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In this experiment, we found that the bioactive compound from water lettuce (*Pistia stratiotes*) successfully extracted by 70% ethanol and water solvents. The 70% ethanol extract shows a high yield of total polyphenol and flavonoids contents when compared to water extract. This extract also exhibits more effective antioxidant activity than water extract. These results indicated that 70% ethanol extract is the potential to use as a source for the developing antioxidant agent.

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We believe that this manuscript is appropriate for publication by the Food Research journal because it is suitable for the journal's aims and scope. This manuscript has not been published and is not under consideration for publication elsewhere.

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Sincerely
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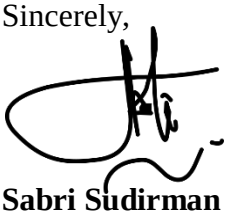

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Total polyphenol and flavonoid contents and antioxidant activities of water lettuce (*Pistia stratiotes*) leave extracts

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Abstract

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases. Under oxidative stress conditions, the human body needs more antioxidants that can externally be supplied from foods or supplements. This study aimed to investigate the total polyphenol content and flavonoids compounds and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*). The dried water lettuce was extracted by maceration. The yield of total phenolic of 70% ethanol extract (238.36±28.51 mg gallic acid equivalent (GAE)/g of dry sample) higher than water extract (70.66±29.43 mg GEA/g of dry sample). The 70% ethanol extract (209.65±7.71 mg quercetin equivalent (QE)/ g of dry sample) also possesses a higher level of flavonoids content compared to water extract (140.98±1.68 mg QE/ g of dry sample).

As analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods, 70% ethanol extract exhibits more powerful antioxidant compared to water extract. The half-maximum inhibitory concentration (IC_{50}) of 70% ethanol and water extracts were about 459.08±25.38 µg/mL and 1086.44±186.53 µg/mL , respectively and the ferric reduction power were about 118.43±2.17 mM Fe^{2+} equivalent/g of dry sample and 71.76±0.45 mM Fe^{2+} equivalent/g of dry sample, respectively. Based on these results, 70% ethanol extract is the potential source for the developing antioxidant agent.

Keywords: antioxidant, bioactive compounds, polyphenol, water lettuce

1. Introduction

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases such as cancer, diabetes, aging disease, cardiovascular, and inflammatory diseases (Lobo *et al.*, 2010). They are characterized by unpaired electrons in their outer layer and highly reactivity to other molecules such as lipids, proteins, and DNA. Then, this reaction cause damage to some tissues in the human body (Aruoma, 1998). Source of free radicals in the human body resulting from normal metabolic processes (endogenous sources) and exogenous sources such as exposure to cigarette smoking, air pollutants, X-rays, and industrial chemicals (Phaniendra *et al.*, 2014).

Under normal conditions, the human body has the ability to reduce the harmfulness of free radicals due to the presence of endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase (Pham-Huy *et al.*, 2008). However, if the exposure of free radicals higher than antioxidant power or under oxidative stress conditions (Pizzino *et al.*, 2017), the human body needs more antioxidants that can externally be supplied from foods and supplements. The exogeneous antioxidants can be supplied from natural products or plant extracts which containing bioactive active compound such as carotenoids (lutein and β-carotene), vitamins (ascorbic acid and γ-tocopherol), and phenolic compounds (flavonoids and phenolic acids) (Roehrs *et al.*, 2011; Da Costa *et al.*, 2012).

Water lettuce (*P. stratiotes*) is a macrophyte plant that lives on the surface of tropical freshwater. Water lettuce leaves and stems contain water (92.9%), carbohydrates (2.6%), protein (1.4%), fat (0.3%), crude fiber (0.9%), and minerals (1.9%) especially phosphorus and potassium (Tulika and Mala, 2015). Vitamins, stigmasterol, and palmitic acid are also found in the leaves of this plant (Khare, 2005; Liu *et al.*, 2008). Recently research on these plant extracts has shown the presence of bioactive compounds such as phenols and tannins which extracted by using n-hexane, ethyl acetate, and methanol (Tulika and Mala, 2015; Sudirman *et al.*, 2017a; Sudirman *et al.*, 2017b). A recently study reported that methanol fractions of this plant extract exhibited great antioxidant activity (Herpandi *et al.*, 2021). However, safer and food grade solvents or green solvents are recommended use during the extraction process such as ethanol and water, especially for human applications such as food supplements (Chemat *et al.*, 2019). Additionally, different concentrations of ethanol/water have used to extract polyphenol compounds (Sun *et al.*, 2015), whereas 70% (v/v) ethanol has widely used for this extraction methods and exhibit the highest polyphenol content and antioxidant activity (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). Therefore, this study aimed to investigate the total polyphenol content (TPC) and total flavonoid content (TFC) and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*).

2. Materials and Methods

2.1. Preparation and extraction process

The water lettuce (*P. stratiotes*) was harvested from Sukaraja Village, Ogan Ilir Regency, South Sumatra, Indonesia. The fresh plant was cleaned and kept the leave for future experiment. The extraction process was followed a previously methods (Chew *et al.*, 2011; Sudirman *et al.*, 2017b). Briefly, the fresh leave was dried by oven at 45 °C for 16 h. After drying process, the sample then was grinded into powder form. It was taken (10 g) and mixed either with 200 mL (1:20, w/v) of 70% ethanol (EtOH70) or distilled water (dH_2O) for maceration process with stirring (120 rpm) at room temperature for 3 h. After maceration process, the liquid phase (filtrate) was separated from residue by filtering and using

a filter paper (Whatman no.42). The filtrate was kept in a collection bottle. Whereas, the residue was taken and repeated the extraction process under the same condition with the first extraction process by adding fresh solvent. Five extractions were performed in total. The filtrates were mixed and evaporated by rotary vacuum evaporator at 50 °C and resulting in concentrated ethanol and water extracts. The concentrated extract was collected into new collection tubes and dried by using a freeze dyer (Biobase BK-FD10S, Shandong, China) to obtain final EtOH70 and dH_2O extracts. The percentage of extraction yield (%) was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried sample}} \times 100\%$$

2.2. Total polyphenol content and flavonoids analysis

TPC and TFC were measured according to previous methods (Chandra *et al.*, 2014). TPC was analyzed by using Folin-Ciocalteu's phenol reagent. Briefly, 0.2 mL of extract (10 mg/mL) were mixed with the Folin-Ciocalteu's reagent (1:1, v/v). After 5 min, in the solution was added 1 mL of saturated sodium carbonate (8% w/v in water), then added by distilled water up to 3 mL in total. The mixture was kept in the dark and allowed to reaction at room temperature for 30 min. After reaction time, the mixture was centrifugated at 3,000 rpm for 30 min and the supernatant was measured the absorbance at 750 nm in a spectrophotometer. The TPC was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Whereas, the TFC was determined by aluminum chloride colorimetric method. Briefly, the quercetin standard stock solution (5 mg/mL in methanol) was diluted to make serial concentrations. Then, 0.6 mL of standard or extract (10 mg/mL) solutions were mixed with 0.6 mL 2% aluminum chloride. The mixture was kept to reaction in room temperature for 60 min. After reaction time, the absorbance was measured at 420 nm. The TFC was expressed as mg quercetin equivalent (QE) per g of dry sample.

