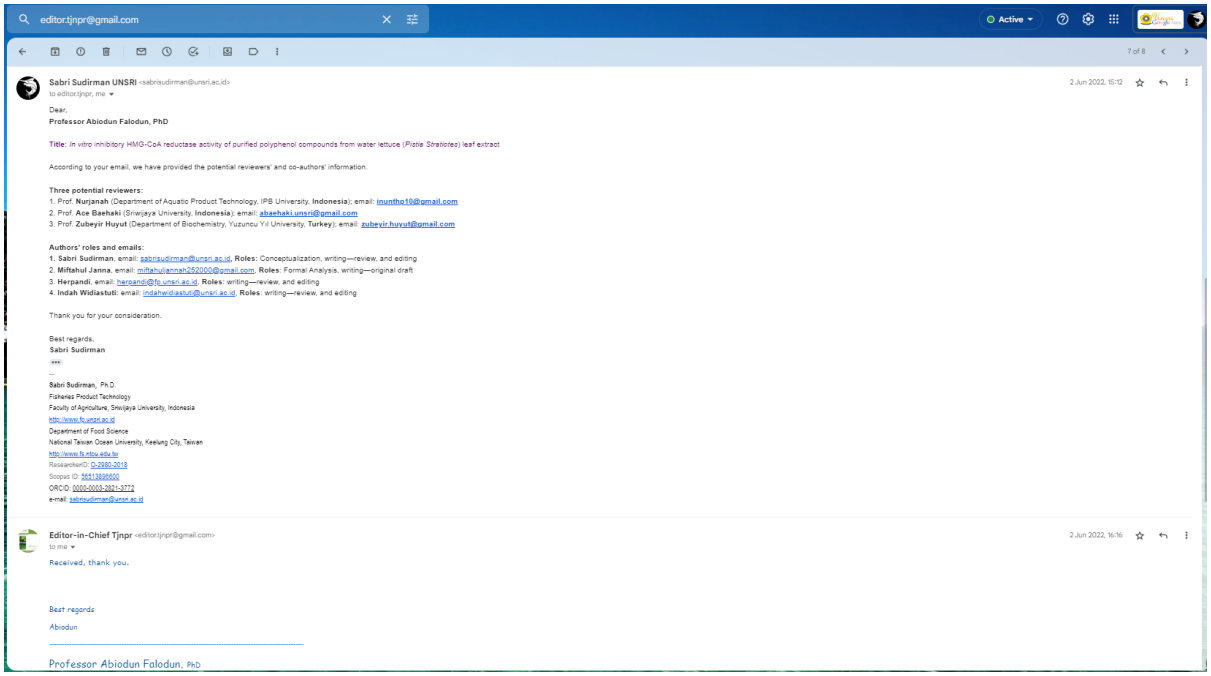

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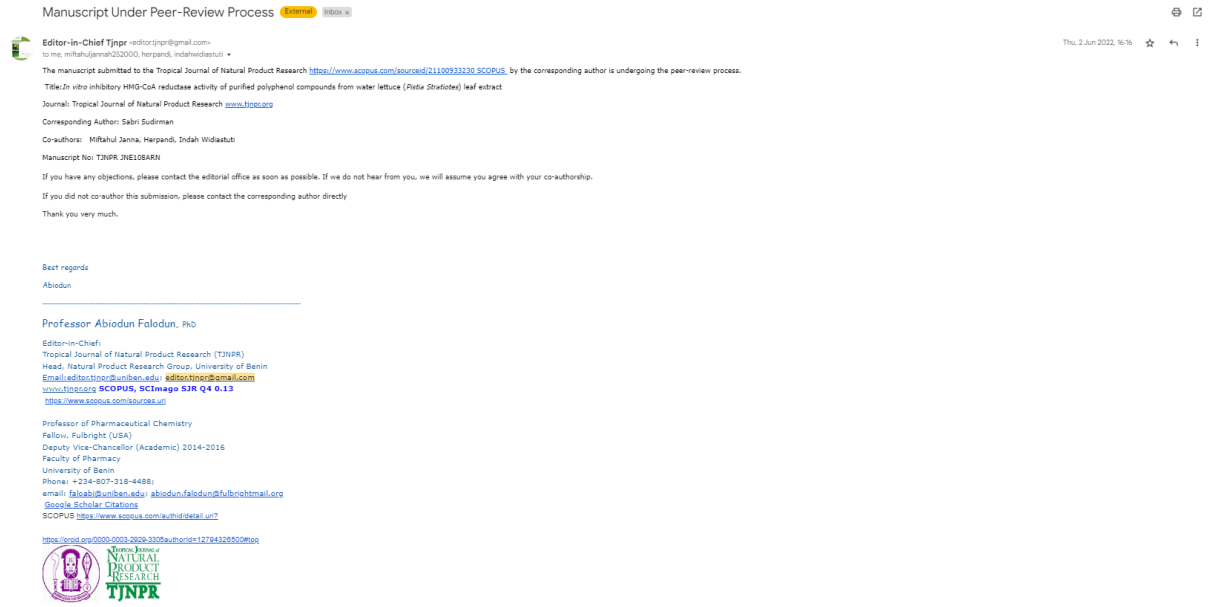

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Title:*In vitro* inhibitory HMG-CoA reductase activity of purified polyphenol compounds from water lettuce (*Pistia Stratiotes*) leaf extract

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Corresponding Author: Sabri Sudirman

Co-authors: Miftahul Janna, Herpandi, Indah Widiastuti

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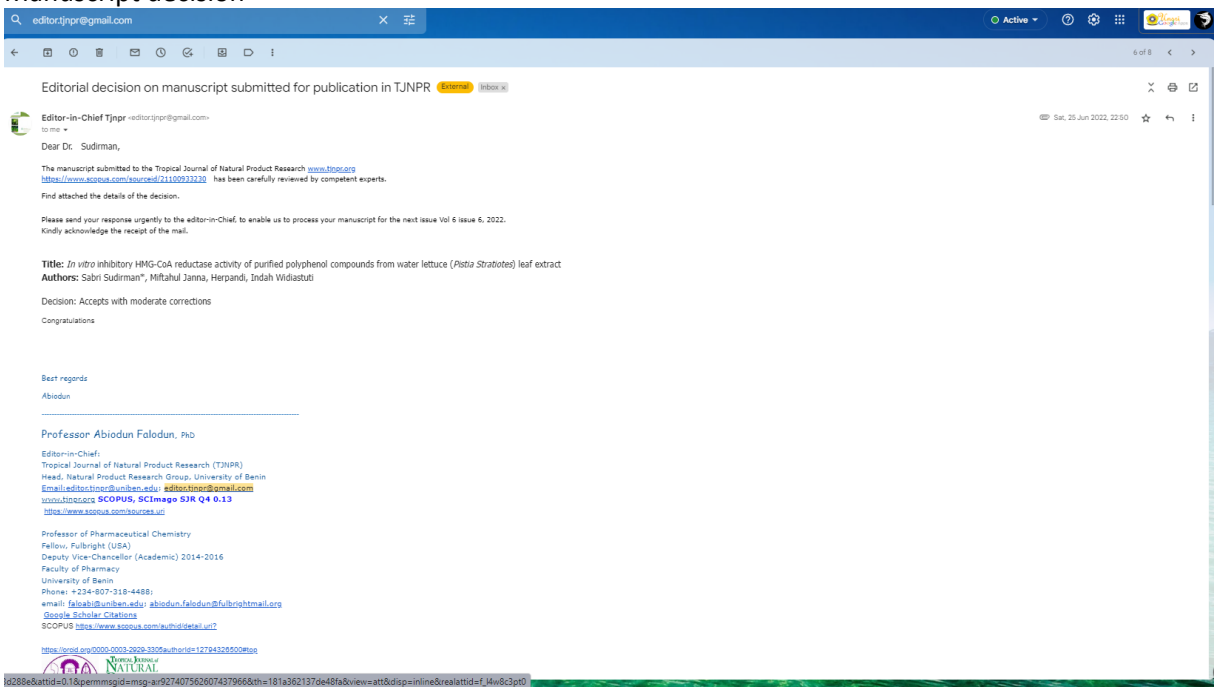

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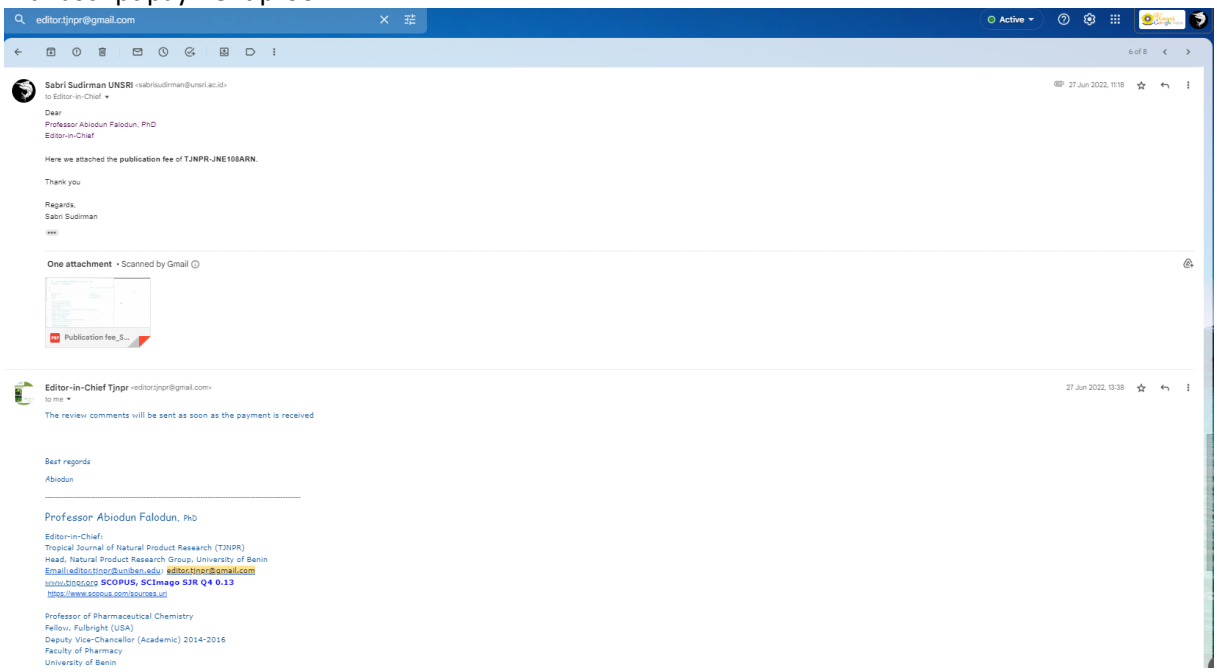

