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

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The peer-review process will commence immediately, as the manuscript will be passed to an editor for initial assessment as soon as possible.

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

Best regards,
Abiodun

Professor Abiodun Falodun, PhD
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COVER LETTER

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Indonesia

[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.

Please address all correspondence concerning this manuscript to me at sabrisudirman@unsri.ac.id.

Thank you for your consideration of this manuscript.

Sincerely,



Sabri Sudirman

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

3

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10 **Abstract**

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

27

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

29 **Introduction**

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

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

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

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

56

57 **Materials and Methods**

58 *Sample preparation and extraction*

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

72

73 *Purification process*

74 The purification process was performed by solid-phase extraction (SPE) and a
75 HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described
76 by a previous method.¹¹ Briefly, 2 mL of dH₂O and then 2 mL of methanol were rinsed for
77 cartridge preconditioned. Then, 2 mL of crude extract was loaded into the cartridge. The
78 sample was eluted by using 2 mL of n-hexane and then 2 mL of 1 N H₂SO₄. The cartridge

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

81

82 *Total polyphenol and flavonoid analysis*

83 The total polyphenol and flavonoid contents were analyzed according to Chandra *et*
84 *al.*¹² Total polyphenol content was analyzed by using Folin-Ciocalteu's phenol reagent.
85 Briefly, 50 mg dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this
86 solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture
87 was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL by dH₂O. The
88 mixture then was incubated at room temperature and in dark conditions for 30 min. After that,
89 the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150
90 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the
91 total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry
92 sample.

93 Total flavonoid content was analyzed by using the aluminum chloride method. Briefly,
94 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed
95 with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min.
96 After reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the
97 absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific,
98 Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC)
99 was expressed as mg quercetin equivalent (QE) per g of dry sample.

100

101 *HMG-CoA reductase inhibitory activity assay*

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

104 Merck) and performed according to the manufacture protocol. Briefly, 50 mg of each extract
 105 (crude and purified extract) was dissolved in Assay buffer. The solution was centrifuged at
 106 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of
 107 each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the
 108 Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a
 109 spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of
 110 inhibitor were calculated according to these formulas:

111

$$112 \text{ Enzyme activity (Units/mgP)} = \frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blanko}}) \times \text{TV}}{12,44 \times V \times 0,6 \times \text{LP}}$$

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

115

$$116 \text{ Inhibition (\%)} = \frac{\text{Enzyme activity without sample} \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample} \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample} \left(\frac{\text{Unit}}{\text{mgP}}\right)} \times 100\%$$

117

118 **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 reductase 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

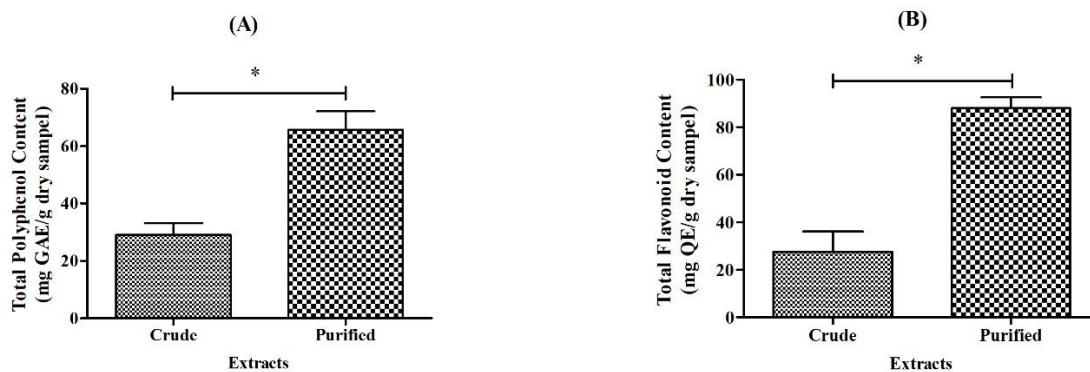
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120 Results and Discussion

121 *Total polifenol and flavonoid content*

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

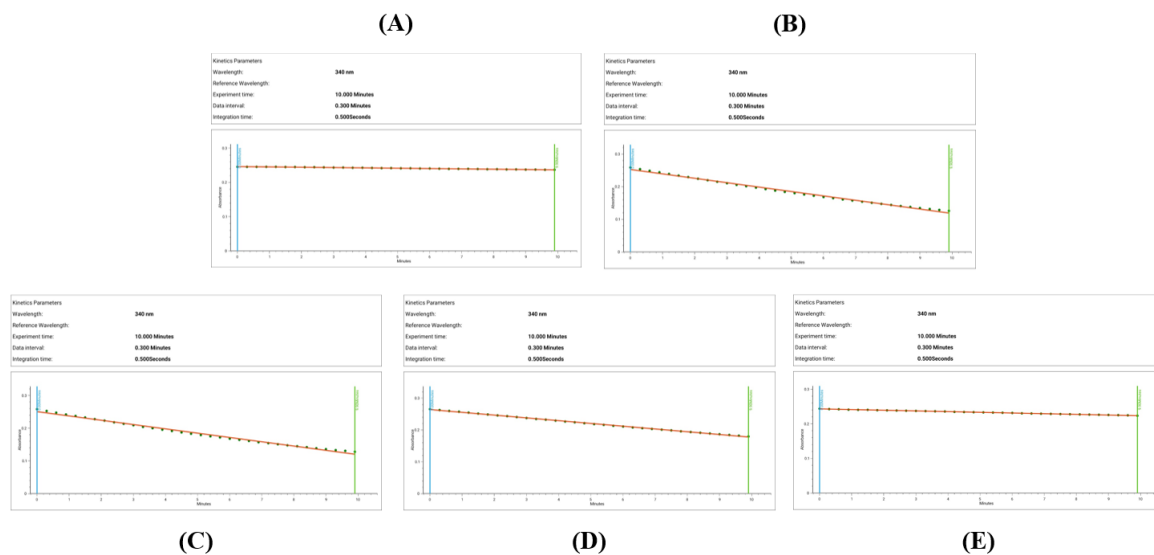
124 Total polyphenol contents (TPC) were about 29.03±4.15 mg GAE/g dry sample (crude
125 extract) and 65.63±6.51 mg GAE/g dry sample (purified extract). Whereas, total flavonoids
126 were about 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample
127 (purified extract). The purified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



138
139 **Figure 1:** Total polyphenol (A) and flavonoid (B) content of crude and purified extract of
140 water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean ± SD (n=3). Statistically
141 significance at *p<0.05 versus purified extract.