2.3. Antioxidant activities assay

The antioxidant activity of the extracts were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. DPPH assay was performed according

105 to the previously described method (Chew *et al.*, 2011). Briefly, the extracts were dissolved in dH_2O to
106 make a serial concentration (0 – 500 µg/mL). 1 mL of extract solutions were mixed with 1 mL of 0.2
107 mM DPPH. The mixture was incubated at 37°C for 30 min. After incubation time, the absorbance was
108 measured at 517 nm. Ascorbic acid was used as a positive control. The percentage of inhibition was
109 calculated according to the following equation:

110 $$\text{Percentage (\%) of inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

111 Whereas: $A_{control}$, absorbance of negative control ; A_{sample} , absorbance of sample.

112 The FRAP method was performed according to the previously described method (Halvorsen *et*
113 *al.*, 2002). Briefly, 0.1 mL of extract solution (20 mg/mL) was reacted with 3 mL of FRAP reagent (2.5
114 mL buffer acetate, pH 3.6; 2.6 mL of 10 mmol/L 2,4,6-tripyridil-striazine [TPTZ]; 2.5 mL of 20 mmol/L
115 $FeCl_3.6H_2O$ were mixed before used). The mixture solution was incubated at room temperature for 30
116 min. $FeSO_4 \cdot 7H_2O$ containing Fe^{2+} was used as a calibration standard. After incubation time, the
117 absorbance was measured at 596 nm. The FRAP value was expressed as mM Fe^{2+} equivalent/g of dry
118 sample.
119
120 **3. Results**
121 *3.1. Yield of extraction*
122 The yields of 70% ethanol (EtOH70) and distilled water (dH2O) extracts have no significant
123 difference (p>0.05) as shown in **Figure 1**. The yield of EtOH70 and dH2O extracts were about
124 16.80±1.01% and 16.45±1.06%, respectively.
125 *3.2. Total phenolic and flavonoids contents*
126 The TPC of EtOH70 extract was significantly (p<0.05) higher than dH_2O extract as shown in
127 **Figure 2A**. The TPC EtOH70 extract was about 238.36±28.51 mg GAE/g. Similar to the TPC, the
128 EtOH70 extract also contains significantly higher flavonoids content (p<0.05, 209.65±7.71 mg QE/g)
129 when compared to dH_2O extract as shown in **Figure 2B**.
130 *3.3. Antioxidant activities of water lettuce extracts*

The antioxidant activity of the extracts was determined by DPPH and FRAP methods as shown in **Table 1.** The EtOH70 extract possessed more effective antioxidant activity as indicated by low half-maximal inhibitory concentration (IC_{50}) and this value was significantly different (p<0.05) when compared to dH_2O extract. As a positive control, ascorbic acid showed more lower IC_{50} when compared to these extracts. The antioxidant activity of EtOH70 extract was also significantly (p<0.05) higher than antioxidant potential of dH_2O extract according to FRAP assay **(Table 2).**

4. Discussion

In this present study, we successfully extract the phenolic and flavonoid compounds from water lettuce (*P. stratiotes*). These bioactive compounds were successfully extracted by maceration with a stirring either with EtOH70 or distilled dH_2O as extraction solvents. Maceration method has widely used to extract some bioactive compounds including polyphenols. Maceration is a traditional method and simple procedure for polyphenol extraction from natural products and suitable for thermolabile compounds (Jovanović *et al.*, 2017; Lezoul *et al.*, 2020). As reported by previous literature that polyphenol compounds are thermolabile . Therefore, maceration is a suitable method to extract polyphenol compounds from plant materials (Sharma *et al.*, 2015; Jovanović *et al.*, 2017). In the present study, the yields extraction has showed no difference between 70% ethanol and water (**Figure 1**). A previous study reported that the extraction yield of some leaves of pepino (*Solanum muricatum*) plant by using a maceration method with 70% ethanol and water as solvents were about 17.09% and 18.63%, respectively (Lezoul *et al.*, 2020). According to the present data, we successfully extracted the bioactive compound from the leave of water lettuce.

The levels of bioactive compounds such as total polyphenolic content and flavonoids of 70% ethanol extract higher compared to water extract (Figure 2). A previous study also reported that the 70% ethanol is the best solvent for polyphenol extraction as indicated by the high yield in the extract (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). The mixture of ethanol-water is suited to penetrate plant matrix including hydrophobic areas (Vongsak *et al.*, 2013). Additionally, 70%

ethanol is polar solvent although slightly less polar than water (Kim and Lee, 2003). Similar to some previous studies, 70% ethanol extracts also showed a high level of total polyphenolic and flavonoids contents compared to water extract in some medical plants (Haq *et al.*, 2019; Lezoul *et al.*, 2020).

As shown in **Tables 1 and 2**, the ETOH70 extract exhibits more effective antioxidant activities when compared to water extract. The ethanol extract shows low half-maximum inhibitory concentration (IC_{50}) and high ferric reduction power as analyzed by DPPH and FRAP methods, respectively. A previous study also reported that 70% ethanol exhibited high antioxidant activity in the DPPH method compared to water extract (Hwang and Nhuan, 2014; Haq *et al.*, 2019). The principle of DPPH assay is the donated hydrogen atom or electron transfer by antioxidant to the DPPH radicals and resulted in color changed from violet to colorless or yellow in the reaction mixture. Whereas, FRAP principle is based on the ability of antioxidant to reduce ferric iron by electron transfer method (Alam *et al.*, 2013).

The majority of plant compounds such as polyphenol compounds act as antioxidants by hydrogen atom transfer (HAT) or single electron transfer (SET) mechanism to neutralize the free radicals and resulted in the reduction of the harmfulness of free radicals (Lee *et al.*, 2015). The hydrogen donating mechanism of these compounds is involved in the preventing of free radical production (Ajila *et al.*, 2007). Flavonoids can be acts as chelating metal ions and prevent their participation in free radical production (Cano *et al.*, 2008; Lee *et al.*, 2015).

5. Conclusion

The bioactive compounds from water lettuce (*P. stratiotes*) by the maceration method at room temperature with 70% ethanol and water solvents. The 70% ethanol extract possessed higher yield of total phenolic and flavonoids compounds compared to water extract. These bioactive compounds act as great antioxidant activities. Therefore, 70% ethanol extract exhibited more effective antioxidant properties compared to water extract. Therefore, 70% ethanol extract is the potential to use as a source for the developing antioxidant agent.