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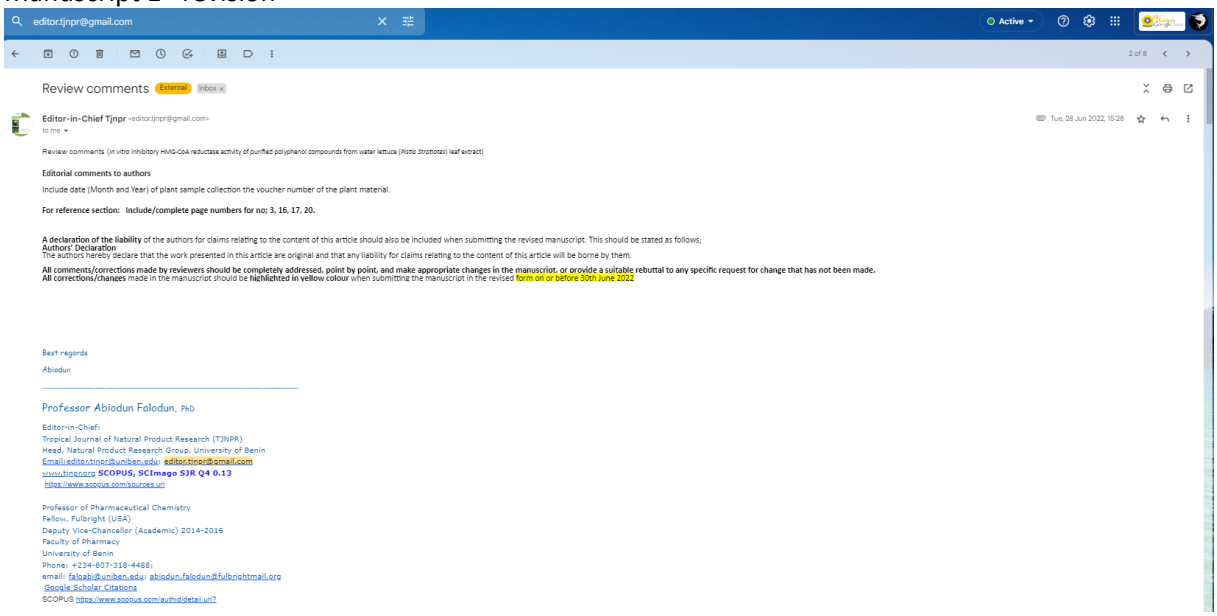

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1 ***In vitro* inhibitory HMG-CoA reductase activity of purified polyphenol compounds from**

2 **water lettuce (*Pistia Stratiotes*) leaf extract**

3

Abstract

The 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase is an enzyme that plays a role in the synthesis of cholesterol. Synthetic anti-cholesterol drugs have side effects, so natural HMG-CoA reductase inhibitors such as from plant origin are needed. This study aimed to determine the inhibitory HMG-CoA reductase activity of polyphenol compounds from water lettuce (*Pistia stratiotes*) leaf extract. The research was conducted experimentally in a laboratory with a treatment consisting of two levels (crude and purified extracts) and repeated three times. Total polyphenols, flavonoids, and HMG-CoA reductase inhibitory activity assay were carried out by *in vitro* analysis. The results obtained were analyzed quantitatively, followed by using an independent sample t-test and presented in graphical form. The results showed that the total polyphenols in each extract were 29.03 mg GAE/g dry sample (crude extract) and 65.63 mg GAE/g dry sample (purified extract). The total flavonoids were 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample (purified extract). The inhibitory activity of the HMG-CoA reductase enzyme showed that the purified extract could inhibit (34.74%) higher than the crude extract (2.61%). The purified extract of water lettuce (*Pistia stratiotes*) has higher levels of polyphenols and flavonoids and inhibits the HMG-CoA reductase enzyme more effectively than crude extract.

Keywords: Anti-hypercholesterolemia, HMG-CoA reductase, Polyphenol, Water lettuce.

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Introduction

The lifestyle of today's society is experiencing significant changes. This change such as highest fast food consumption and this is also accompanied by a lack of physical activity including exercise. This condition causes a metabolic imbalance in the body that can cause the accumulation of fat and increase cholesterol. A previous study reported that cholesterol is a very important compound in human life. However, the cholesterol showed adverse effects if its quantity is too high, especially for low-density lipoprotein-cholesterol (LDL-C).[1] High cholesterol levels or hypercholesterolemia cause some diseases related to cardiovascular diseases (CVDs), such as atherosclerosis, stroke, and heart disease.[2]

Pharmacological management is a treatment for reducing cholesterol levels, such as the consumption of statin drugs to decrease cholesterol levels in the body by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase enzyme. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis.[3] However, the use of this drug has shown some adverse effects, such as headache, muscle pain, and digestive system problems.[4, 5] Therefore, research to find an alternative inhibitor of the HMG-CoA reductase is an emerging field, such as by using plant extract as either functional food or food supplement.

Water lettuce (*Pistia stratiotes*) is an aquatic plant that contains some bioactive compounds, such as polyphenols, flavonoids, and tannins.[6, 7] A previous study also reported polyphenol compounds show lowering cholesterol activity by inhibiting the HMG-CoA reductase enzyme.[8] Additionally, flavonoids also possess the potential anti-cholesterol activity and prevent CVDs.[9] Polyphenol extraction with organic solvent was obtained as a crude extract which is still composed of other non-polyphenol components, such as lipid, sugar, and organic acids. Thereby, a purification process is required to remove these compounds.[10] Based on this condition, we hypothesized that the polyphenol extract showed different activity

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before and after purification. Therefore, this study aimed to investigate the polyphenol content from water lettuce (*Pistia stratiotes*) and its HMG-CoA reductase activity.

Materials and Methods

Sample preparation and extraction

The water lettuce (*Pistia stratiotes*) was harvested from Sukaraja Village, South Sumatra, Indonesia. The leaf was cleaned and dried by oven at 45ºC for 24 h. After the drying process, the sample was ground and kept for the extraction process. The polyphenol compound was extracted by the maceration method by using 70% ethanol as a solvent at room temperature for 3 h.[7] Briefly, 20 mg of sample and 200 mL of 70% ethanol were mixed in the Erlenmeyer flask, then stirred by using a magnetic stirrer. After 3 h, the filtrate and residue were separated by using a filter paper (Whatman 42). The filtrate was kept in a new collection tube, then the residue was extracted by a fresh solvent under the same condition as the first extraction and five extractions were performed in total. filtrate-mixed was then evaporated by using a vacuum rotary evaporator at 40ºC to obtain the concentrated extract. Half of the concentrated extract was dried by using a freeze dryer to obtain polyphenol extract in powder form (crude extract). On the other hand, one was purified by using a HyperSep Retain PEP cartridge to obtain purified extract.

Purification process

The purification process was performed by solid-phase extraction (SPE) and a HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described by a previous method.[11] Briefly, 2 mL of dH_2O and then 2 mL of methanol were rinsed for cartridge preconditioned. Then, 2 mL of crude extract was loaded into the cartridge. The sample was eluted by using 2 mL of n-hexane and then 2 mL of 1 N H_2SO_4. The cartridge

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was washed with absolute methanol to obtain purified polyphenol extract in an aqueous form. Then, it was dried with a freeze dryer to obtain the powder form of purified extract.

Total polifenol and flavonoid analysis

The total polyphenol and flavonoid contents were analyzed according to Chandra *et al*.[12] Total polyphenol content was analyzed by using Folin-Ciocalteu's phenol reagent. Briefly, 50 mg dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL by dH_2O. The mixture then was incubated at room temperature and in dark conditions for 30 min. After that, the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Total flavonoid content was analyzed by using the aluminum chloride method. Briefly, 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min. After reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC) was expressed as mg quercetin equivalent (QE) per g of dry sample.

HMG-CoA reductase inhibitory activity assay

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory activity was measured by using commercial kits from Sigma-Aldrich Co. (CS1090-1KT,

Merck) and performed according to the manufacture protocol. Briefly, 50 mg of each extract (crude and purified extract) was dissolved in Assay buffer. The solution was centrifuged at 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated according to these formulas:

$$\text{Enzyme activity (Units/mgP)} = \frac{(\Delta A340/\text{min}_{\text{sample}} - \Delta A340/\text{min}_{\text{blanko}}) \times TV}{12{,}44 \times V \times 0{,}6 \times LP}$$

Where: TV = Total volume of the reaction in ml (1 ml for cuvettes and 0.2 ml for plates); V = volume of enzyme used in the assay (ml); LP = Light path in cm (1 for cuvettes).

$$\text{Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)} \times 100\%$$

Table 1. Reaction Mixed of HMG-CoA reductase inhibitory activity (for 1 mL of cuvette)

Sample	Assay buffer	Crude extract (CE	Purified extract (PE)	Pravastatin (Prava)	NADPH	HMG-CoA	HMG-CoA Reductase
Blanko	920 µL	-	-	-	20 µL	60 µL	-
HMG-CoA reduktase activity	915 µL	-	-	-	20 µL	60 µL	5 µL
CE inhibition	910 µL	5 µL	-	-	20 µL	60 µL	5 µL
PE inhibition	910 µL	-	5 µL	-	20 µL	60 µL	5 µL

Prava inhibition	910 µL	-	-	5 µL	20 µL	60 µL	5 µL

Results and Discussion

Total polifenol and flavonoid content

In the present study, we found that purified extract shows significantly higher total polyphenol and flavonoid contents when compared to crude extract as shown in **Figure 1**. Total polyphenol contents (TPC) were about 29.03±4.15 mg GAE/g dry sample (crude extract) and 65.63±6.51 mg GAE/g dry sample (purified extract). Whereas, total flavonoids were about 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample (purified extract). The purified extract is majorly composed of polyphenol and flavonoid compounds due to unwanted compounds being removed during the purification process. A previous study reported that the quality or bioactivity of crude extract was increased after purification.[13] A solid-phase extraction (SPE) method was used for the purification process due to its rapid, simple, and economic.[14] The n-hexane was used to remove non-polar from the crude extract and a low concentrate of sulfuric acid was used to remove non-polyphenol polar compounds, such as sugar and organic acids.[11] A previous study reported that the total polyphenol of crude extract of *Inga edulis* increased from 496.5 mg GAE/g to 518.8 mg GAE/ after purification process.[15] Additionally, the luteolin (a flavonoid compound) increased from 0.68 mg/g to 3.52 mg/g and some flavonoids compounds of olive pomace also increased after purification process.[16]

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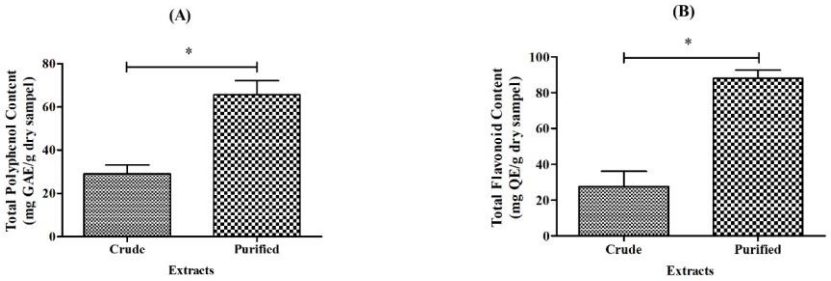

Figure 1: Total polyphenol **(A)** and flavonoid **(B)** content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (n=3). Statistically significance at *p<0.05 versus purified extract.