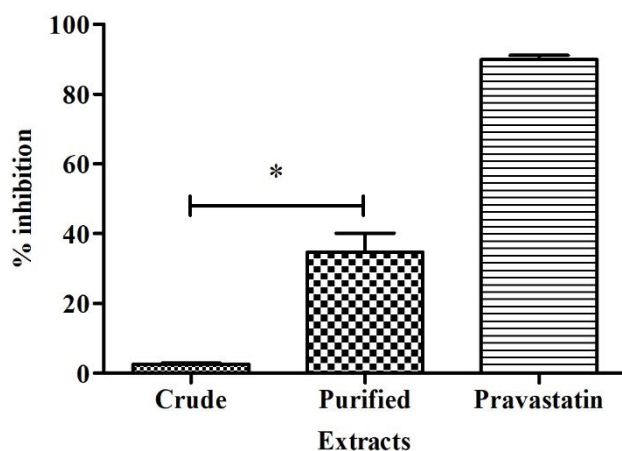
142
143 *HMG-CoA reductase inhibitory activity*

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



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

153



154

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

158

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

173

174 **Conclusion**

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

180

181 **DECLARATIONS**

182 **Acknowledgment**

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187

188 **Conflict of interest**

189 No conflict of interest associated with this work.

190

191 **Contribution of authors**

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

195

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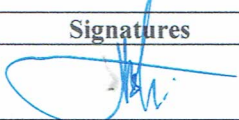
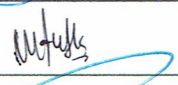


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No one who has contributed significantly to the work has been denied authorship and those who helped have been duly acknowledged.

I/we also agree to the authorship of the article in the following sequence:

	Author's names	Signatures	Date
1	Sabri Sudirman		2-06-2022
2	Miftahul Janna		2-06-2022
3	Herpandi		2-06-2022
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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

4 **Abstract**

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

21
22 **Keywords:** Anti-hypercholesterolemia, HMG-CoA reductase, Polyphenol, Water lettuce.

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23 Introduction

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

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

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

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

50

51 **Materials and Methods**

52 *Sample preparation and extraction*

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

66

67 *Purification process*

68 The purification process was performed by solid-phase extraction (SPE) and a
69 HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described
70 by a previous method.¹¹ Briefly, 2 mL of dH₂O and then 2 mL of methanol were rinsed for
71 cartridge preconditioned. Then, 2 mL of crude extract was loaded into the cartridge. The
72 sample was eluted by using 2 mL of n-hexane and then 2 mL of 1 N H₂SO₄. The cartridge

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

75

76 *Total polyphenol and flavonoid analysis*

77 The total polyphenol and flavonoid contents were analyzed according to Chandra *et*
78 *al.*¹² Total polyphenol content was analyzed by using Folin-Ciocalteu's phenol reagent.
79 Briefly, 50 mg dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this
80 solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture
81 was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL by dH₂O. The
82 mixture then was incubated at room temperature and in dark conditions for 30 min. After that,
83 the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150
84 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the
85 total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry
86 sample.

87 Total flavonoid content was analyzed by using the aluminum chloride method. Briefly,
88 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed
89 with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min.
90 After reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the
91 absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific,
92 Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC)
93 was expressed as mg quercetin equivalent (QE) per g of dry sample.

94

95 *HMG-CoA reductase inhibitory activity assay*

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

98 Merck) and performed according to the manufacture protocol. Briefly, 50 mg of each extract
 99 (crude and purified extract) was dissolved in Assay buffer. The solution was centrifuged at
 100 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of
 101 each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the
 102 Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a
 103 spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of
 104 inhibitor were calculated according to these formulas:

105

$$106 \text{ Enzyme activity (Units/mgP)} = \frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blanko}}) \times TV}{12,44 \times V \times 0,6 \times LP}$$

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

109

$$110 \text{ Inhibition (\%)} = \frac{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample } \left(\frac{\text{Unit}}{\text{mgP}}\right)} \times 100\%$$

111

112 **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 reductase 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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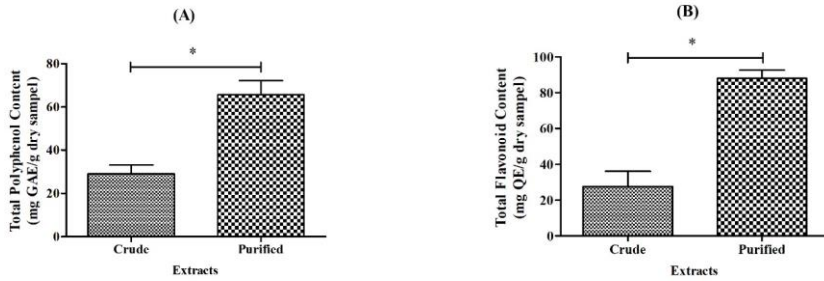
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114 **Results and Discussion**

115 *Total polyphenol and flavonoid content*

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

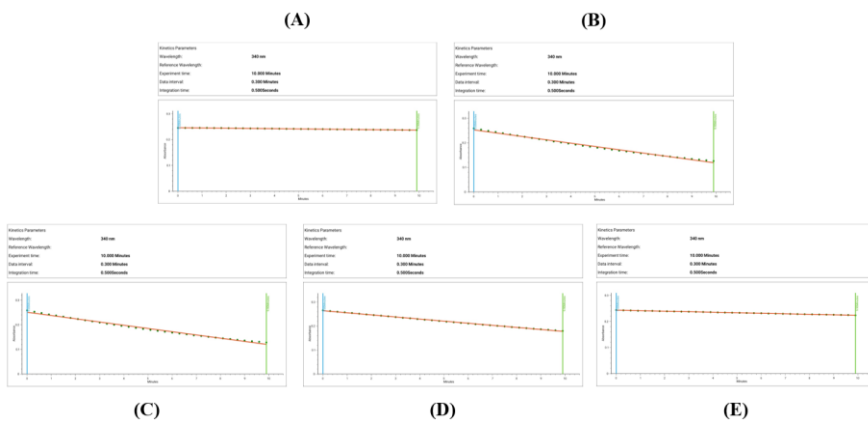
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132
 133 **Figure 1:** Total polyphenol (A) and flavonoid (B) content of crude and purified extract of
 134 water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean \pm SD ($n=3$). Statistically
 135 significance at $*p<0.05$ versus purified extract.