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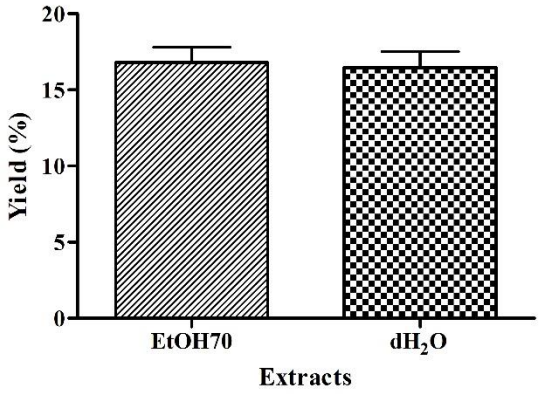
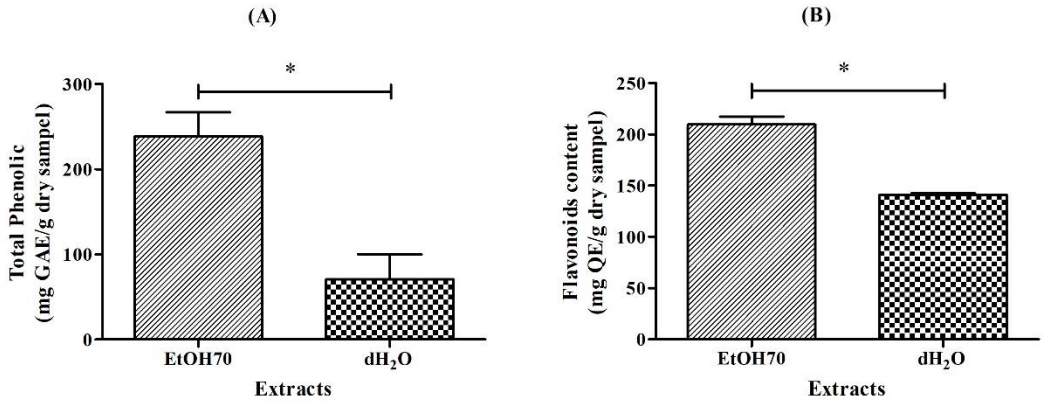


Figure 1. Extraction yield of water lettuce (*Pistia stratiotes*) extracts. EtOH70, 70% ethanol; dH_2O, distilled water.



273

274 **Figure 2.** Total phenolic **(A)** and flavonoids **(B)** contents of water lettuce (*Pistia stratiotes*) extracts.

275 Statistically significance at *p<0.05 versus ethanol extract. EtOH70, 70% ethanol; dH_2O, distilled

276 water.

277 **Table 1.** The antioxidant activity by DPPH assay of 70% ethanol (EtOH) and distilled water (dH_2O)

278 extracts of water lettuce (*Pistia stratiotes*).

Antioxidant assay	EtOH70 extract (µg/mL)	dH_2O extract (µg/mL)	Ascorbic acid (µg/mL)
DPPH [a] (IC_{50})	459.08±25.38	1086.44±186.53*	10.85±0.52*

279 [a] DPPH, 2,2-diphenyl-1-picrylhydrazyl; Statistically significance at *p<0.05 versus ethanol extract.

280 EtOH70, 70% ethanol; dH_2O, distilled water.

Table 2. The antioxidant activity by FRAP assay of 70% ethanol (EtOH70) and distilled water (dH_2O) extracts of water lettuce (*Pistia stratiotes*)

Antioxidant assay	EtOH70 extract (mM Fe^{2+} eq/g of dry sample)	dH_2O extract (mM Fe^{2+} eq/g of dry sample)
FRAP [a]	118.43±2.17	71.76±0.45*

[a] FRAP, Ferric reducing antioxidant power; Statistically significance at *p<0.05 versus ethanol extract. EtOH70, 70% ethanol; dH_2O, distilled water.

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Manuscript Type *(Please Bold)*	**Original Article** Review Short Communication Technical Notes
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Authors: Sudirman, S., Herpandi, Safitri, E., Apriani, E.F. and Taqwa, F.H.

Manuscript title: Total polyphenol and flavonoid contents and antioxidant activities of water lettuce (*Pistia stratiotes*) leave extracts

Manuscript ID: FR-2021-484

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According to reviewer comments, we have revised our manuscript.
The manuscript revision is attached.

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Total polyphenol and flavonoid contents and antioxidant activities of water lettuce (*Pistia stratiotes*) leave extracts

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Abstract

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases. Under oxidative stress conditions, the human body needs more antioxidants that can be externally obtained ~~be supplied~~ from foods or supplements. This study aimed to investigate the total polyphenol content ~~–~~and flavonoids compounds and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*). The dried water lettuce was extracted by maceration. The yield of total polyphenol~~ic~~ content of 70% ethanol extract (238.36±28.51 mg gallic acid equivalent (GAE)/g of dry sample) was higher than water extract (70.66±29.43 mg GEA/g of dry sample). The 70% ethanol extract (209.65±7.71 mg quercetin equivalent (QE)/ g of dry sample) also possesses a higher level of flavonoids content compared to water extract

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(140.98±1.68 mg QE/ g of dry sample). As analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods, 70% ethanol extract exhibiteds more powerful antioxidant activities compared to water extract. The half-maximum inhibitory concentration (IC_{50}) of 70% ethanol and water extracts were about 459.08±25.38 µg/mL and 1086.44±186.53 µg/mL , respectively and the ferric reduction power were about 118.43±2.17 mM Fe^{2+} equivalent/g of dry sample and 71.76±0.45 mM Fe^{2+} equivalent/g of dry sample, respectively. Based on these results, it is understood that 70% ethanol extract is the potential source for the developingas antioxidant agent.

Keywords: antioxidant, bioactive compounds, polyphenol, water lettuce

1. Introduction

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases such as cancer, diabetes, aging disease, cardiovascular, and inflammatory diseases (Lobo *et al.*, 2010). They are characterized by unpaired electrons in their outer layer and are highly reactivity reactive to other molecules such as lipids, proteins, and DNA. Then, this reaction cause damage to some tissues in the human body (Aruoma, 1998). Source of free radicals in the human body resulting from normal metabolic processes (endogenous sources) and exogenous sources such as exposure to cigarette smoking, air pollutants, X-rays, and industrial chemicals (Phaniendra *et al.*, 2014).

Under normal conditions, the human body has the ability to reduce the harmfulness of free radicals due to the presence of endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase (Pham-Huy *et al.*, 2008). However, if the exposure of free radicals is higher than antioxidant power or under oxidative stress conditions (Pizzino *et al.*, 2017), the human body needs more antioxidants that can externally be obtained supplied from foods and supplements. The exogeneous antioxidants can be obtained supplied from natural products or plant extracts which containing bioactive active compounds such as carotenoids (lutein and β-carotene), vitamins (ascorbic acid and γ-tocopherol), and phenolic compounds (flavonoids and phenolic acids) (Roehrs *et al.*, 2011; Da Costa *et al.*, 2012).