HMG-CoA reductase inhibitory activity

Figure 2 showed the representative reduction of absorbance at 340 nm during the measurement of the HMG-CoA reductase activity assay. After the calculation, we found that purified extract (34.74±5.40%) showed HMG-CoA reductase activity significantly higher than crude extract (2.61±0.28%) as shown in **Figure 3**.

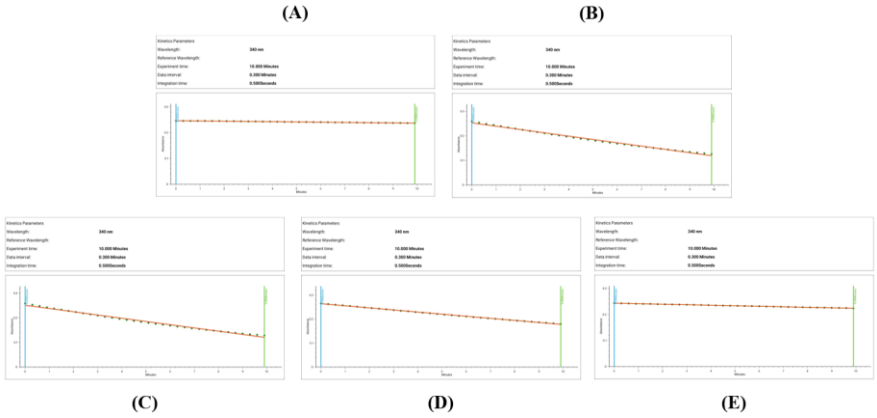

Figure 2: Representative of HMG-CoA reductase activity and inhibitory activity of sample: (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D)

Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were measured at 340 nm for 10 min by a spectrophotometer.

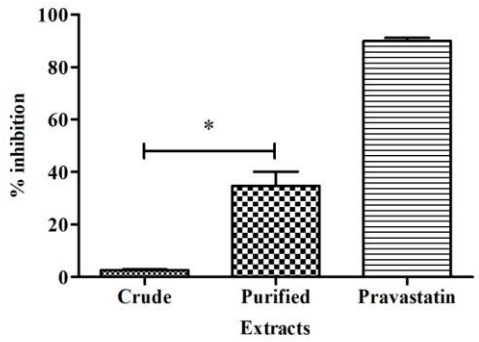

Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean ± SD (n=3). Statistically significance at *p<0.05 versus purified extract.

High inhibition of purified extract to HMG-CoA reductase due to this extract composed of high concentrate polyphenol and flavonoid compounds. A previous study reported that polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to inhibit HMG-CoA reductase activity.[17] A previous reference also reported that isoflavon (a flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during cholesterol synthesis.[18] Chen *et al*. also reported that catechin (a polyphenol compound) successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.[19] Additionally, Islam *et al*. reported that polyphenol and flavonoid compounds can block the electron transfer on the substrate HMG-CoA and bind on HMG-CoA reductase on the $NADP^+$ binding site.[8] The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis. This enzyme catalyzed HMG-CoA to Coenzyme A and mevalonate. The

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mevalonate then was converted to cholesterol by the mevalonate pathway.[3] Therefore, inhibited HMG-CoA reductase is an effective way to reduce cholesterol level in human and animal experiments.[20]

Conclusion

The purified extract showed a high polyphenol and flavonoid contents when compared to before the purification process (crude extract). The 3-hydroxy-3-methylglutaryl Coenzyme-A reductase inhibitory activity of the purified extract is also high when compared to crude extract. Therefore, a purified extract of polyphenol compounds from water lettuce (*Pistia stratiotes*) has a potential alternative to develop as an anti-hypercholesterolemia agent.

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DECLARATIONS

Acknowledgment

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Conflict of interest

No conflict of interest associated with this work.

Contribution of authors

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In vitro inhibitory HMG-CoA reductase activity of purified polyphenol compounds from water lettuce (*Pistia Stratiotes*) leaf extract

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Abstract

The 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase is an enzyme that plays a role in the synthesis of cholesterol. Synthetic anti-cholesterol drugs have side effects, so natural HMG-CoA reductase inhibitors such as from plant origin are desired. This study aimed to determine the inhibitory HMG-CoA reductase activity of polyphenol compounds from water lettuce (*Pistia stratiotes*) leaf extract. The research was conducted experimentally in a laboratory with a treatment consisting of two levels (crude and purified extracts) and replicated three times. Total polyphenols, flavonoids, and HMG-CoA reductase inhibitory activity assay were carried out by *in vitro* analysis. The results obtained were analyzed quantitatively, followed by using an independent sample t-test and presented in graphical form. The results showed that the total polyphenols in each extract were 29.03 mg GAE/g dry sample (crude extract) and 65.63 mg GAE/g dry sample (purified extract). The total flavonoids were 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample (purified extract). The inhibitory activity of the HMG-CoA reductase enzyme showed that the purified extract could inhibit (34.74%) higher than the crude extract (2.61%). The purified extract of water lettuce (*Pistia stratiotes*) has higher levels of polyphenols and flavonoids and inhibits the HMG-CoA reductase enzyme more effectively than crude extract.

Keywords: Anti-hypercholesterolemia, HMG-CoA reductase, Polyphenol, Water lettuce.

Introduction

The lifestyle of today's society is experiencing significant changes such changes include increased consumption of fast food and lack of physical activity including exercise. This condition causes a metabolic imbalance in the body that can cause the accumulation of fat and increase cholesterol. A previous study reported that cholesterol is a very important compound in human life. However, the cholesterol showed adverse effects if its quantity is too high, especially for low-density lipoprotein-cholesterol (LDL-C).[1] High cholesterol levels or hypercholesterolemia cause some diseases related to cardiovascular diseases (CVDs), such as atherosclerosis, stroke, and heart disease.[2]

Pharmacological management is a treatment for reducing cholesterol levels, such as the consumption of statin drugs to decrease cholesterol levels in the body by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase enzyme. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis.[3] However, the use of this drug has shown some adverse effects, such as headache, muscle pain, and digestive system problems.[4, 5] Therefore, research to find an alternative inhibitor of the HMG-CoA reductase is an emerging field, such as by using plant extract as either functional food or food supplement.

Water lettuce (*Pistia stratiotes*) is an aquatic plant that contains some bioactive compounds, such as polyphenols, flavonoids, and tannins.[6, 7] A previous study also reported polyphenol compounds show lowering cholesterol activity by inhibiting the HMG-CoA reductase enzyme.[8] Additionally, flavonoids also possess the potential anti-cholesterol activity and prevent CVDs.[9] Polyphenol extraction with organic solvent was obtained as a crude extract which is still composed of other non-polyphenol components, such as lipid, sugar, and organic acids. Thereby, a purification process is required to remove these compounds.[10] Based on this condition, we hypothesized that the polyphenol extract showed different activity

before and after purification. Therefore, this study aimed to investigate the polyphenol content from water lettuce (*Pistia stratiotes*) and its HMG-CoA reductase activity.

Materials and Methods

Sample preparation and extraction

The water lettuce (*Pistia stratiotes*) was collected in September 2021 from Sukaraja Village, South Sumatra, Indonesia and was authenticated at Microbiology and Biotechnology Laboratory of Fisheries Product Technology, Universitas Sriwijaya. The leaf was cleaned with distilled water and dried by oven at 45ºC until constant weight is reached. After the drying process, the sample was milled to size of 40 mesh and kept for the extraction process. The polyphenol compound was extracted by the maceration method by using 70% ethanol as a solvent at room temperature for 3 h.[7] Briefly, 20 mg of sample and 200 mL of 70% ethanol were mixed in the Erlenmeyer flask, then stirred by using a magnetic stirrer. After 3 h, the filtrate and residue were separated by using a filter paper (Whatman No. 42). The filtrate was kept in a new collection tube, then the residue was extracted by a fresh solvent under the same condition as the first extraction and five extractions were performed in total. filtrate-mixed was then evaporated by using a vacuum rotary evaporator at 40ºC to obtain the concentrated extract. Half of the concentrated extract was dried by using a freeze dryer to obtain polyphenol extract in powder form (crude extract). On the other hand, one was purified by using a HyperSep Retain PEP cartridge to obtain purified extract.

Purification process

The purification process was performed by solid-phase extraction (SPE) and a HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described by a previous method.[11] Briefly, 2 mL of dH_2O and then 2 mL of methanol were rinsed for

cartridge preconditioned. Then, 2 mL of crude extract was loaded into the cartridge. The sample was eluted by using 2 mL of n-hexane and then 2 mL of 1 N H_2SO_4. The cartridge was washed with absolute methanol to obtain purified polyphenol extract in an aqueous form. Then, it was dried with a freeze dryer to obtain the powder form of purified extract.

Total polifenol and flavonoid analysis

The total polyphenol and flavonoid contents were analyzed according to Chandra *et al*.[12] Total polyphenol content was analyzed by using Folin-Ciocalteu's phenol reagent. Briefly, 50 mg dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL by dH_2O. The mixture then was incubated at room temperature and in dark conditions for 30 min. After that, the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Total flavonoid content was analyzed by using the aluminum chloride method. Briefly, 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min. After reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC) was expressed as mg quercetin equivalent (QE) per g of dry sample.

HMG-CoA reductase inhibitory activity assay

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory activity was measured by using commercial kits from Sigma-Aldrich Co. (CS1090-1KT, Merck) and performed according to the manufacture protocol. Briefly, 50 mg of each extract (crude and purified extract) was dissolved in Assay buffer. The solution was centrifuged at 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated according to these formulas:

$$\text{Enzyme activity (Units/mgP)} = \frac{(\Delta A340/\text{min}_{\text{sample}} - \Delta A340/\text{min}_{\text{blanko}}) \text{ x TV}}{12{,}44 \text{ x V x } 0{,}6 \text{ x LP}}$$

Where: TV = Total volume of the reaction in ml (1 ml for cuvettes and 0.2 ml for plates); V = volume of enzyme used in the assay (ml); LP = Light path in cm (1 for cuvettes).

$$\text{Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{ Enzyme activity with sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)} \text{ x } 100\%$$

Table 1. Reaction Mixed of HMG-CoA reductase inhibitory activity (for 1 mL of cuvette)

Sample	Assay buffer	Crude extract (CE	Purified extract (PE)	Pravastatin (Prava)	NADPH	HMG-CoA	HMG-CoA Reductase
Blanko	920 µL	-	-	-	20 µL	60 µL	-
HMG-CoA reduktase activity	915 µL	-	-	-	20 µL	60 µL	5 µL
CE inhibition	910 µL	5 µL	-	-	20 µL	60 µL	5 µL
PE inhibition	910 µL	-	5 µL	-	20 µL	60 µL	5 µL
Prava inhibition	910 µL	-	-	5 µL	20 µL	60 µL	5 µL