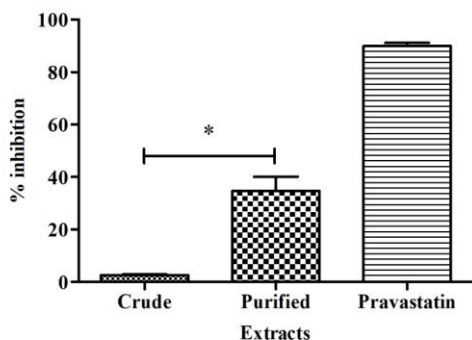
136
 137 *HMG-CoA reductase inhibitory activity*

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



142
 143 **Figure 2:** Representative of HMG-CoA reductase activity and inhibitory activity of sample:
 144 (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D)

145 Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive
146 control). These activities were measured at 340 nm for 10 min by a spectrophotometer.
147



148
149 **Figure 3:** HMG-CoA reductase inhibitory activities of crude extract, purified extract, and
150 pravastatin (as positive control). Data are shown as the mean \pm SD ($n=3$). Statistically
151 significance at $*p<0.05$ versus purified extract.

152
153 High inhibition of purified extract to HMG-CoA reductase due to this extract composed
154 of high concentrate polyphenol and flavonoid compounds. A previous study reported that
155 polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to
156 inhibit HMG-CoA reductase activity.¹⁷ A previous reference also reported that isoflavon (a
157 flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during
158 cholesterol synthesis.¹⁸ Chen *et al.* also reported that catechin (a polyphenol compound)
159 successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.¹⁹

160 Additionally, Islam *et al.* reported that polyphenol and flavonoid compounds can block the
161 electron transfer on the substrate HMG-CoA and bind on HMG-CoA reductase on the NADP⁺
162 binding site.⁸ The HMG-CoA reductase is an important enzyme that is involved in cholesterol
163 synthesis. This enzyme catalyzed HMG-CoA to Coenzyme A and mevalonate. The

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

167

168 **Conclusion**

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

174

175 **DECLARATIONS**

176 **Acknowledgment**

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178 Indonesia Directorate General of Higher Education, Research and Technology Project,
179 Ministry of Education, Culture, Research, and Technology, SPPK No.
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181

182 **Conflict of interest**

183 No conflict of interest associated with this work.

184

185 **Contribution of authors**

186 The authors declare that this work was done by the authors named in this article and all
187 liabilities pertaining to claims relating to the content of this article will be borne by the
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189

190 **REFERENCES**

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

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10 **Abstract**

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

27

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

29 **Introduction**

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

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

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

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

56

57 **Materials and Methods**

58 *Sample preparation and extraction*

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

74

75 *Purification process*

76 The purification process was performed by solid-phase extraction (SPE) and a
77 HyperSep Retain PEP cartridge (Part. No. 60107-212, Thermofisher Scientific) as described
78 by a previous method.¹¹ Briefly, 2 mL of dH₂O and then 2 mL of methanol were rinsed for

79 cartridge preconditioned. Then, 2 mL of crude extract was loaded into the cartridge. The
80 sample was eluted by using 2 mL of n-hexane and then 2 mL of 1 N H₂SO₄. The cartridge
81 was washed with absolute methanol to obtain purified polyphenol extract in an aqueous form.
82 Then, it was dried with a freeze dryer to obtain the powder form of purified extract.

83

84 *Total polyphenol and flavonoid analysis*

85 The total polyphenol and flavonoid contents were analyzed according to Chandra *et*
86 *al.*¹² Total polyphenol content was analyzed by using Folin-Ciocalteu's phenol reagent.
87 Briefly, 50 mg dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of this
88 solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the mixture
89 was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL by dH₂O. The
90 mixture then was incubated at room temperature and in dark conditions for 30 min. After that,
91 the absorbance was measured at 765 nm by a spectrophotometer (Genesys 150
92 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and the
93 total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of dry
94 sample.

95 Total flavonoid content was analyzed by using the aluminum chloride method. Briefly,
96 50 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed
97 with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min.
98 After reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that, the
99 absorbance was measured at 420 nm by a spectrophotometer (Genesys 150 ThermoScientific,
100 Massachusetts, USA). The quercetin was used as a standard and total flavonoid content (TFC)
101 was expressed as mg quercetin equivalent (QE) per g of dry sample.

102

103 *HMG-CoA reductase inhibitory activity assay*

104 The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory
 105 activity was measured by using commercial kits from Sigma-Aldrich Co. (CS1090-1KT,
 106 Merck) and performed according to the manufacture protocol. Briefly, 50 mg of each extract
 107 (crude and purified extract) was dissolved in Assay buffer. The solution was centrifuged at
 108 3,000 rpm for 5 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of
 109 each extract and 0.5 µL of pravastatin were pipetted into reaction tubes according to the
 110 Reaction Mixed (**Table 1**). The reaction was observed at 340 nm by using a
 111 spectrophotometer every 20 s for 10 min. The HMG-CoA reductase activity and percentage of
 112 inhibitor were calculated according to these formulas:

113

$$114 \text{ Enzyme activity (Units/mgP)} = \frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blanko}}) \times TV}{12,44 \times V \times 0,6 \times LP}$$

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

117

$$118 \text{ Inhibition (\%)} = \frac{\text{Enzyme activity without sample} \left(\frac{\text{Unit}}{\text{mgP}}\right) - \text{Enzyme activity with sample} \left(\frac{\text{Unit}}{\text{mgP}}\right)}{\text{Enzyme activity without sample} \left(\frac{\text{Unit}}{\text{mgP}}\right)} \times 100\%$$

119

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

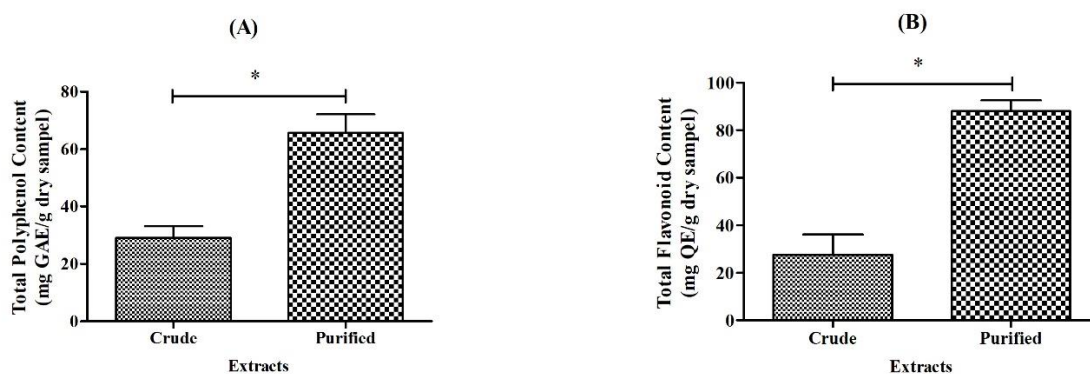
Sample	Assay buffer	Crude extract (CE)	Purified extract (PE)	Pravastatin (Prava)	NADPH	HMG-CoA	HMG-CoA Reductase
Blanko	920 µL	-	-	-	20 µL	60 µL	-
HMG-CoA reductase activity	915 µL	-	-	-	20 µL	60 µL	5 µL
CE inhibition	910 µL	5 µL	-	-	20 µL	60 µL	5 µL
PE inhibition	910 µL	-	5 µL	-	20 µL	60 µL	5 µL
Prava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