Water lettuce (*P. stratiotes*) is a macrophyte plant that lives on the surface of tropical freshwater. Water lettuce leaves and stems contain water (92.9%), carbohydrates (2.6%), protein (1.4%), fat (0.3%), crude fiber (0.9%), and minerals (1.9%) especially phosphorus and potassium (Tulika and Mala, 2015). Vitamins, stigmasterol, and palmitic acid are also found in the leaves of this plant (Khare, 2005; Liu *et al.*, 2008). Recently research on these plant extracts has shown the presence of bioactive compounds such as phenols and tannins ~~which~~ that can be extracted by using n-hexane, ethyl acetate, and methanol (Tulika and Mala, 2015; Sudirman *et al.*, 2017a; Sudirman *et al.*, 2017b). A recently study reported that methanol fractions of this plant extract exhibited great antioxidant activity (Herpandi *et al.*, 2021). However, safer and food-grade solvents or green solvents are recommended for the ~~use during the~~ extraction process such as ethanol and water, especially for human applications such as food supplements (Chemat *et al.*, 2019). Additionally, different concentrations of ethanol/water have been used to extract polyphenol compounds (Sun *et al.*, 2015), whereas 70% (v/v) ethanol has widely been used for this extraction method~~s~~ and exhibit the highest polyphenol content and antioxidant activity (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). Therefore, this study aimed to investigate the total polyphenol content (TPC) and total flavonoid content (TFC) and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*).

2. Materials and ~~Methods~~methods

2.1. Preparation and extraction process

The water lettuce (*P. stratiotes*) was harvested from Sukaraja Village, Ogan Ilir Regency, South Sumatra, Indonesia. The fresh plant was cleaned and kept the leaves for future experiments. The extraction process was ~~followed~~ conducted according to a previously reported methods (Chew *et al.*, 2011; Sudirman *et al.*, 2017b). Briefly, the fresh leaves was dried by oven at 45 °C for 16 h. After the drying process, the sample~~s~~ ~~then~~ was ~~grinded~~ ground into powder form. A 10 g sample was ~~. It was taken (10 g) and~~ mixed either with 200 mL (1:20, w/v) of 70% ethanol (70EtOH~~70~~) or distilled water (dH_2O) for maceration process with stirring (120 rpm) at room temperature for 3 h. After the maceration process, the liquid phase (filtrate) was separated

from residue by filtering and using ~~a~~ filter paper (Whatman no.42). The filtrate was kept in a collection bottle. Whereas, the residue was taken and repeated the extraction process under the same condition ~~with~~ as the first extraction process by adding fresh solvent. Five extractions were performed in total. The filtrates were mixed and evaporated by a rotary vacuum evaporator at 50 °C and resulting in concentrated ethanol and water extracts. The concentrated extract was collected into new collection tubes and dried by using a freeze dryer (Biobase BK-FD10S, Shandong, China) to obtain final 70EtOH~~70~~ and dH_2O extracts. The percentage of extraction yield (%) was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried sample}} \times 100\%$$

2.2. Total polyphenol content and flavonoids analysis

TPC and TFC were measured according to previous methods (Chandra *et al.*, 2014). TPC was analyzed by using Folin-Ciocalteu's phenol reagent. Briefly, 0.2 mL of extract (10 mg/mL) were mixed with the Folin-Ciocalteu's reagent (1:1, v/v). After 5 min, ~~in the solution was added~~ 1 mL of saturated sodium carbonate (8% w/v in water) was added, followed by the addition of ~~then added by~~ distilled water up to 3 mL in total. The mixture was kept in the dark ~~and allowed to reaction~~ at room temperature for 30 min. After the reaction time, the mixture was centrifugated at 3,000 rpm for 30 min and the supernatant was taken then measured ~~the absorbance~~ at 750 nm ~~in~~with a spectrophotometer. The TPC was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Whereas, the TFC was determined by the aluminum chloride colorimetric method. Briefly, the quercetin standard stock solution (5 mg/mL in methanol) was diluted to make serial concentrations. Then, 0.6 mL of standard or extract (10 mg/mL) solutions were mixed with 0.6 mL 2% aluminum chloride. The mixture was kept to react~~ion in~~ at room temperature for 60 min. After the reaction time, the absorbance was measured at 420 nm. The TFC was expressed as mg quercetin equivalent (QE) per g of dry sample.

2.3. Antioxidant activities assay

The antioxidant activity of the extracts were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. DPPH assay was performed according

to the previously described method (Chew *et al.*, 2011). Briefly, the extracts were dissolved in dH_2O to make a serial concentration (0 – 500 µg/mL). 1 mL of extract solutions were mixed with 1 mL of 0.2 mM DPPH. The mixture was incubated at 37°C for 30 min. After incubation time, the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. The percentage of inhibition was calculated according to the following equation:

$$\text{Percentage (\%) of inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Whereas: $A_{control}$, absorbance of negative control ; A_{sample} , absorbance of sample.

The FRAP method was performed according to the previously described method (Halvorsen *et al.*, 2002). Briefly, 0.1 mL of extract solution (20 mg/mL) was reacted with 3 mL of FRAP reagent (2.5 mL buffer acetate, pH 3.6; 2.6 mL of 10 mmol/L 2,4,6-tripyridil-striazine [TPTZ]; 2.5 mL of 20 mmol/L $FeCl_3.6H_2O$ were mixed before used). The mixture solution was incubated at room temperature for 30 min. $FeSO_4 \cdot 7H_2O$ containing Fe^{2+} was used as a calibration standard. After incubation time, the absorbance was measured at 596 nm. The FRAP value was expressed as mM Fe^{2+} equivalent/g of dry sample.

3. Results and discussion

3.1. Yield of extraction

In this present study, we successfully extract the bioactive compounds from water lettuce (*P. stratiotes*) by maceration with stirring either with 70% ethanol (70EtOH) or distilled water ~~distilled~~ (dH_2O) as extraction solvents. The yields of ~~70% ethanol~~ (70EtOH~~70~~) and ~~distilled water~~ (dH_2O) extracts have no significant difference ($p>0.05$) as shown in **Figure 1**. The yield of 70EtOH~~70~~ and dH_2O extracts were about 16.80±1.01% and 16.45±1.06%, respectively. Maceration method has widely been used to extract some bioactive compounds including polyphenols. Maceration is a traditional method and simple procedure for polyphenol extraction from natural products and is suitable for thermolabile compounds (Jovanović *et al*., 2017; Lezoul *et al*., 2020). ~~As reported by previous literature that p~~Polyphenols ~~compounds~~ are thermolabile (Maghsoudlou *et al*., 2019; Roselló-Soto *et al*., 2019)~~.~~ Therefore, maceration is a suitable method to extract polyphenol compounds from plant

materials (Sharma *et al*., 2015; Jovanović *et al*., 2017). A previous study reported that the extraction yield of some leaves of pepino (*Solanum muricatum*) plant by using a maceration method with 70% ethanol and water as solvents were about 17.09% and 18.63%, respectively (Lezoul *et al*., 2020). Additionally, Sun *et al*. (2020) also reported that the mixture of ethanol-water solvents shows a high extraction yield from *Apis mellifera* compared to water alonesolvent.