Results and Discussion

Total polifenol and flavonoid content

In the present study, we found that purified extract shows significantly higher total polyphenol and flavonoid contents when compared to crude extract as shown in **Figure 1**. Total polyphenol contents (TPC) were about 29.03±4.15 mg GAE/g dry sample (crude extract) and 65.63±6.51 mg GAE/g dry sample (purified extract). Whereas, total flavonoids were about 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample (purified extract). The purified extract is majorly composed of polyphenol and flavonoid compounds due to unwanted compounds being removed during the purification process. A previous study reported that the quality or bioactivity of crude extract was increased after purification.[13] The bioactivity will increase after purification because unwanted and undesirable substances would have been screened out thereby improving the overall quality of the crude extract.[14] A solid-phase extraction (SPE) method was used for the purification process due to its rapid, simple, and economic.[15] The n-hexane was used to remove non-polar from the crude extract and a low concentrate of sulfuric acid was used to remove non-polyphenol polar compounds, such as sugar and organic acids.[11] A previous study reported that the total polyphenol of crude extract of *Inga edulis* increased from 496.5 mg GAE/g to 518.8 mg GAE/ after purification process.[16] Additionally, the luteolin (a flavonoid compound) increased from 0.68 mg/g to 3.52 mg/g and some flavonoids compounds of olive pomace also increased after purification process.[17]

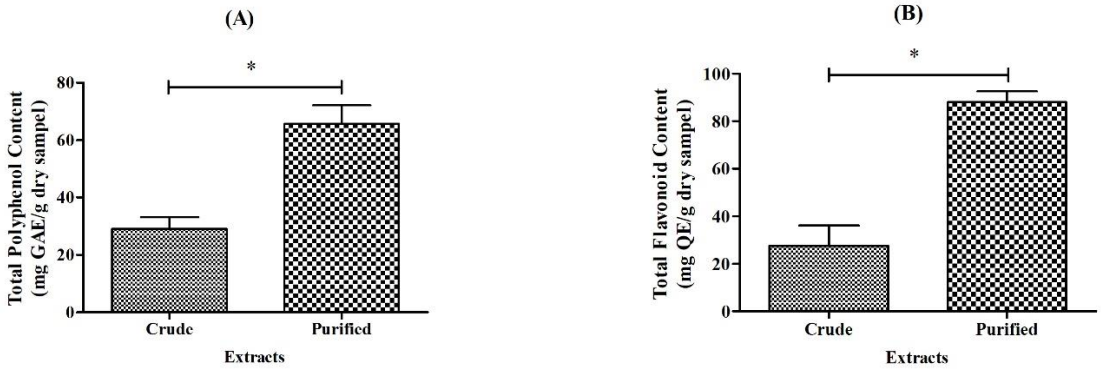

Figure 1: Total polyphenol **(A)** and flavonoid **(B)** content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (n=3). Statistically significance at *p<0.05 versus purified extract.

HMG-CoA reductase inhibitory activity

Figure 2 showed the representative reduction of absorbance at 340 nm during the measurement of the HMG-CoA reductase activity assay. After the calculation, we found that purified extract (34.74±5.40%) showed HMG-CoA reductase activity significantly higher than crude extract (2.61±0.28%) as shown in **Figure 3**.

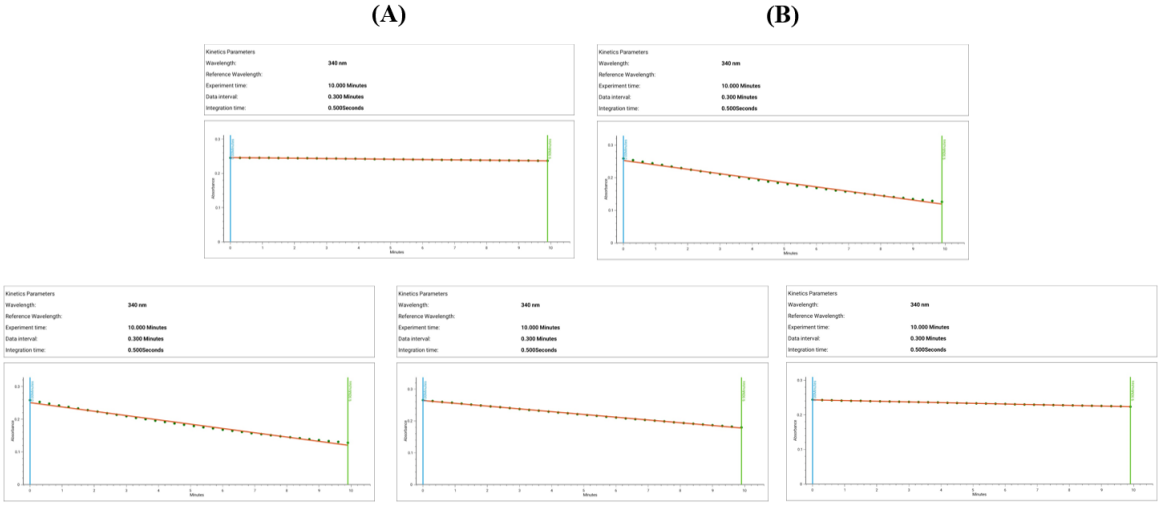

Figure 2: Representative of HMG-CoA reductase activity and inhibitory activity of sample: (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D) Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were measured at 340 nm for 10 min by a spectrophotometer.

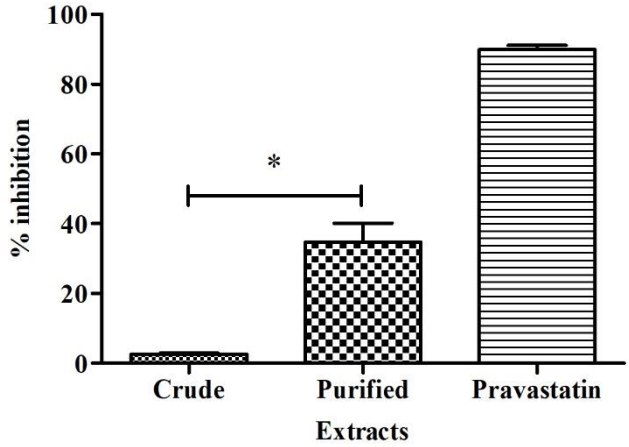

Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean ± SD (n=3). Statistically significance at *p<0.05 versus purified extract.

High inhibition of purified extract to HMG-CoA reductase due to this extract composed of high concentrate polyphenol and flavonoid compounds. A previous study reported that polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to inhibit HMG-CoA reductase activity.[18] A previous reference also reported that isoflavon (a flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during cholesterol synthesis.[19] Chen *et al*. also reported that catechin (a polyphenol compound) successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.[20] Additionally, Islam *et al*.[8] reported that polyphenol and flavonoid compounds can block the electron transfer on the substrate HMG-CoA and bind on HMG-CoA reductase on the $NADP^+$ binding site. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis. This enzyme catalyzed HMG-CoA to Coenzyme A and mevalonate. The mevalonate then was converted to cholesterol by the mevalonate pathway.[3] Therefore, inhibited HMG-CoA reductase is an effective way to reduce cholesterol level in human and animal experiments.[21]

Conclusion

The purified extract showed a high polyphenol and flavonoid contents when compared to before the purification process (crude extract). The 3-hydroxy-3-methylglutaryl Coenzyme-A reductase inhibitory activity of the purified extract was also high when compared to crude extract. Therefore, a purified extract of polyphenol compounds from water lettuce (*Pistia stratiotes*) has a potential alternative to develop as an anti-hypercholesterolemia agent.

DECLARATIONS

Acknowledgment

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Conflict of interest

No conflict of interest associated with this work.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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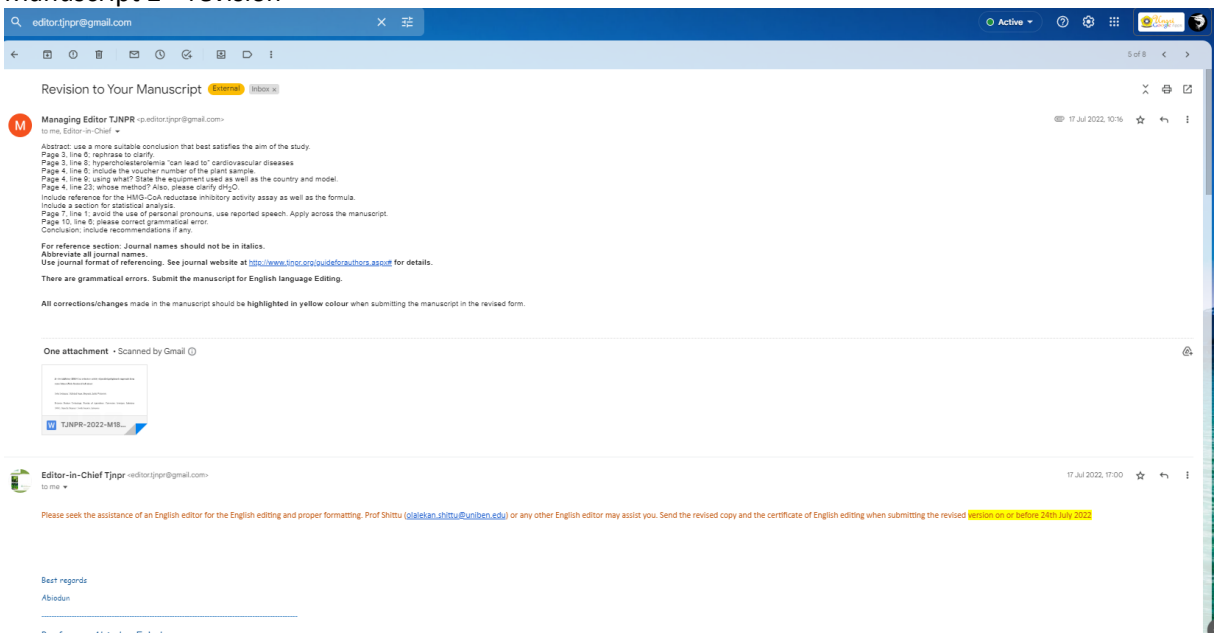

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Page 4, line 6: include the voucher number of the plant sample.
Page 4, line 9: using what? State the equipment used as well as the country and model.
Page 4, line 23: whose method? Also, please clarify dH_2O.
Include reference for the HMG-CoA reductase inhibitory activity assay as well as the formula.
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Water lettuce (*Pistia stratiotes*) is an aquatic plant that contains some bioactive compounds, such as polyphenols, flavonoids, and tannins.[6, 7] A previous study also reported polyphenol compounds show lowering cholesterol activity by inhibiting the HMG-CoA reductase enzyme.[8] It was also reported that flavonoid possess the potential anti-cholesterol activity and prevent CVDs.[9] Since polyphenol extraction with organic solvent was obtained as a crude extract composed of other non-polyphenol components, such as lipid, sugar, and organic acids, a purification process is required to remove these compounds.[10] Based on this condition, the authors hypothesized that the polyphenol extract showed different activity

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Where: TV = Total volume of the reaction in ml (1 ml for cuvettes and 0.2 ml for plates); V = volume of enzyme used in the assay (ml); LP = Light path in cm (1 for cuvettes).