121

122 Results and Discussion

123 *Total polifenol and flavonoid content*

124 In the present study, we found that purified extract shows significantly higher total
125 polyphenol and flavonoid contents when compared to crude extract as shown in **Figure 1**.
126 Total polyphenol contents (TPC) were about 29.03 ± 4.15 mg GAE/g dry sample (crude
127 extract) and 65.63 ± 6.51 mg GAE/g dry sample (purified extract). Whereas, total flavonoids
128 were about 27.58 mg QE/g dry sample (crude extract) and 88.02 mg QE/g dry sample
129 (purified extract). The purified extract is majorly composed of polyphenol and flavonoid
130 compounds due to unwanted compounds being removed during the purification process. A
131 previous study reported that the quality or bioactivity of crude extract was increased after
132 purification.¹³ The bioactivity will increase after purification because unwanted and
133 undesirable substances would have been screened out thereby improving the overall quality of
134 the crude extract.¹⁴ A solid-phase extraction (SPE) method was used for the purification
135 process due to its rapid, simple, and economic.¹⁵ The n-hexane was used to remove non-polar
136 from the crude extract and a low concentrate of sulfuric acid was used to remove non-
137 polyphenol polar compounds, such as sugar and organic acids.¹¹ A previous study reported
138 that the total polyphenol of crude extract of *Inga edulis* increased from 496.5 mg GAE/g to
139 518.8 mg GAE/ after purification process.¹⁶ Additionally, the luteolin (a flavonoid compound)
140 increased from 0.68 mg/g to 3.52 mg/g and some flavonoids compounds of olive pomace also
141 increased after purification process.¹⁷

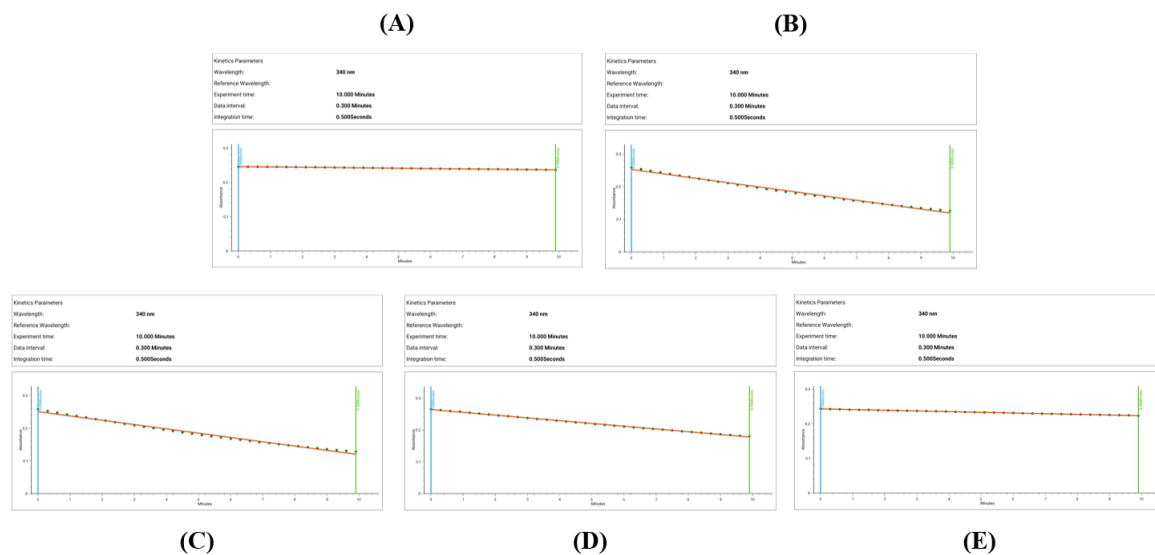


143 **Figure 1:** Total polyphenol (A) and flavonoid (B) content of crude and purified extract of
144 water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean \pm SD ($n=3$). Statistically
145 significance at $*p<0.05$ versus purified extract.

146

147 *HMG-CoA reductase inhibitory activity*

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



152

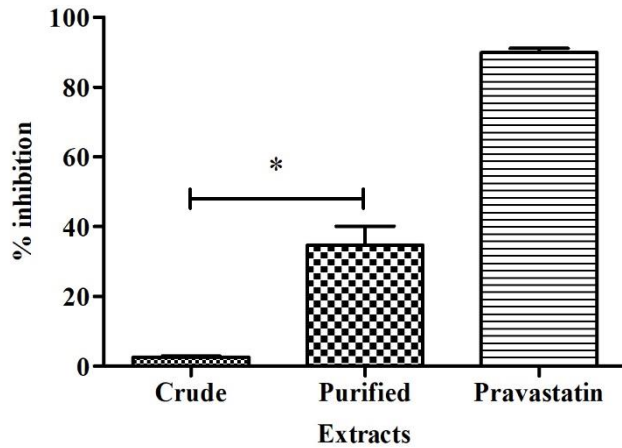
153 **Figure 2:** Representative of HMG-CoA reductase activity and inhibitory activity of sample:

154 (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D)

155 Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive

156 control). These activities were measured at 340 nm for 10 min by a spectrophotometer.

157



158

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

162

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

177

178 **Conclusion**

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

184

185 **DECLARATIONS**

186 **Acknowledgment**

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188 Indonesia Directorate General of Higher Education, Research and Technology Project,
189 Ministry of Education, Culture, Research, and Technology, SPPK No.
190 142/E5/PG.02.00PT/2022.

191

192 **Conflict of interest**

193 No conflict of interest associated with this work.

194

195 **Authors' Declaration**

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

198

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Manuscript 2nd revision

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Revision to Your Manuscript

Managing Editor TJNPR

to Mr. Editor-in-Chief

Abstract: use a more suitable conclusion that best satisfies the aim of the study.
Page 3, line 6: rephrase to clarify.
Page 3, line 8: hypercholesterolemia 'can lead to' cardiovascular diseases
Page 4, line 6: include theoucher number of the plant sample.
Page 4, line 8: using what? State the equipment used as well as the country and model.
Page 4, line 23: whose method? Also, please clarify OH_2 .
Include reference for the HMG-CoA reductase inhibitors activity assay as well as the formula.
Include a section for statistical analysis.
Page 7, line 3: avoid the use of personal pronouns, use reported speech. Apply across the manuscript.
Page 10, line 8, please correct grammatical error.
Conclusion: include recommendations if any.