3.2. Total phenolic and flavonoids contents

The TPC of 70EtOH70 extract was significantly (p<0.05) higher than dH_2O extract as shown in **Figure 2A**. The TPC 70EtOH70 extract was about 238.36±28.51 mg GAE/g of dry sample. Similar to the TPC, the 70EtOH70 extract also contains significantly higher flavonoids content (p<0.05, 209.65±7.71 mg QE/g of dry sample) when compared to dH_2O extract as shown in **Figure 2B**. A previous study also reported that the 70% ethanol is the best solvent for polyphenol extraction as indicated by the high yield in the extract (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). The mixture of ethanol-water is suited to penetrate plant matrix including hydrophobic areas (Vongsak *et al.*, 2013). Additionally, 70% ethanol is polar solvent although slightly less polar than water (Kim and Lee, 2003). Similar to some previous studies, Previous studies reported that 70% ethanol extracts also showed a high level of total polyphenolic and flavonoids contents compared to water extract in some medical plants (Haq *et al.*, 2019; Lezoul *et al.*, 2020).

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3.3. Antioxidant activities of water lettuce extracts

The antioxidant activity of the extracts was determined by DPPH and FRAP methods as shown in **Table 1.** The 70EtOH70 extract possessed more effective antioxidant activity as indicated by low half-maximal inhibitory concentration (IC_{50}) and this value was significantly different (p<0.05) when compared to dH_2O extract. As a positive control, ascorbic acid showed more lower IC_{50} when compared to these extracts. The antioxidant activity of 70EtOH70 extract was also significantly (p<0.05) higher than the antioxidant potential of dH_2O extract according to FRAP assay **(Table 2).** A previous study also reported that 70% ethanol exhibited high antioxidant activity in the DPPH

method compared to water extract (Hwang and Nhuan, 2014; Haq *et al.*, 2019). Sun *et al*. (2020) also reported that ethanol extracts from ~~*Apis mellifera* shows~~ strong antioxidant activity compared to water extract.

The majority of plant compounds such as polyphenol compounds act as antioxidants by hydrogen atom transfer (HAT) or single electron transfer (SET) mechanism to neutralize the free radicals and resulted in the reduction of the harmfulness of free radicals (Lee *et al.*, 2015). The hydrogen donating mechanism of these compounds is involved in the preventing of free radical production (Ajila *et al.*, 2007). Polyphenol antioxidant can provide a hydrogen atom to free radical substrate and ~~changed it to~~generate a non-radical substrate (Zeb, 2020). Flavonoids can be acts as chelating metal ions and prevent their participation in free radical production (Cano *et al.*, 2008; Lee *et al.*, 2015). A previous study also reported that

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~~5.~~4. Conclusion

The bioactive compounds from water lettuce (*P. stratiotes*) have been succesfully extracted by the maceration method at room temperature with 70% ethanol and distilled water solvents. The 70% ethanol extract possessed a higher yield of total phenolic and flavonoids compounds ~~compared to water extract. These bioactive compounds~~and showed ~~act as~~ great antioxidant activities. ~~Therefore, 70% ethanol extract exhibited more effective antioxidant properties compared to water extract.~~ Therefore, 70% ethanol extract ~~is~~ can be used as~~the potential to use as a~~ source material for the developing a food supplement, especially as an antioxidant agent.

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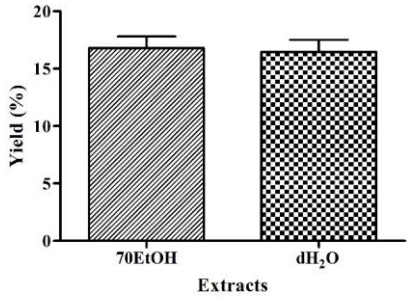

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290 **Figure 1**. Extraction yield of water lettuce (*Pistia stratiotes*) extracts. 70EtOH~~70~~, 70% ethanol; dH_2O,

291 distilled water.

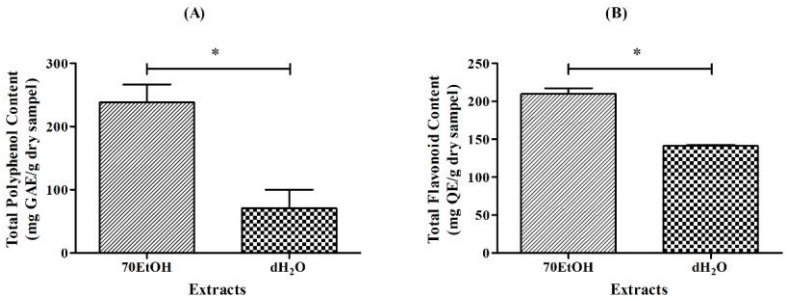

Figure 2. Total polyphenol~~ic~~ **(A)** and flavonoid~~s~~ **(B)** contents of water lettuce (*Pistia stratiotes*) extracts. Statistically significance at *p<0.05 versus 70% ethanol extract. 70EtOH~~70~~, 70% ethanol; dH_2O, distilled water.

Table 1. The antioxidant activity by DPPH assay of 70% ethanol ~~(EtOH)~~ and distilled water ~~(dH_2O)~~ extracts of water lettuce (*Pistia stratiotes*).

Antioxidant assay	70EtOH~~70~~ extract (µg/mL)	dH_2O extract (µg/mL)	Ascorbic acid (µg/mL)
DPPH [a] (IC_{50})	459.08±25.38	1086.44±186.53*	10.85±0.52*

[a] DPPH, 2,2-diphenyl-1-picrylhydrazyl; Statistically significance at *p<0.05 versus ethanol extract. 70EtOH~~70~~, 70% ethanol; dH_2O, distilled water.

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Table 2. The antioxidant activity by FRAP assay of 70% ethanol ~~(EtOH70)~~ and distilled water ~~(dH_2O)~~ extracts of water lettuce (*Pistia stratiotes*)

Antioxidant assay	70EtOH~~70~~ extract (mM Fe^{2+} eq/g of dry sample)	dH_2O extract (mM Fe^{2+} eq/g of dry sample)
FRAP [a]	118.43±2.17	71.76±0.45*

[a] FRAP, Ferric reducing antioxidant power; Statistically significance at *p<0.05 versus ethanol extract.

70EtOH~~70~~, 70% ethanol; dH_2O, distilled water.

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Manuscript Title	:	Total polyphenol and flavonoid contents and antioxidant activities of water lettuce (*Pistia stratiotes*) leave extracts
Authors	:	Sudirman, S., Herpandi, Safitri, E., Apriani, E.F. and Taqwa F.H.