$$\text{Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)} \times 100\%$$

Table 1: Reaction Mixed of HMG-CoA reductase inhibitory activity (for 1 mL of cuvette)

Sample	Assay buffer	Crude extract (CE	Purified extract (PE)	Pravastatin (Prava)	NADPH	HMG-CoA	HMG-CoA Reductase
Blanko	920 µL	-	-	-	20 µL	60 µL	-
HMG-CoA reduktase activity	915 µL	-	-	-	20 µL	60 µL	5 µL
CE inhibition	910 µL	5 µL	-	-	20 µL	60 µL	5 µL
PE inhibition	910 µL	-	5 µL	-	20 µL	60 µL	5 µL
Prava inhibition	910 µL	-	-	5 µL	20 µL	60 µL	5 µL

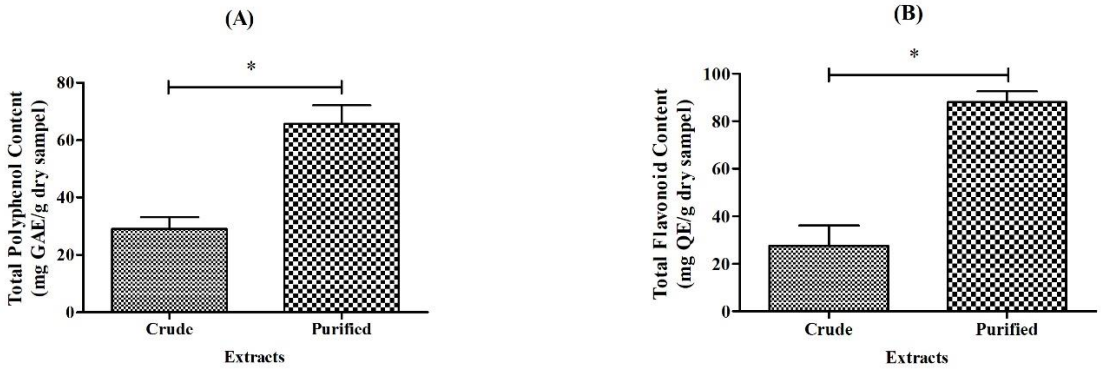
Statistical analysis

All data are expressed as the mean ± standard deviation (SD) and analyzed by one-way ANOVA with Duncan's post-hoc test ($p<0.05$) using SPSS (v.22.0; IBM Corp., Armonk, NY, USA).

Results and Discussion

Total polifenol and flavonoid content

The results showed that the purified extract has significantly higher total polyphenol and flavonoid contents compared to the crude extract as shown in **Figure 1**. Total polyphenol contents (TPC) of the crude and purified extracts were about 29.03±4.15 mg GAE/g dry sample and 65.63±6.51 mg GAE/g dry sample. Meanwhile, total flavonoids were about 27.58 mg QE/g dry sample and 88.02 mg QE/g dry sample, respectively. It was also found that the purified extract is majorly composed of polyphenol and flavonoid compounds due to unwanted compounds are being removed during the purification process. A previous study reported that the quality or bioactivity of crude extract was increased after purification.[13] This is because unwanted and undesirable substances have been removed, thereby improving the overall quality of the crude extract.[14] A solid-phase extraction (SPE) method was used for the purification process due to its rapid, simple, and economic.[15] The n-hexane was applied for the removal of non-polar from the crude extract and a low concentrate of sulfuric acid was used to remove non-polyphenol polar compounds, such as sugar and organic acids.[11] A previous study reported that the total polyphenol of crude extract of *Inga edulis* increased from 496.5 mg GAE/g to 518.8 mg GAE/ after purification process.[16] Furthermore, the luteolin (a flavonoid compound) increased from 0.68 mg/g to 3.52 mg/g and some flavonoids compounds of olive pomace also improved after the purification process.[17]



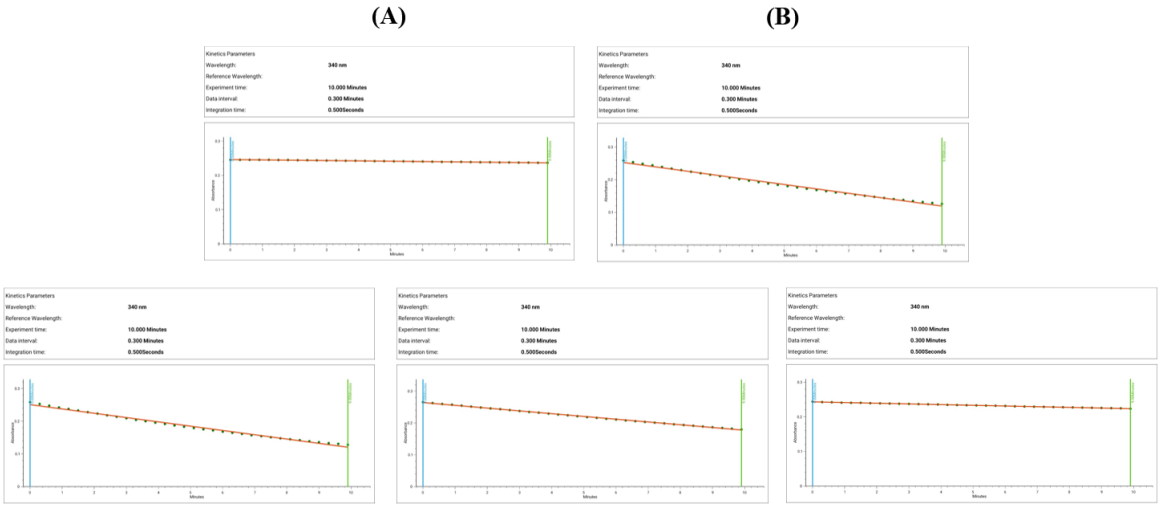
147

148 **Figure 1:** Total polyphenol **(A)** and flavonoid **(B)** content of crude and purified extract of
149 water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (*n*=3). Statistically
150 significance at **p*<0.05 versus purified extract.

151

152 *HMG-CoA reductase inhibitory activity*

153 **Figure 2** showed the representative reduction of absorbance at 340 nm during the
154 measurement of the HMG-CoA reductase activity assay. After the calculation, it was found
155 that purified extract with a value 34.74±5.40% showed HMG-CoA reductase activity
156 significantly higher than crude extract (2.61±0.28%) as shown in **Figure 3**.



157

158 **Figure 2:** Representative of HMG-CoA reductase activity and inhibitory activity of sample:
159 (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D)

Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were measured at 340 nm for 10 min by a spectrophotometer.

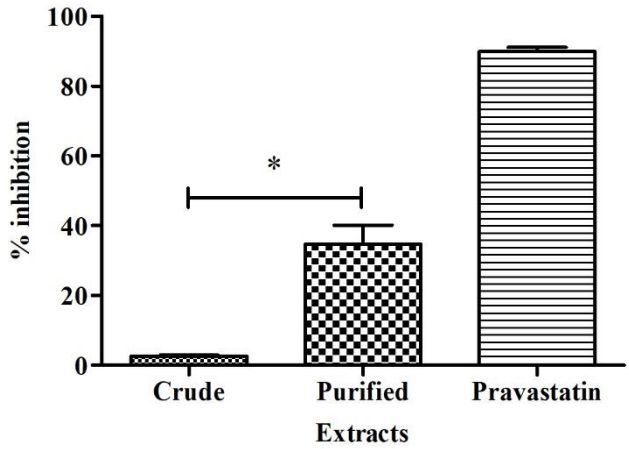


Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean ± SD (n=3). Statistically significance at *p<0.05 versus purified extract.

The increased inhibition of purified extract to HMG-CoA reductase due to it composed of high concentrations of polyphenol and flavonoid compounds. A previous study reported that polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to inhibit HMG-CoA reductase activity.[18] A previous reference also reported that isoflavon (a flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during cholesterol synthesis.[19] According to Chen *et al*., catechin (a polyphenol compound) successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.[20] Additionally, Islam *et al*.[8] reported that these compounds can block the electron transfer on the substrate HMG-CoA and bind on HMG-CoA reductase on the $NADP^+$ binding site. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis. This enzyme catalyzed HMG-CoA to Coenzyme A and mevalonate. The mevalonate then was

converted to cholesterol by the mevalonate pathway.[3] Therefore, inhibited HMG-CoA reductase is an effective way to reduce cholesterol levels in human and animal experiments.[21]

Conclusion

The purified extract showed a high polyphenol and flavonoid contents when compared to before the purification process (crude extract). The 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitory activity of the purified extract was also high when compared to crude extract. Therefore, the purified extract of polyphenol compounds from water lettuce (*Pistia stratiotes*) has a potential alternative to develop as an anti-hypercholesterolemia agent.

DECLARATIONS

Acknowledgment

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Conflict of interest

No conflict of interest associated with this work.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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In vitro inhibitory HMG-CoA reductase activity of purified polyphenol compounds from water lettuce (*Pistia Stratiotes*) leaf extract

Sabri Sudirman*, Miftahul Janna, Herpandi, Indah Widiastuti

Fisheries Product Technology, Faculty of Agriculture, Universitas Sriwijaya, Indralaya 30862, Ogan Ilir Regency, South Sumatra, Indonesia

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Abstract

The 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase is an enzyme that plays a role in the synthesis of cholesterol. The side effect of synthetic anti-cholesterol drugs has led to the demand for natural HMG-CoA reductase inhibitors such as plant extract. Therefore, this study aimed to determine the inhibitory HMG-CoA reductase activity of polyphenol compounds from water lettuce (*Pistia stratiotes*) leaf extract. The research was conducted using the crude and purified extracts of water lettuce. The total polyphenols, flavonoids, and HMG-CoA reductase inhibitory activity assay were carried out by *in vitro* analysis. The values obtained were analyzed quantitatively, followed by the use of an independent sample t-test and presented in graphical form. The results showed that the total polyphenols in each crude and purified extracts were 29.03 mg GAE/g dry sample and 65.63 mg GAE/g dry sample, respectively, while the total flavonoids were 27.58 mg QE/g dry sample and 88.02 mg QE/g dry sample, respectively. The inhibitory activity of the HMG-CoA reductase enzyme showed that the purified extract showed percentage inhibition of 34.74% which was higher than that of the crude extract which was 2.61%. This indicated that the purified extract of water lettuce has higher levels of polyphenols and flavonoids that can inhibit the HMG-CoA reductase enzyme more effectively than the crude extract.

Keywords: Anti-hypercholesterolemia, HMG-CoA reductase, Polyphenol, Water lettuce.

Introduction

Currently, society's lifestyle is experiencing significant changes such as high consumption of fast food and lack of physical activity including exercise. This condition usually leads to a metabolic imbalance in the body that can cause the accumulation of fat and increase cholesterol. A previous study reported that cholesterol is a very important compound in human life. However, high cholesterol is closely linked with many other medical problems, especially elevated levels of low-density lipoprotein-cholesterol (LDL-C).[1] It was also discovered that high cholesterol levels or hypercholesterolemia can cause cardiovascular diseases (CVDs), such as atherosclerosis, stroke, and heart disease.[2]

Pharmacological management such as the consumption of statin drugs is a treatment for reducing cholesterol levels in the body by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase enzyme. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis.[3] However, the use of this drug has shown some adverse effects, which include headache, muscle pain, and digestive system problems.[4, 5] Therefore, investigations are being carried out using plant extract as a functional or food supplement to discover an alternative inhibitor of the HMG-CoA reductase.