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Please seek the assistance of an English editor for the English editing and proper formatting. Prof Shittu (olajekan.shittu@uniben.edu) or any other English editor may assist you. Send the revised copy and the certificate of English editing when submitting the revised **manuscript on or before 24th July 2022**.

Best regards
Abiodun

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10 **Abstract**

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24 the purified extract can inhibit with 34.74% higher than the crude extract which is 2.61%.
25 This indicated that the purified extract of water lettuce (*Pistia stratiotes*) has higher levels of
26 polyphenols and flavonoids that can inhibit the HMG-CoA reductase enzyme more effectively
27 than crude extract.

28

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

30 **Introduction**

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

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

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

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

56

57 **Materials and Methods**

58 *Sample preparation and extraction*

59 The water lettuce (*Pistia stratiotes*) was collected in September 2021 from Sukaraja
60 Village, South Sumatra, Indonesia. The sample collected was authenticated at Microbiology
61 and Biotechnology Laboratory of Fisheries Product Technology, Universitas Sriwijaya
62 (FPT0015092022). The leaf was rinsed with distilled water and oven-dried at 45°C until
63 constant weight is reached. After the drying process, the sample was milled to a size of 40
64 mesh using a grinding machine (Microphyte disintegrator B-One DM-120M) and kept for the
65 extraction process. The polyphenol compound was extracted by the maceration method using
66 70% ethanol as a solvent at room temperature for 3 hours.⁷ Briefly, 20 mg of sample and 200
67 mL of 70% ethanol were mixed in the Erlenmeyer flask, then stirred using a magnetic stirrer.
68 After 3 hours, the filtrate and residue were separated with a filter paper (Whatman No. 42).
69 The filtrate was kept in a new collection tube and the residue was extracted by a fresh solvent
70 under the same condition as the first extraction, which was carried out five repetitions. The
71 filtrate-mixed was evaporated using a vacuum rotary evaporator at 40°C to obtain the
72 concentrated extract. Half of the concentrated extract was dried using a freeze dryer to obtain
73 polyphenol extract in powder form (crude extract). Meanwhile, purified extract was obtained
74 through a HyperSep Retain PEP cartridge.

75

76 *Purification process*

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

79 by Pérez-Magariño *et al.*¹¹ Briefly, 2 mL of distilled water and then 2 mL of methanol was
80 rinsed for cartridge preconditioned. Subsequently, 2 mL of crude extract was loaded into the
81 cartridge and the sample was eluted by using 2 mL of n-hexane and 2 mL of 1 N H₂SO₄. The
82 cartridge was washed with absolute methanol to obtain purified polyphenol extract in an
83 aqueous form and dried with a freeze dryer to obtain the powder form of purified extract.

84

85 *Total polyphenol and flavonoid analysis*

86 The total polyphenol and flavonoid contents were analyzed according to Chandra *et*
87 *al.*¹² Moreover, total polyphenol content was analyzed using Folin-Ciocalteu's phenol
88 reagent. Briefly, 50 mg of dried extract was dissolved in 10 mL of methanol. Then 0.2 mL of
89 this solution was mixed with 0.2 mL Folin-Ciocalteu's phenol reagent. After 5 min, the
90 mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL with
91 distilled water. The mixture was incubated at room temperature and in dark conditions for 30
92 min. Subsequently, the absorbance was measured at 765 nm by a spectrophotometer (Genesys
93 150 ThermoScientific, Massachusetts, USA). The gallic acid (GA) was used as a standard and
94 the total polyphenol content (TPC) was expressed as mg gallic acid equivalent (GAE) per g of
95 dry sample.

96 Total flavonoid content was analyzed using the aluminum chloride method. Briefly, 50
97 mg extract was dissolved in 10 mL of methanol. Then, 1 mL of extract solution was mixed
98 with 1 mL of 2% aluminum chloride and allowed to react at room temperature for 60 min.
99 After the reaction time, 1 mL of mixture was made up to 10 mL and kept for 5 min. After that,
100 the absorbance was measured at 420 nm by a spectrophotometer (Genesys 150
101 ThermoScientific, Massachusetts, USA). The quercetin was used as a standard and total
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103

104 *HMG-CoA reductase inhibitory activity assay*

105 The 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitory
 106 activity was measured using commercial kits from Sigma-Aldrich Co. (CS1090-1KT, Merck)
 107 and performed according to the manufacturers' protocol. Briefly, 50 mg of each crude and
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 109 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and
 110 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed
 111 (Table 1). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for
 112 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated
 113 according to these formulas:

114

115 Enzyme activity (Units/mgP) =
$$\frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blanko}}) \times \text{TV}}{12,44 \times V \times 0,6 \times \text{LP}}$$

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

118

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

120

121 **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 reductase 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

122

123 *Statistical analysis*

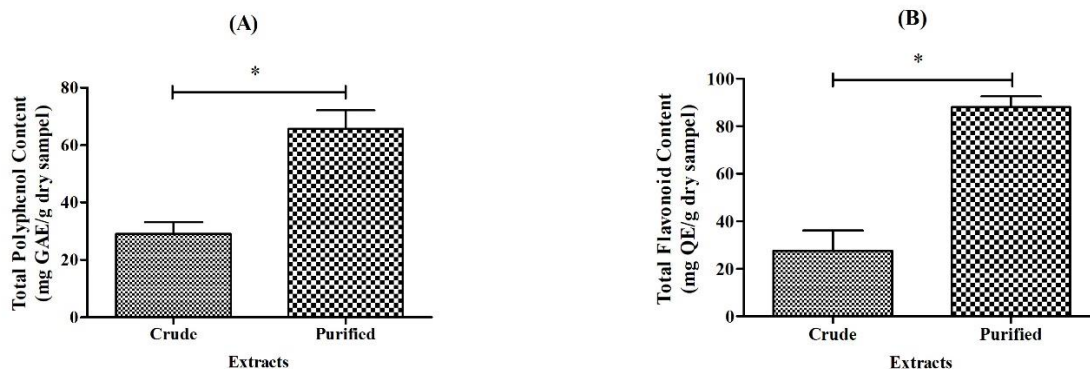
124 All data are expressed as the mean \pm standard deviation (SD) and analyzed by one-way
125 ANOVA with Duncan's post-hoc test ($p < 0.05$) using SPSS (v.22.0; IBM Corp., Armonk, NY,
126 USA).