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Yours sincerely,

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FOOD RESEARCH

FULL PAPER

Total polyphenol and flavonoid contents and antioxidant activities of water lettuce (*Pistia stratiotes*) leave extracts

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Abstract

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases. Under oxidative stress conditions, the human body needs more antioxidants that can be externally obtained from foods or supplements. This study aimed to investigate the total polyphenol content and flavonoid compounds and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*). The dried water lettuce was extracted by maceration. The yield of total polyphenol content of 70% ethanol extract (238.36±28.51 mg gallic acid equivalent (GAE)/g of dry sample) was higher than water extract (70.66±29.43 mg GEA/g of dry sample). The 70% ethanol extract (209.65±7.71 mg quercetin equivalent (QE)/g of dry sample) also possesses a higher level of flavonoid content compared to water extract (140.98±1.68 mg QE/g of dry sample). As analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods, 70% ethanol extract exhibited more powerful antioxidant activities compared to water extract. The half-maximum inhibitory concentration (IC_{50}) of 70% ethanol and water extracts were about 459.08±25.38 µg/mL and 1086.44±186.53 µg/mL, respectively and the ferric reduction power was about 118.43±2.17 mM Fe^{2+} equivalent/g of dry sample and 71.76±0.45 mM Fe^{2+} equivalent/g of dry sample, respectively. Based on these results, it is understood that 70% ethanol extract is the potential source of an antioxidant agent.

1. Introduction

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases such as cancer, diabetes, ageing disease, cardiovascular, and inflammatory diseases (Lobo *et al.*, 2010). They are characterized by unpaired electrons in their outer layer and are highly reactive to other molecules such as lipids, proteins, and DNA. Then, this reaction cause damage to some tissues in the human body (Aruoma, 1998). Source of free radicals in the human body resulting from normal metabolic processes (endogenous sources) and exogenous sources such as exposure to cigarette smoking, air pollutants, X-rays, and industrial chemicals (Phaniendra *et al.*, 2014).

Under normal conditions, the human body has the ability to reduce the harmfulness of free radicals due to the presence of endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase (Pham-Huy *et al.*, 2008). However, if the exposure of free radicals is higher than antioxidant power or under oxidative stress conditions (Pizzino *et al.*, 2017), the human body needs more antioxidants that can externally be obtained from foods and supplements. The exogenous antioxidants can be obtained from natural products or plant extracts containing bioactive active compounds such as carotenoids (lutein and β-carotene), vitamins (ascorbic acid and γ-tocopherol), and phenolic compounds (flavonoids and phenolic acids) (Roehrs *et al.*, 2011; Da Costa *et al.*, 2012).

Water lettuce (*P. stratiotes*) is a macrophyte plant that lives on the surface of tropical freshwater. Water lettuce leaves and stems contain water (92.9%), carbohydrates (2.6%), protein (1.4%), fat (0.3%), crude fibre (0.9%), and minerals (1.9%), especially phosphorus and potassium (Tulika and Mala, 2015). Vitamins, stigmasterol, and palmitic acid are also found in the leaves of this plant (Khare, 2005; Liu *et al.*, 2008).

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FULL PAPER

Recently research on these plant extracts has shown the presence of bioactive compounds such as phenols and tannins that can be extracted by using n-hexane, ethyl acetate, and methanol (Tulika and Mala, 2015; Sudirman , Herpandi, Lestari *et al.*, 2017; Sudirman, Herpandi, Nopianti *et al.*, 2017). A recent study reported that methanol fractions of this plant extract exhibited great antioxidant activity (Herpandi *et al.*, 2021). However, safer and food-grade solvents or green solvents are recommended for the extraction process such as ethanol and water, especially for human applications such as food supplements (Chemat *et al.*, 2019). Additionally, different concentrations of ethanol/ water have been used to extract polyphenol compounds (Sun *et al.*, 2015), whereas 70% (v/v) ethanol has widely been used for this extraction method and exhibits the highest polyphenol content and antioxidant activity (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). Therefore, this study aimed to investigate the total polyphenol content (TPC) and total flavonoid content (TFC) and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*).

2. Materials and methods

2.1 Preparation and extraction process

The water lettuce (*P. stratiotes*) was harvested from Sukaraja Village, Ogan Ilir Regency, South Sumatra, Indonesia. The fresh plant was cleaned and kept leaves for future experiments. The extraction process was conducted according to a previously reported method (Chew *et al.*, 2011; Sudirman, Herpandi, Nopianti *et al.,* 2017). Briefly, the fresh leaves were dried in an oven at 45°C for 16 hrs. After the drying process, the samples were ground into powder form. A 10 g sample was mixed either with 200 mL (1:20, w/v) of 70% ethanol (70EtOH) or distilled water (dH_2O) for the maceration process with stirring (120 rpm) at room temperature for 3 hrs. After the maceration process, the liquid phase (filtrate) was separated from residue by filtering and using filter paper (Whatman no. 42). The filtrate was kept in a collection bottle. Whereas, the residue was taken and repeated in the extraction process under the same condition as the first extraction process by adding fresh solvent. A total of five extractions were performed. The filtrates were mixed and evaporated by a rotary vacuum evaporator at 50°C and resulting in concentrated ethanol and water extracts. The concentrated extract was collected into new collection tubes and dried by using a freeze dryer (Biobase BK-FD10S, Shandong, China) to obtain final 70EtOH and dH_2O extracts. The percentage of extraction yield (%) was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried sample}} \times 100\%$$

2.2 Total polyphenol content and flavonoids analysis

TPC and TFC were measured according to previous methods (Chandra *et al.*, 2014). TPC was analyzed by using Folin-Ciocalteu's phenol reagent. Briefly, 0.2 mL of extract (10 mg/mL) were mixed with the Folin-Ciocalteu's reagent (1:1, v/v). After 5 mins, 1 mL of saturated sodium carbonate (8% w/v in water) was added, followed by the addition of distilled water up to 3 mL in total. The mixture was kept in the dark at room temperature for 30 mins. After the reaction time, the mixture was centrifugated at 3,000 rpm for 30 mins and the supernatant was taken and then measured at 750 nm with a spectrophotometer. The TPC was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Whereas, the TFC was determined by the aluminium chloride colourimetric method. Briefly, the quercetin standard stock solution (5 mg/mL in methanol) was diluted to make serial concentrations. Then, 0.6 mL of standard or extract (10 mg/mL) solutions were mixed with 0.6 mL 2% aluminum chloride. The mixture was kept to react at room temperature for 60 mins. After the reaction time, the absorbance was measured at 420 nm. The TFC was expressed as mg quercetin equivalent (QE) per g of dry sample.

2.3 Antioxidant activities assay

The antioxidant activity of the extracts was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. DPPH assay was performed according to the previously described method (Chew *et al.*, 2011). Briefly, the extracts were dissolved in dH_2O to make a serial concentration (0 – 500 µg/mL). 1 mL of extract solutions were mixed with 1 mL of 0.2 mM DPPH. The mixture was incubated at 37°C for 30 mins. After incubation time, the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. The percentage of inhibition was calculated according to the following equation:

$$\text{Percentage (\%) of inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Whereas $A_{control}$ is the absorbance of negative control and A_{sample} is the absorbance of sample.

The FRAP method was performed according to the previously described method (Halvorsen *et al.*, 2002). Briefly, 0.1 mL of extract solution (20 mg/mL) was reacted with 3 mL of FRAP reagent (2.5 mL buffer acetate, pH 3.6; 2.6 mL of 10 mmol/L 2,4,6-tripyridyl-

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striazine [TPTZ]; 2.5 mL of 20 mmol/L $FeCl_3.6H_2O$ were mixed before used). The mixture solution was incubated at room temperature for 30 min. $FeSO_4 \cdot 7H_2O$ containing Fe^{2+} was used as a calibration standard. After incubation time, the absorbance was measured at 596 nm. The FRAP value was expressed as mM Fe^{2+} equivalent/g of dry sample.