Water lettuce (*Pistia stratiotes*) is an aquatic plant that contains some bioactive compounds, such as polyphenols, flavonoids, and tannins.[6, 7] A previous study also reported polyphenol compounds show lowering cholesterol activity by inhibiting the HMG-CoA reductase enzyme.[8] It was also reported that flavonoid possess the potential anti-cholesterol activity and prevent CVDs.[9] Since polyphenol extraction with organic solvent was obtained as a crude extract composed of other non-polyphenol components, such as lipid, sugar, and organic acids, a purification process is required to remove these compounds.[10] Based on this condition, the authors hypothesized that the polyphenol extract showed different activity

before and after purification. Therefore, this study aimed to investigate the polyphenol content from water lettuce (*Pistia stratiotes*) and its HMG-CoA reductase activity.

Materials and Methods

Sample preparation and extraction

The water lettuce (*Pistia stratiotes*) was collected in September 2021 from Sukaraja Village, South Sumatra, Indonesia. The sample collected was authenticated at Microbiology and Biotechnology Laboratory of Fisheries Product Technology, Universitas Sriwijaya (FPT0015092022). The leaf was rinsed with distilled water and oven-dried at 45ºC until constant weight is reached. After the drying process, the sample was milled to a size of 40 mesh using a grinding machine (Microphyte disintegrator B-One DM-120M) and kept for the extraction process. The polyphenol compound was extracted by the maceration method using 70% ethanol as a solvent at room temperature for 3 hours.[7] Briefly, 20 mg of sample and 200 mL of 70% ethanol were mixed in the Erlenmeyer flask, then stirred using a magnetic stirrer. After 3 hours, the filtrate and residue were separated with a filter paper (Whatman No. 42). The filtrate was kept in a new collection tube and the residue was extracted by a fresh solvent under the same condition as the first extraction, which was carried out five repetitions. The filtrate-mixed was evaporated using a vacuum rotary evaporator at 40ºC to obtain the concentrated extract. Half of the concentrated extract was dried using a freeze dryer to obtain polyphenol extract in powder form (crude extract). Meanwhile, purified extract was obtained through a HyperSep Retain PEP cartridge.

Purification process

The purification process was performed by solid-phase extraction (SPE) and a HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described

by Pérez-Magariño *et al.*[11] Briefly, 2 mL of distilled water and then 2 mL of methanol was rinsed for cartridge preconditioned. Subsequently, 2 mL of crude extract was loaded into the cartridge and the sample was eluted by using 2 mL of n-hexane and 2 mL of 1 N H_2SO_4. The cartridge was washed with absolute methanol to obtain purified polyphenol extract in an aqueous form and dried with a freeze dryer to obtain the powder form of purified extract.

Total polyphenol and flavonoid analysis

The total polyphenol and flavonoid contents were analyzed according to Chandra *et al.*[12] Moreover, total polyphenol content was analyzed using Folin-Ciocalteu's phenol reagent. Briefly, 50 mg of dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL with distilled water. The mixture was incubated at room temperature and in dark conditions for 30 min. Subsequently, the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Total flavonoid content was analyzed using the aluminum chloride method. Briefly, 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min. After the reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC) was expressed as mg quercetin equivalent (QE) per g of dry sample.

HMG-CoA reductase inhibitory activity assay

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory activity was measured using commercial kits from Sigma-Aldrich Co. (CS1090-1KT, Merck) and performed according to the manufacturers' protocol. Briefly, 50 mg of each crude and purified extract was dissolved in assay buffer. The solution was centrifuged at 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated according to these formulas:

$$\text{Enzyme activity (Units/mgP)} = \frac{(\Delta A340/\text{min}_{\text{sample}} - \Delta A340/\text{min}_{\text{blanko}}) \text{ x TV}}{12{,}44 \text{ x V x } 0{,}6 \text{ x LP}}$$

Where: TV = Total volume of the reaction in ml (1 ml for cuvettes and 0.2 ml for plates); V = volume of enzyme used in the assay (ml); LP = Light path in cm (1 for cuvettes).

$$\text{Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)} \text{ x } 100\%$$

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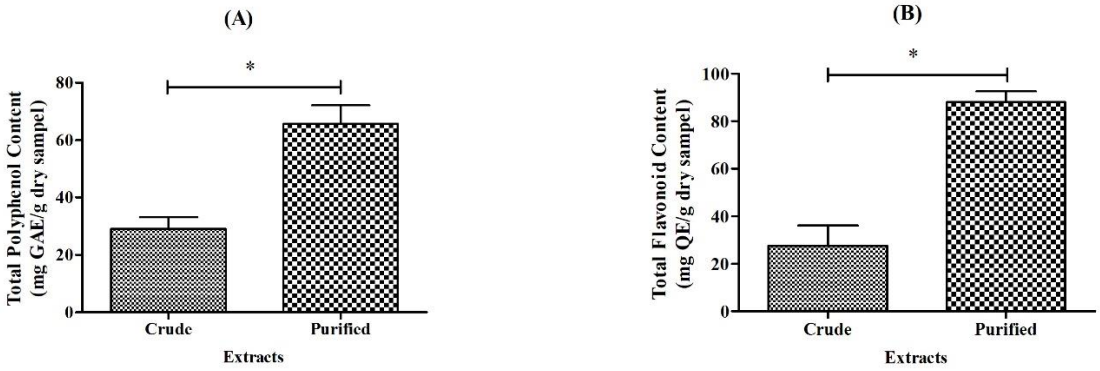
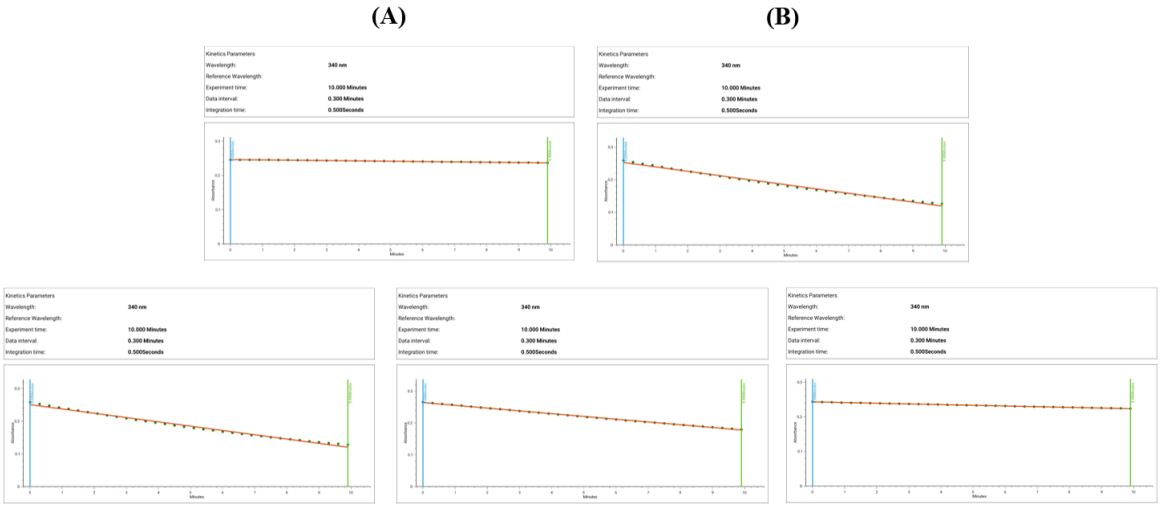


Figure 1: Total polyphenol **(A)** and flavonoid **(B)** content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (*n*=3). Statistically significance at **p*<0.05 versus purified extract.

HMG-CoA reductase inhibitory activity

Figure 2 showed the representative reduction of absorbance at 340 nm during the measurement of the HMG-CoA reductase activity assay. After the calculation, it was found that purified extract with a value 34.74±5.40% showed HMG-CoA reductase activity significantly higher than crude extract (2.61±0.28%) as shown in **Figure 3**.



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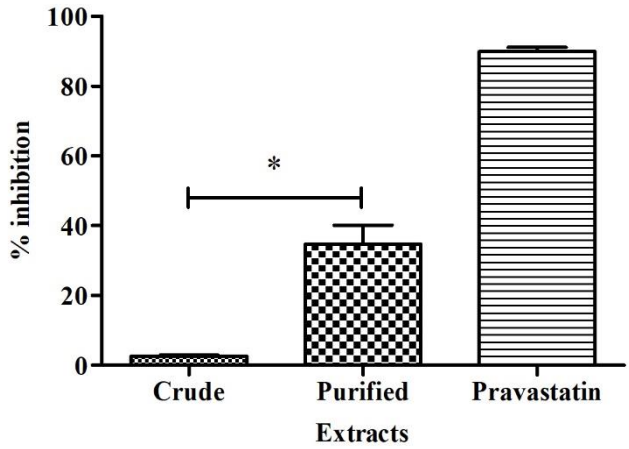

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Conclusion

The purified extract of water lettuce showed a high polyphenol and flavonoid contents when compared to the crude extract. The 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitory activity of the purified extract was also higher when compared to crude extract. Therefore, the purified extract of polyphenol compounds from water lettuce (*Pistia stratiotes*) has a potential to be developed as an alternative anti-hypercholesterolemia agent.