127

128 **Results and Discussion**

129 *Total polyphenol and flavonoid content*

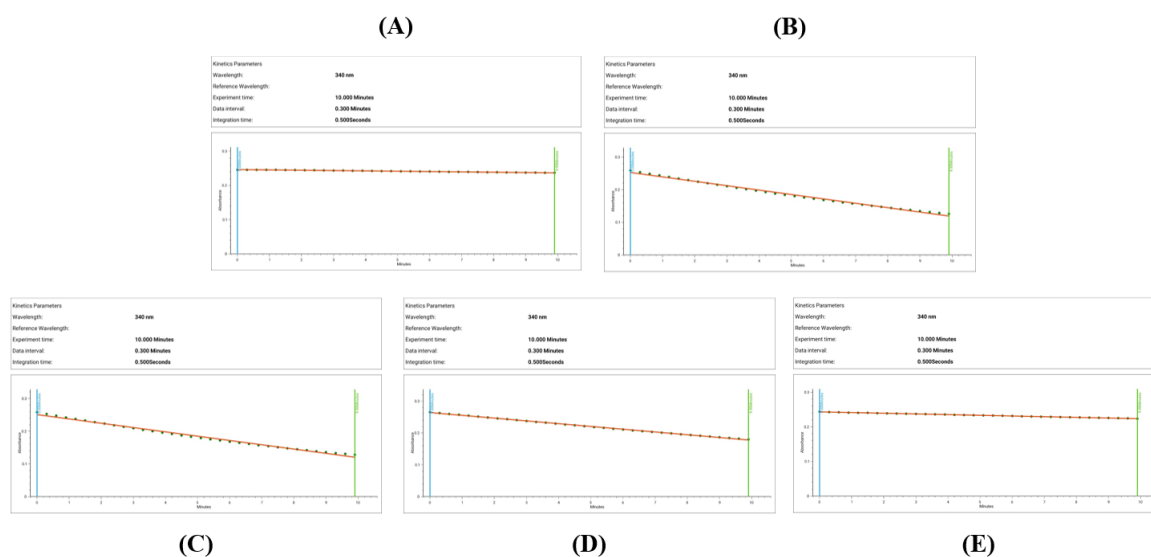
130 The results showed that the purified extract has significantly higher total polyphenol
131 and flavonoid contents compared to the crude extract as shown in **Figure 1**. Total polyphenol
132 contents (TPC) of the crude and purified extracts were about 29.03 ± 4.15 mg GAE/g dry
133 sample and 65.63 ± 6.51 mg GAE/g dry sample. Meanwhile, total flavonoids were about 27.58
134 mg QE/g dry sample and 88.02 mg QE/g dry sample, respectively. It was also found that the
135 purified extract is majorly composed of polyphenol and flavonoid compounds due to
136 unwanted compounds are being removed during the purification process. A previous study
137 reported that the quality or bioactivity of crude extract was increased after purification.¹³ This
138 is because unwanted and undesirable substances have been removed, thereby improving the
139 overall quality of the crude extract.¹⁴ A solid-phase extraction (SPE) method was used for the
140 purification process due to its rapid, simple, and economic.¹⁵ The n-hexane was applied for
141 the removal of non-polar from the crude extract and a low concentrate of sulfuric acid was
142 used to remove non-polyphenol polar compounds, such as sugar and organic acids.¹¹ A
143 previous study reported that the total polyphenol of crude extract of *Inga edulis* increased
144 from 496.5 mg GAE/g to 518.8 mg GAE/ after purification process.¹⁶ Furthermore, the
145 luteolin (a flavonoid compound) increased from 0.68 mg/g to 3.52 mg/g and some flavonoids
146 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 \pm SD ($n=3$). Statistically
 150 significance at $*p < 0.05$ versus purified extract.

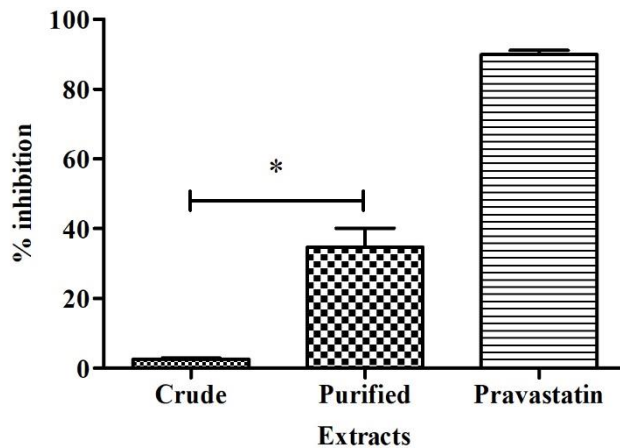
151
 152 *HMG-CoA reductase inhibitory activity*

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 $34.74 \pm 5.40\%$ showed HMG-CoA reductase activity
 156 significantly higher than crude extract ($2.61 \pm 0.28\%$) as shown in **Figure 3**.



157 (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D)
 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)

160 Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive
161 control). These activities were measured at 340 nm for 10 min by a spectrophotometer.
162



163
164 **Figure 3:** HMG-CoA reductase inhibitory activities of crude extract, purified extract, and
165 pravastatin (as positive control). Data are shown as the mean \pm SD ($n=3$). Statistically
166 significance at $*p<0.05$ versus purified extract.

167
168 The increased inhibition of purified extract to HMG-CoA reductase due to it composed
169 of high concentrations of polyphenol and flavonoid compounds. A previous study reported
170 that polyphenols and flavonoids from Malabar spinach (*Basella alba*) leaf have the ability to
171 inhibit HMG-CoA reductase activity.¹⁸ A previous reference also reported that isoflavon (a
172 flavonoid compound) inhibits HMG-CoA reductase activity as an inhibitor competitive during
173 cholesterol synthesis.¹⁹ According to Chen *et al.*, catechin (a polyphenol compound)
174 successfully reduced cholesterol levels through *in vitro* and *in vivo* experiments.²⁰
175 Additionally, Islam *et al.*⁸ reported that these compounds can block the electron transfer on
176 the substrate HMG-CoA and bind on HMG-CoA reductase on the NADP⁺ binding site. The
177 HMG-CoA reductase is an important enzyme that is involved in cholesterol synthesis. This
178 enzyme catalyzed HMG-CoA to Coenzyme A and mevalonate. The mevalonate then was

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

181

182 **Conclusion**

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

188

189 **DECLARATIONS**

190 **Acknowledgment**

191 This work was financially supported by the Universitas Sriwijaya within the framework of the
192 Indonesia Directorate General of Higher Education, Research and Technology Project,
193 Ministry of Education, Culture, Research, and Technology, SPPK No.
194 142/E5/PG.02.00PT/2022.