3. Results and discussion

3.1 Yield of extraction

In this present study, we successfully extract the bioactive compounds from water lettuce (*P. stratiotes*) by maceration with stirring either with 70% ethanol (70EtOH) or distilled water (dH_2O) as extraction solvents. The yields of 70EtOH and dH_2O extracts have no significant difference (p>0.05) as shown in Figure 1. The yield of 70EtOH and dH_2O extracts were about 16.80±1.01% and 16.45±1.06%, respectively. The maceration method has widely been used to extract some bioactive compounds including polyphenols. Maceration is a traditional method and simple procedure for polyphenol extraction from natural products and is suitable for thermolabile compounds (Jovanović *et al*., 2017; Lezoul *et al*., 2020). Polyphenols are thermolabile (Maghsoudlou *et al*., 2019; Roselló-Soto *et al*., 2019). Therefore, maceration is a suitable method to extract polyphenol compounds from plant materials (Sharma *et al*., 2015; Jovanović *et al*., 2017). A previous study reported that the extraction yield of some leaves of the Pepino (*Solanum muricatum*) plant by using a maceration method with 70% ethanol and water as solvents were about 17.09% and 18.63%, respectively (Lezoul *et al.*, 2020). Additionally, Sun *et al*. (2015) also reported that the mixture of ethanol-water solvent shows a high extraction yield from *Apis mellifera* compared to water solvent.

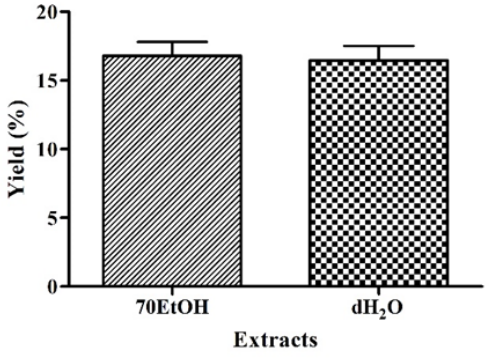

Figure 1. Extraction yield of water lettuce (*Pistia stratiotes*) extracts. 70EtOH: 70% ethanol, dH_2O: distilled water.

3.2 Total phenolic and flavonoids contents

The TPC of 70EtOH extract was significantly (p<0.05) higher than dH_2O extract as shown in Figure 2A. The TPC 70EtOH extract was about 238.36±28.51 mg GAE/g of dry sample. Similar to the TPC, the 70EtOH extract also contains significantly higher flavonoid content (p<0.05, 209.65±7.71 mg QE/g of dry sample) when compared to dH_2O extract as shown in Figure 2B. A previous study also reported that 70% ethanol is the best solvent for polyphenol extraction as indicated by the high yield in the extract (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). The mixture of ethanol-water is suited to penetrate plant matrix including hydrophobic areas (Vongsak *et al.*, 2013). Additionally, 70% ethanol is a polar solvent although slightly less polar than water (Kim and Lee, 2003). Previous studies reported that 70% ethanol extracts also showed a high level of total polyphenolic and flavonoid contents compared to water extract in some medical plants (Haq *et al.*, 2019; Lezoul *et al.*, 2020).

3.3 Antioxidant activities of water lettuce extracts

The antioxidant activity of the extracts was determined by DPPH and FRAP methods as shown in Table 1. The 70EtOH extract possessed more effective antioxidant activity as indicated by low half-maximal inhibitory concentration (IC_{50}) and this value was significantly different (p<0.05) when compared to dH_2O extract. As a positive control, ascorbic acid showed

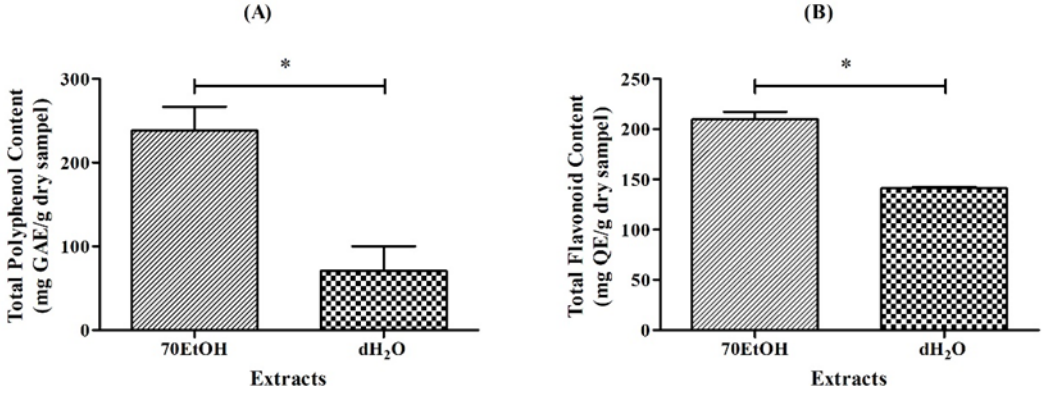

Figure 2. Total polyphenol (A) and flavonoid (B) contents of water lettuce (*Pistia stratiotes*) extracts. Statistically significance at *p<0.05 versus 70% ethanol extract. 70EtOH: 70% ethanol, dH_2O: distilled water.

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Table 1. The antioxidant activity by DPPH assay of 70% ethanol and distilled water extracts of water lettuce (*Pistia stratiotes*)

Antioxidant assay	70EtOH extract (µg/mL)	dH_2O extract (µg/mL)	Ascorbic acid (µg/mL)
DPPH (IC_{50})	459.08±25.38	1086.44±186.53*	10.85±0.52*

Statistically significance at *p<0.05 versus ethanol extract. DPPH: 2,2-diphenyl-1-picrylhydrazyl, 70EtOH: 70% ethanol, dH_2O: distilled water.

lower IC_{50} when compared to these extracts. The antioxidant activity of 70EtOH extract was also significantly (p<0.05) higher than the antioxidant potential of dH_2O extract according to the FRAP assay (Table 2). A previous study also reported that 70% ethanol exhibited high antioxidant activity in the DPPH method compared to water extract (Hwang and Nhuan, 2014; Haq *et al.*, 2019). Sun *et al*. (2015) also reported that ethanol extracts show strong antioxidant activity compared to water extracts.

Table 2. The antioxidant activity by FRAP assay of 70% ethanol and distilled water extracts of water lettuce (*Pistia stratiotes*)

Antioxidant assay	70EtOH extract (mM Fe^{2+} eq/g of dry sample)	dH_2O extract (mM Fe^{2+} eq/g of dry sample)
FRAP	118.43±2.17	71.76±0.45*

Statistically significance at *p<0.05 versus ethanol extract. FRAP: Ferric reducing antioxidant power, 70EtOH: 70% ethanol, dH_2O: distilled water.