DECLARATIONS

Acknowledgment

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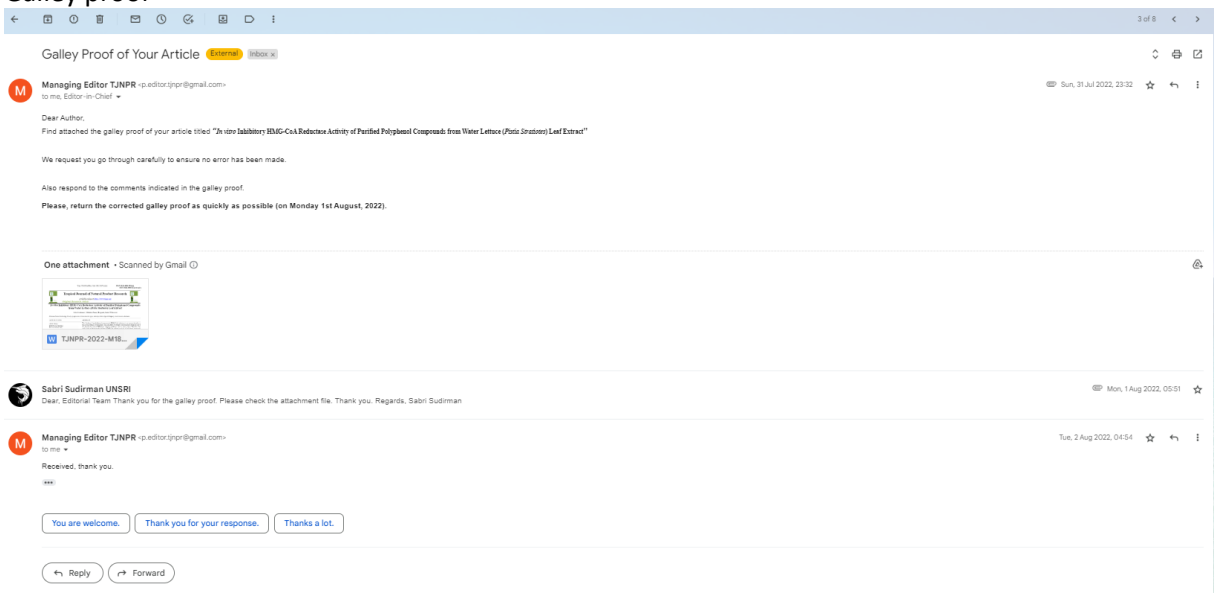

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Original Research Article

In vitro Inhibitory HMG-CoA Reductase Activity of Purified Polyphenol Compounds from Water Lettuce (*Pistia Stratiotes*) Leaf Extract

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ABSTRACT

The 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase is an enzyme that plays a role in the synthesis of cholesterol. The side effect of synthetic anti-cholesterol drugs has led to the demand for natural HMG-CoA reductase inhibitors such as plant extract. Therefore, this study aimed to determine the inhibitory HMG-CoA reductase activity of polyphenol compounds from water lettuce (*Pistia stratiotes*) leaf extract. The research was conducted using the crude and purified extracts of water lettuce. The total polyphenols, flavonoids, and HMG-CoA reductase inhibitory activity assay were carried out by *in vitro* analysis. The values obtained were analyzed quantitatively, followed by the use of an independent sample t-test and presented in graphical form. The results showed that the total polyphenols in each crude and purified extracts were 29.03 mg GAE/g dry sample and 65.63 mg GAE/g dry sample, respectively, while the total flavonoids were 27.58 mg QE/g dry sample and 88.02 mg QE/g dry sample, respectively. The inhibitory activity of the HMG-CoA reductase enzyme showed that the purified extract showed percentage inhibition of 34.74% which was higher than that of the crude extract which was 2.61%. This indicated that the purified extract of water lettuce has higher levels of polyphenols and flavonoids that can inhibit the HMG-CoA reductase enzyme more effectively than the crude extract.

Keywords: Anti-hypercholesterolemia, HMG-CoA reductase, Polyphenol, Water lettuce.

Introduction

Currently, society's lifestyle is experiencing significant changes such as high consumption of fast food and lack of physical activity including exercise. This condition usually leads to a metabolic imbalance in the body that can cause the accumulation of fat and increase cholesterol. A previous study reported that cholesterol is a very important compound in human life. However, high cholesterol is closely linked with many other medical problems, especially elevated levels of low-density lipoprotein-cholesterol (LDL-C).[1] It was also discovered that high cholesterol levels or hypercholesterolemia can cause cardiovascular diseases (CVDs), such as atherosclerosis, stroke, and heart disease.[2]

Pharmacological management such as the consumption of statin drugs is a treatment for reducing cholesterol levels in the body by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase enzyme. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis.[3] However, the use of this drug has shown some adverse effects, which include headache, muscle pain, and digestive system problems.[4, 5] Therefore, investigations are being carried out using plant extract as a functional or food supplement to discover an alternative inhibitor of the HMG-CoA reductase.

Water lettuce (*Pistia stratiotes*) is an aquatic plant that contains some bioactive compounds, such as polyphenols, flavonoids, and tannins.[6, 7] A previous study also reported polyphenol compounds show lowering cholesterol activity by inhibiting the HMG-CoA reductase enzyme.[8]

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It was also reported that flavonoid possess the potential anti-cholesterol activity and prevent CVDs.[9] Since polyphenol extraction with organic solvent was obtained as a crude extract composed of other non-polyphenol components, such as lipid, sugar, and organic acids, a purification process is required to remove these compounds.[10] Based on this condition, the authors hypothesized that the polyphenol extract showed different activity before and after purification. Therefore, this study aimed to investigate the polyphenol content from water lettuce (*Pistia stratiotes*) and its HMG-CoA reductase activity.

Materials and Methods

Sample preparation and extraction

The water lettuce (*Pistia stratiotes*) was collected in September 2021 from Sukaraja Village, South Sumatra, Indonesia. The sample collected was authenticated at Microbiology and Biotechnology Laboratory of Fisheries Product Technology, Universitas Sriwijaya (FPT0015092022). The leaf was rinsed with distilled water and oven-dried at 45ºC until constant weight is reached. After the drying process, the sample was milled to a size of 40 mesh using a grinding machine (Microphyte disintegrator B-One DM-120M) and kept for the extraction process. The polyphenol compound was extracted by the maceration method using 70% ethanol as a solvent at room temperature for 3 hours.[7] Briefly, 20 mg of sample and 200 mL of 70% ethanol were mixed in the Erlenmeyer flask, then stirred using a magnetic stirrer. After 3 hours, the filtrate and residue were separated with a filter paper (Whatman No. 42). The filtrate was kept in a new collection tube and the residue was extracted by a fresh solvent under the same condition as the first extraction, which was carried out five repetitions. The filtrate-mixed was evaporated using a vacuum rotary evaporator at 40ºC to obtain the concentrated extract. Half of the concentrated extract was dried using a freeze dryer to obtain polyphenol extract in powder form (crude extract). Meanwhile, purified extract was obtained through a HyperSep Retain PEP cartridge.

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Purification process

The purification process was performed by solid-phase extraction (SPE) and a HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described by Pérez-Magariño *et al.*[11] Briefly, 2 mL of distilled water and then 2 mL of methanol was rinsed for cartridge preconditioned. Subsequently, 2 mL of crude extract was loaded into the cartridge and the sample was eluted by using 2 mL of n-hexane and 2 mL of 1 N H_2SO_4. The cartridge was washed with absolute methanol to obtain purified polyphenol extract in an aqueous form and dried with a freeze dryer to obtain the powder form of purified extract.

Total polyphenol and flavonoid analysis

The total polyphenol and flavonoid contents were analyzed according to Chandra *et al*.[12] Moreover, total polyphenol content was analyzed using Folin-Ciocalteu's phenol reagent. Briefly, 50 mg of dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL with distilled water. The mixture was incubated at room temperature and in dark conditions for 30 min. Subsequently, the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Total flavonoid content was analyzed using the aluminum chloride method. Briefly, 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min. After the reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC) was expressed as mg quercetin equivalent (QE) per g of dry sample.

HMG-CoA reductase inhibitory activity assay

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory activity was measured using commercial kits from Sigma-Aldrich Co. (CS1090-1KT, Merck) and performed according to the manufacturers' protocol. Briefly, 50 mg of each crude and purified extract was dissolved in assay buffer. The solution was centrifuged at 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated according to these formulas:

$$= \frac{(\Delta A340/min_{sample} - \Delta A340/min_{blanko}) \times TV}{12{,}44 \times V \times 0{,}6 \times LP} \quad \text{Enzyme activity (Units/mgP)}$$

Where: TV = Total volume of the reaction in ml (1 ml for cuvettes and 0.2 ml for plates); V = volume of enzyme used in the assay (ml); LP = Light path in cm (1 for cuvettes).

$$\text{Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{Unit}{mgP}\right) - \text{Enzyme activity with sample } \left(\frac{Unit}{mgP}\right)}{\text{Enzyme activity without sample } \left(\frac{Unit}{mgP}\right)} \times 100\%$$

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) and analyzed by one-way ANOVA with Duncan's post-hoc test ($p<0.05$) using SPSS (v.22.0; IBM Corp., Armonk, NY, USA).

Table 1: Reaction Mixture of HMG-CoA reductase inhibitory activity (for 1 mL of cuvette)

Sample	Assay buffer	Crude extract (CE	Purified extract (PE)	Pravastatin (Prava)	NADPH	HMG-CoA	HMG-CoA Reductase
Blanko	920 µL	-	-	-	20 µL	60 µL	-
HMG-CoA reduktase activity	915 µL	-	-	-	20 µL	60 µL	5 µL
CE inhibition	910 µL	5 µL	-	-	20 µL	60 µL	5 µL
PE inhibition	910 µL	-	5 µL	-	20 µL	60 µL	5 µL
Prava inhibition	910 µL	-	-	5 µL	20 µL	60 µL	5 µL

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Results and Discussion

Total polifenol and flavonoid content

The results showed that the purified extract has significantly higher total polyphenol and flavonoid contents compared to the crude extract as shown in Figure 1. Total polyphenol contents (TPC) of the crude and purified extracts were about 29.03 ± 4.15 mg GAE/g dry sample and 65.63 ± 6.51 mg GAE/g dry sample. Meanwhile, total flavonoids were about 27.58 mg QE/g dry sample and 88.02 mg QE/g dry sample, respectively. It was also found that the purified extract is majorly composed of polyphenol and flavonoid compounds due to unwanted compounds are being removed during the purification process. A previous study reported that the quality or bioactivity of crude extract was increased after purification.[13] This is because unwanted and undesirable substances have been removed, thereby improving the overall quality of the crude extract.[14] A solid-phase extraction (SPE) method was used for the purification process due to its rapid, simple, and economic.[15] The n-hexane was applied for the removal of non-polar from the crude extract and a low concentrate of sulfuric acid was used to remove non-polyphenol polar compounds, such as sugar and organic acids.[11] A previous study reported that the total polyphenol of crude extract of *Inga edulis* increased from 496.5 mg GAE/g to 518.8 mg GAE/ after purification process.[16] Furthermore, the luteolin (a flavonoid compound) increased from 0.68 mg/g to 3.52 mg/g and some flavonoids compounds of olive pomace also improved after the purification process.[17]

HMG-CoA reductase inhibitory activity

Figure 2 showed the representative reduction of absorbance at 340 nm during the measurement of the HMG-CoA reductase activity assay. After the calculation, it was found that purified extract with a value 34.74±5.40% showed HMG-CoA reductase activity significantly higher than crude extract (2.61±0.28%) as shown in Figure 3.

The increased inhibition of purified extract to HMG-CoA reductase due to it composed of high concentrations of polyphenol and flavonoid compounds. A previous study reported that polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to inhibit HMG-CoA reductase activity.[18] A previous reference also reported that isoflavon (a flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during cholesterol synthesis.[19] According to Chen *et al*., catechin (a polyphenol compound) successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.[20] Additionally, Islam *et al*.[8] reported that these compounds can block the electron transfer on the substrate HMG-CoA and bind on HMG-CoA reductase on the $NADP^+$ binding site. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis. This enzyme catalyzed HMG-CoA to

Coenzyme A and mevalonate. The mevalonate then was converted to cholesterol by the mevalonate pathway.[3] Therefore, inhibited HMG-CoA reductase is an effective way to reduce cholesterol levels in human and animal experiments.[21]

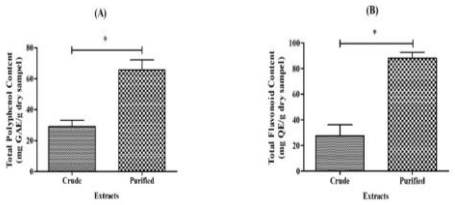

Figure 1: Total polyphenol **(A)** and flavonoid **(B)** content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (*n*=3). Statistically significance at **p*<0.05 versus purified extract.