195

196 **Conflict of interest**

197 No conflict of interest associated with this work.

198

199 **Authors' Declaration**

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

202

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Sabri Sudirman

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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

4 Sabri Sudirman*, Miftahul Janna, Herpandi, Indah Widiastuti

5

6 Fisheries Product Technology, Faculty of Agriculture, Universitas Sriwijaya, Indralaya

7 30862, Ogan Ilir Regency, South Sumatra, Indonesia

8

9 * Corresponding author: **Email:** sabrisudirman@unsri.ac.id; **Tel:** +62 711580934

10 **Abstract**

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

27

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

29 Introduction

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

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

45 Water lettuce (*Pistia stratiotes*) is an aquatic plant that contains some bioactive
46 compounds, such as polyphenols, flavonoids, and tannins.^{6,7} A previous study also reported
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52 condition, the authors hypothesized that the polyphenol extract showed different activity

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55

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68 The filtrate was kept in a new collection tube and the residue was extracted by a fresh solvent
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83

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85 The total polyphenol and flavonoid contents were analyzed according to Chandra *et*
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89 mixture was added with 1 mL of 8% sodium carbonate and then volume up to 3 mL with
90 distilled water. The mixture was incubated at room temperature and in dark conditions for 30
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102

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 106 and performed according to the manufacturers' protocol. Briefly, 50 mg of each crude and
 107 purified extract was dissolved in assay buffer. The solution was centrifuged at 3,000 rpm for 5
 108 min, filtrated and the supernatant was kept for the next steps. Then, 5 µL of each extract and
 109 0.5 µL of pravastatin were pipetted into reaction tubes according to the Reaction Mixed
 110 (Table 1). The reaction was observed at 340 nm by using a spectrophotometer every 20 s for
 111 10 min. The HMG-CoA reductase activity and percentage of inhibitor were calculated
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113

114 Enzyme activity (Units/mgP) =
$$\frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blanko}}) \times \text{TV}}{12,44 \times V \times 0,6 \times \text{LP}}$$

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

117

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

119

120 **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 reductase 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

121

122 *Statistical analysis*

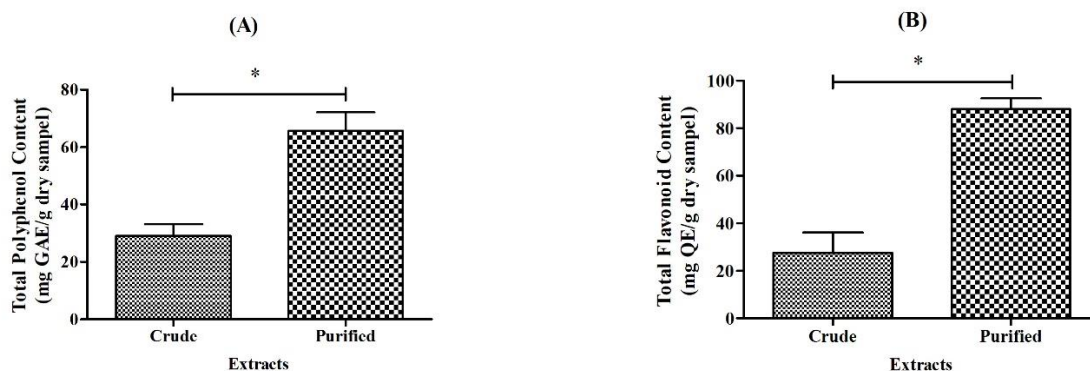
123 All data are expressed as the mean \pm standard deviation (SD) and analyzed by one-way
124 ANOVA with Duncan's post-hoc test ($p < 0.05$) using SPSS (v.22.0; IBM Corp., Armonk, NY,
125 USA).

126

127 **Results and Discussion**

128 *Total polyphenol and flavonoid content*

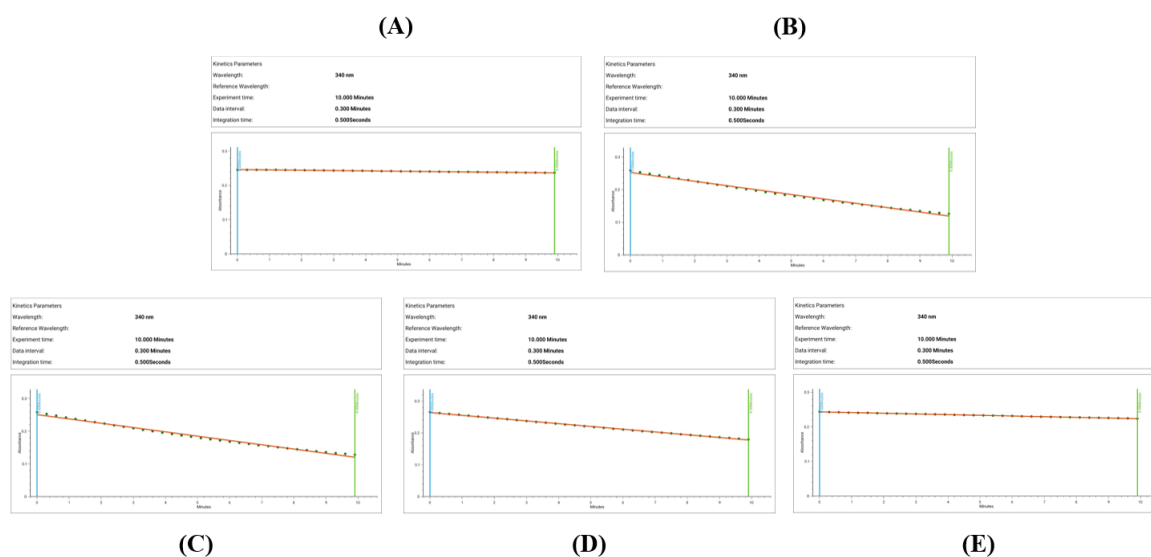
129 The results showed that the purified extract has significantly higher total polyphenol
130 and flavonoid contents compared to the crude extract as shown in **Figure 1**. Total polyphenol
131 contents (TPC) of the crude and purified extracts were about 29.03 ± 4.15 mg GAE/g dry
132 sample and 65.63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



146
 147 **Figure 1:** Total polyphenol (A) and flavonoid (B) content of crude and purified extract of
 148 water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean \pm SD ($n=3$). Statistically
 149 significance at $*p < 0.05$ versus purified extract.

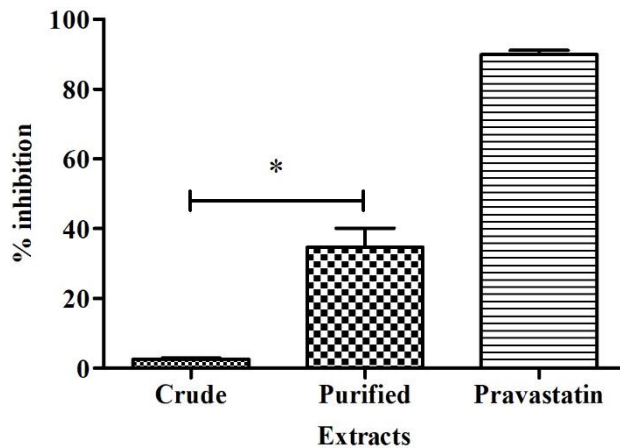
150
 151 *HMG-CoA reductase inhibitory activity*

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



156
 157 **Figure 2:** Representative of HMG-CoA reductase activity and inhibitory activity of sample:
 158 (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D)

159 Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive
160 control). These activities were measured at 340 nm for 10 min by a spectrophotometer.
161



162

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

166

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

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

180

181 **Conclusion**

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

187

188 **DECLARATIONS**

189 **Acknowledgment**

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191 Indonesia Directorate General of Higher Education, Research and Technology Project,
192 Ministry of Education, Culture, Research, and Technology, SPPK No.
193 142/E5/PG.02.00PT/2022.

194

195 **Conflict of interest**

196 No conflict of interest associated with this work.

197

198 **Authors' Declaration**

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

201

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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

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ABSTRACT

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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.

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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 45°C 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 40°C 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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Herpandi

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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 preconditioning. 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 H₂SO₄. 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 Mixture (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:

$$= \frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blank}}) \times TV}{12.44 \times V \times 0.6 \times LP} \quad \text{Enzyme activity (Units/mgP)}$$

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).

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 reductase 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 polyphenol 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.¹⁵ 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/g 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

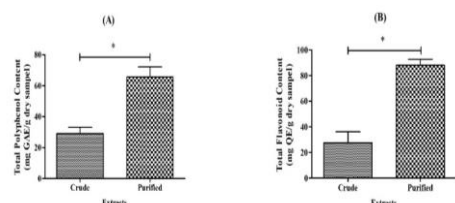


Figure 1: Total polyphenol (A) and flavonoid (B) content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean \pm SD ($n=3$). Statistically significance at $*p<0.05$ versus purified extract.

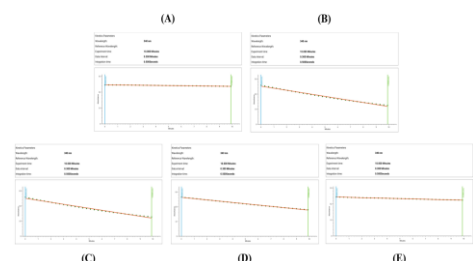


Figure 2: Representative of HMG-CoA reductase activity and inhibitory activity of sample: (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D) Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were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

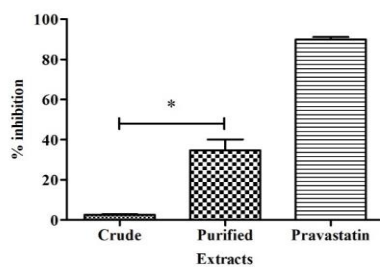


Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean \pm SD ($n=3$). Statistically significance at $*p<0.05$ versus purified extract.

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.

Acknowledgements

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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. 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.

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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 45°C 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 40°C 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 H₂SO₄. 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:

$$= \frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blanko}}) \times TV}{12.44 \times V \times 0.6 \times LP} \text{ Enzyme activity (Units/mgP)}$$

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 reductase 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 polyphenol 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 $34.74 \pm 5.40\%$ showed HMG-CoA reductase activity significantly higher than crude extract ($2.61 \pm 0.28\%$) 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

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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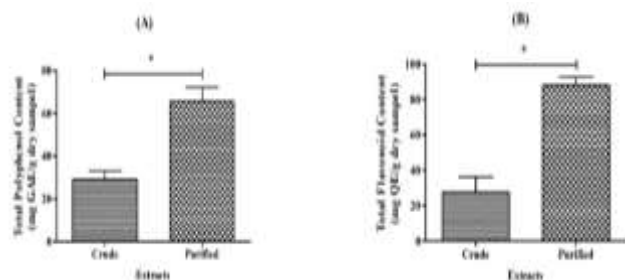


Figure 1: Total polyphenol (A) and flavonoid (B) content of crude and purified extract of water lettuce (*Pistia stratiotes*) leaf. Data are shown as the mean \pm SD ($n=3$). Statistically significance at $*p<0.05$ versus purified extract.

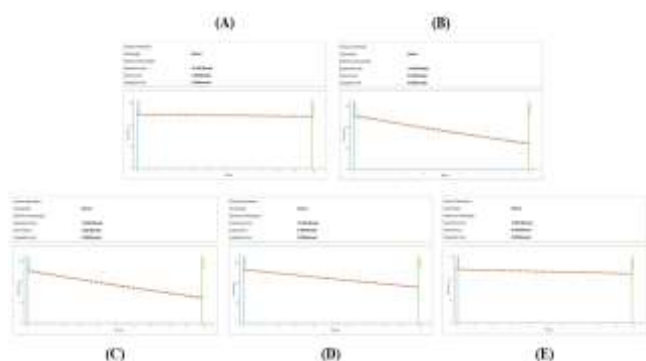


Figure 2: Representative of HMG-CoA reductase activity and inhibitory activity of sample: (A) Blank (B) HMG-CoA reductase activity, (C) Crude extraction inhibitory activity, (D) Purified extract inhibitory activity, and (E) Pravastatin inhibitory activity (as a positive control). These activities were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

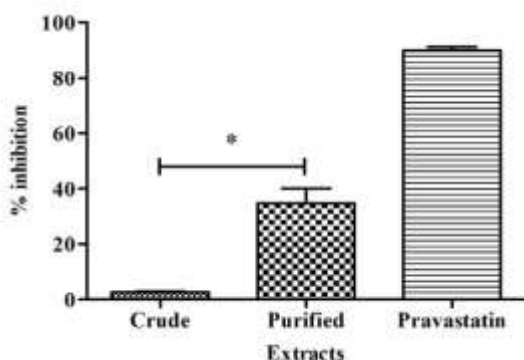


Figure 3: HMG-CoA reductase inhibitory activities of crude extract, purified extract, and pravastatin (as positive control). Data are shown as the mean \pm SD ($n=3$). Statistically significance at $*p<0.05$ versus purified extract.

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

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