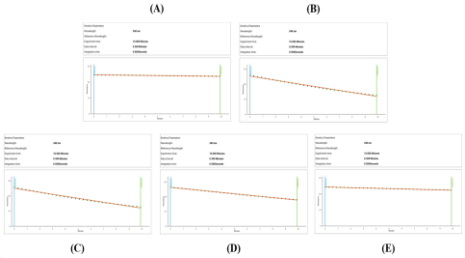

Figure 2: Representative of HMG-CoA reductase activity and inhibitory activity of sample: (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D) Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were measured at 340 nm for 10 min by a spectrophotometer.

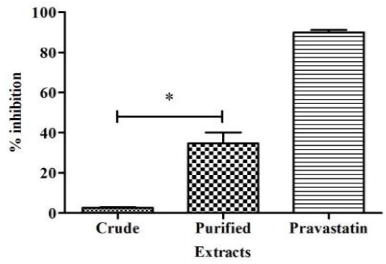

Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean ± SD (*n*=3). Statistically significance at **p*<0.05 versus purified extract.

Conclusion

The purified extract of water lettuce showed a high polyphenol and flavonoid contents when compared to the crude extract. The 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitory activity of the purified extract was also higher when compared to crude extract. Therefore, the purified extract of polyphenol compounds from water lettuce (*Pistia stratiotes*) has a potential to be developed as an alternative anti-hypercholesterolemia agent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Original Research Article

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Materials and Methods

Sample preparation and extraction

The water lettuce (*Pistia stratiotes*) was collected in September 2021 from Sukaraja Village, South Sumatra, Indonesia. The sample collected was authenticated at Microbiology and Biotechnology Laboratory of Fisheries Product Technology, Universitas Sriwijaya (FPT0015092022). The leaf was rinsed with distilled water and oven-dried at 45ºC until constant weight is reached. After the drying process, the sample was milled to a size of 40 mesh using a grinding machine (Microphyte disintegrator B-One DM-120M) and kept for the extraction process. The polyphenol compound was extracted by the maceration method using 70% ethanol as a solvent at room temperature for 3 hours.[7] Briefly, 20 mg of sample and 200 mL of 70% ethanol were mixed in the Erlenmeyer flask, then stirred using a magnetic stirrer. After 3 hours, the filtrate and residue were separated with a filter paper (Whatman No. 42). The filtrate was kept in a new collection tube and the residue was extracted by a fresh solvent under the same condition as the first extraction, which was carried out five repetitions. The filtrate-mixed was evaporated using a vacuum rotary evaporator at 40ºC to obtain the concentrated extract. Half of the concentrated extract was dried using a freeze dryer to obtain polyphenol extract in powder form (crude extract). Meanwhile, purified extract was obtained through a HyperSep Retain PEP cartridge.

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Purification process

The purification process was performed by solid-phase extraction (SPE) and a HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described by Pérez-Magariño *et al.*[11] Briefly, 2 mL of distilled water and then 2 mL of methanol was rinsed for cartridge preconditioned. Subsequently, 2 mL of crude extract was loaded into the cartridge and the sample was eluted by using 2 mL of n-hexane and 2 mL of 1 N H_2SO_4. The cartridge was washed with absolute methanol to obtain purified polyphenol extract in an aqueous form and dried with a freeze dryer to obtain the powder form of purified extract.

Total polyphenol and flavonoid analysis

The total polyphenol and flavonoid contents were analyzed according to Chandra *et al.*[12] Moreover, total polyphenol content was analyzed using Folin-Ciocalteu's phenol reagent. Briefly, 50 mg of dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL with distilled water. The mixture was incubated at room temperature and in dark conditions for 30 min. Subsequently, the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Total flavonoid content was analyzed using the aluminum chloride method. Briefly, 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min. After the reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC) was expressed as mg quercetin equivalent (QE) per g of dry sample.

HMG-CoA reductase inhibitory activity assay

The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory activity was measured using commercial kits from Sigma-Aldrich Co. (CS1090-1KT, Merck) and performed according to the manufacturers' protocol. Briefly, 50 mg of each crude and purified extract was dissolved in assay buffer. The solution was centrifuged at 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated according to these formulas:

$$= \frac{(\Delta A340/min_{sample} - \Delta A340/min_{blanko}) \times TV}{12{,}44 \times V \times 0{,}6 \times LP} \quad \text{Enzyme activity (Units/mgP)}$$

Where: TV = Total volume of the reaction in ml (1 ml for cuvettes and 0.2 ml for plates); V = volume of enzyme used in the assay (ml); LP = Light path in cm (1 for cuvettes).

$$\text{Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)} \times 100\%$$

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) and analyzed by one-way ANOVA with Duncan's post-hoc test ($p<0.05$) using SPSS (v.22.0; IBM Corp., Armonk, NY, USA).

Table 1: Reaction Mixture of HMG-CoA reductase inhibitory activity (for 1 mL of cuvette)

Sample	Assay buffer	Crude extract (CE	Purified extract (PE)	Pravastatin (Prava)	NADPH	HMG-CoA	HMG-CoA Reductase
Blanko	920 µL	-	-	-	20 µL	60 µL	-
HMG-CoA reduktase activity	915 µL	-	-	-	20 µL	60 µL	5 µL
CE inhibition	910 µL	5 µL	-	-	20 µL	60 µL	5 µL
PE inhibition	910 µL	-	5 µL	-	20 µL	60 µL	5 µL
Prava inhibition	910 µL	-	-	5 µL	20 µL	60 µL	5 µL

Results and Discussion

Total polifenol and flavonoid content

The results showed that the purified extract has significantly higher total polyphenol and flavonoid contents compared to the crude extract as shown in Figure 1. Total polyphenol contents (TPC) of the crude and purified extracts were about 29.03 ± 4.15 mg GAE/g dry sample and 65.63 ± 6.51 mg GAE/g dry sample. Meanwhile, total flavonoids were about 27.58 mg QE/g dry sample and 88.02 mg QE/g dry sample, respectively. It was also found that the purified extract is majorly composed of polyphenol and flavonoid compounds due to unwanted compounds are being removed during the purification process. A previous study reported that the quality or bioactivity of crude extract was increased after purification.[13] This is because unwanted and undesirable substances have been removed, thereby improving the overall quality of the crude extract.[14] A solid-phase extraction (SPE) method was used for the purification process due to its rapid, simple, and economic.[15] The n-hexane was applied for the removal of non-polar from the crude extract and a low concentrate of sulfuric acid was used to remove non-polyphenol polar compounds, such as sugar and organic acids.[11] A previous study reported that the total polyphenol of crude extract of *Inga edulis* increased from 496.5 mg GAE/g to 518.8 mg GAE/ after purification process.[16] Furthermore, the luteolin (a flavonoid compound) increased from 0.68 mg/g to 3.52 mg/g and some flavonoids compounds of olive pomace also improved after the purification process.[17]

HMG-CoA reductase inhibitory activity

Figure 2 showed the representative reduction of absorbance at 340 nm during the measurement of the HMG-CoA reductase activity assay. After the calculation, it was found that purified extract with a value 34.74±5.40% showed HMG-CoA reductase activity significantly higher than crude extract (2.61±0.28%) as shown in Figure 3.

The increased inhibition of purified extract to HMG-CoA reductase due to it composed of high concentrations of polyphenol and flavonoid compounds. A previous study reported that polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to inhibit HMG-CoA reductase activity.[18] A previous reference also reported that isoflavon (a flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during cholesterol synthesis.[19] According to Chen *et al.*, catechin (a polyphenol compound) successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.[20] Additionally, Islam *et al.*[8] reported that these compounds can block the electron transfer on the substrate HMG-CoA and bind on HMG-CoA reductase on the $NADP^+$ binding site. The HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis. This enzyme catalyzed HMG-CoA to

Coenzyme A and mevalonate. The mevalonate then was converted to cholesterol by the mevalonate pathway.[3] Therefore, inhibited HMG-CoA reductase is an effective way to reduce cholesterol levels in human and animal experiments.[21]

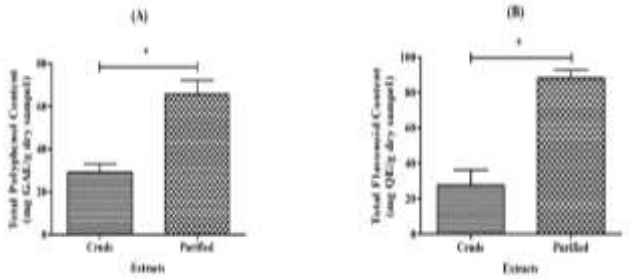

Figure 1: Total polyphenol **(A)** and flavonoid **(B)** content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (*n*=3). Statistically significance at **p*<0.05 versus purified extract.

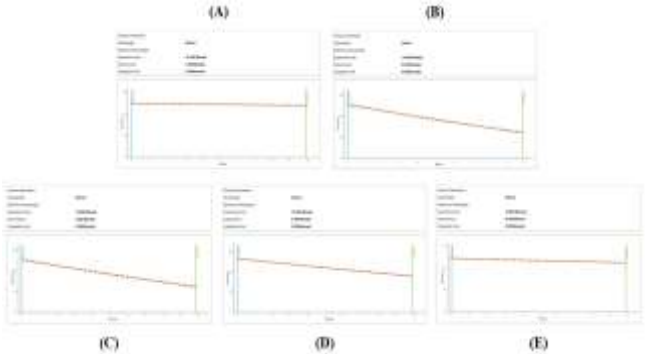

Figure 2: Representative of HMG-CoA reductase activity and inhibitory activity of sample: (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D) Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were measured at 340 nm for 10 min by a spectrophotometer.

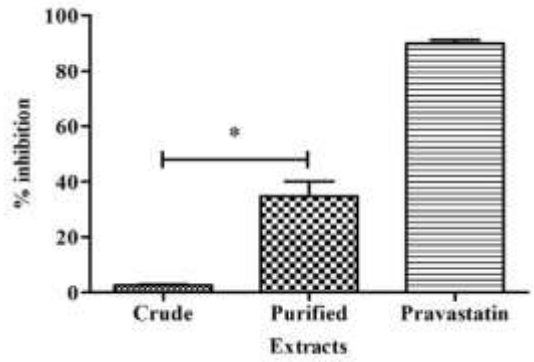

Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean ± SD (*n*=3). Statistically significance at **p*<0.05 versus purified extract.

Conclusion

The purified extract of water lettuce showed a high polyphenol and flavonoid contents when compared to the crude extract. The 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitory activity of the purified extract was also higher when compared to crude extract. Therefore, the purified extract of polyphenol compounds from water lettuce (*Pistia stratiotes*) has a potential to be developed as an alternative anti-hypercholesterolemia agent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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