The majority of plant compounds such as polyphenol compounds act as antioxidants by hydrogen atom transfer (HAT) or single electron transfer (SET) mechanism to neutralize the free radicals and resulted in the reduction of the harmfulness of free radicals (Lee *et al.*, 2015). The hydrogen donating mechanism of these compounds is involved in the prevention of free radical production (Ajila *et al.*, 2007). Polyphenol antioxidants can provide a hydrogen atom to free radical substrate and generate a non-radical substrate (Zeb, 2020). Flavonoids can be acts as chelating metal ions and prevent their participation in free radical production (Cano *et al.*, 2008; Lee *et al.*, 2015).

4. Conclusion

The bioactive compounds from water lettuce (*P. stratiotes*) have been successfully extracted by the maceration method at room temperature with 70% ethanol and distilled water solvents. The 70% ethanol extract possessed a higher yield of total phenolic and flavonoid compounds and showed great antioxidant activities. Therefore, 70% ethanol extract can be used as a source material for developing a food supplement, especially as an antioxidant agent.

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FULL PAPER

Total polyphenol and flavonoid contents and antioxidant activities of water lettuce (*Pistia stratiotes*) leave extracts

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Abstract

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases. Under oxidative stress conditions, the human body needs more antioxidants that can be externally obtained from foods or supplements. This study aimed to investigate the total polyphenol content and flavonoid compounds and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*). The dried water lettuce was extracted by maceration. The yield of total polyphenol content of 70% ethanol extract (238.36±28.51 mg gallic acid equivalent (GAE)/g of dry sample) was higher than water extract (70.66±29.43 mg GEA/g of dry sample). The 70% ethanol extract (209.65±7.71 mg quercetin equivalent (QE)/g of dry sample) also possesses a higher level of flavonoid content compared to water extract (140.98±1.68 mg QE/g of dry sample). As analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods, 70% ethanol extract exhibited more powerful antioxidant activities compared to water extract. The half-maximum inhibitory concentration (IC_{50}) of 70% ethanol and water extracts were about 459.08±25.38 µg/mL and 1086.44±186.53 µg/mL, respectively and the ferric reduction power was about 118.43±2.17 mM Fe^{2+} equivalent/g of dry sample and 71.76±0.45 mM Fe^{2+} equivalent/g of dry sample, respectively. Based on these results, it is understood that 70% ethanol extract is the potential source of an antioxidant agent.

1. Introduction

Free radicals such as reactive oxygen species and reactive nitrogen species are involved in the development of various chronic diseases such as cancer, diabetes, ageing disease, cardiovascular, and inflammatory diseases (Lobo *et al.*, 2010). They are characterized by unpaired electrons in their outer layer and are highly reactive to other molecules such as lipids, proteins, and DNA. Then, this reaction cause damage to some tissues in the human body (Aruoma, 1998). Source of free radicals in the human body resulting from normal metabolic processes (endogenous sources) and exogenous sources such as exposure to cigarette smoking, air pollutants, X-rays, and industrial chemicals (Phaniendra *et al.*, 2014).

Under normal conditions, the human body has the ability to reduce the harmfulness of free radicals due to the presence of endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase (Pham-Huy *et al.*, 2008). However, if the exposure of free radicals is higher than antioxidant power or under oxidative stress conditions (Pizzino *et al.*, 2017), the human body needs more antioxidants that can externally be obtained from foods and supplements. The exogenous antioxidants can be obtained from natural products or plant extracts containing bioactive active compounds such as carotenoids (lutein and β-carotene), vitamins (ascorbic acid and γ-tocopherol), and phenolic compounds (flavonoids and phenolic acids) (Roehrs *et al.*, 2011; Da Costa *et al.*, 2012).

Water lettuce (*P. stratiotes*) is a macrophyte plant that lives on the surface of tropical freshwater. Water lettuce leaves and stems contain water (92.9%), carbohydrates (2.6%), protein (1.4%), fat (0.3%), crude fibre (0.9%), and minerals (1.9%), especially phosphorus and potassium (Tulika and Mala, 2015). Vitamins, stigmasterol, and palmitic acid are also found in the leaves of this plant (Khare, 2005; Liu *et al.*, 2008).

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Recently research on these plant extracts has shown the presence of bioactive compounds such as phenols and tannins that can be extracted by using n-hexane, ethyl acetate, and methanol (Tulika and Mala, 2015; Sudirman , Herpandi, Lestari *et al.*, 2017; Sudirman, Herpandi, Nopianti *et al.*, 2017). A recent study reported that methanol fractions of this plant extract exhibited great antioxidant activity (Herpandi *et al.*, 2021). However, safer and food-grade solvents or green solvents are recommended for the extraction process such as ethanol and water, especially for human applications such as food supplements (Chemat *et al.*, 2019). Additionally, different concentrations of ethanol/ water have been used to extract polyphenol compounds (Sun *et al.*, 2015), whereas 70% (v/v) ethanol has widely been used for this extraction method and exhibits the highest polyphenol content and antioxidant activity (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). Therefore, this study aimed to investigate the total polyphenol content (TPC) and total flavonoid content (TFC) and antioxidant activities of 70% ethanol and water extracts of water lettuce (*Pistia stratiotes*).

2. Materials and methods

2.1 Preparation and extraction process

The water lettuce (*P. stratiotes*) was harvested from Sukaraja Village, Ogan Ilir Regency, South Sumatra, Indonesia. The fresh plant was cleaned and kept leaves for future experiments. The extraction process was conducted according to a previously reported method (Chew *et al.*, 2011; Sudirman, Herpandi, Nopianti *et al.,* 2017). Briefly, the fresh leaves were dried in an oven at 45°C for 16 hrs. After the drying process, the samples were ground into powder form. A 10 g sample was mixed either with 200 mL (1:20, w/v) of 70% ethanol (70EtOH) or distilled water (dH_2O) for the maceration process with stirring (120 rpm) at room temperature for 3 hrs. After the maceration process, the liquid phase (filtrate) was separated from residue by filtering and using filter paper (Whatman no. 42). The filtrate was kept in a collection bottle. Whereas, the residue was taken and repeated in the extraction process under the same condition as the first extraction process by adding fresh solvent. A total of five extractions were performed. The filtrates were mixed and evaporated by a rotary vacuum evaporator at 50°C and resulting in concentrated ethanol and water extracts. The concentrated extract was collected into new collection tubes and dried by using a freeze dryer (Biobase BK-FD10S, Shandong, China) to obtain final 70EtOH and dH_2O extracts. The percentage of extraction yield (%) was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried sample}} \times 100\%$$

2.2 Total polyphenol content and flavonoids analysis

TPC and TFC were measured according to previous methods (Chandra *et al.*, 2014). TPC was analyzed by using Folin-Ciocalteu's phenol reagent. Briefly, 0.2 mL of extract (10 mg/mL) were mixed with the Folin-Ciocalteu's reagent (1:1, v/v). After 5 mins, 1 mL of saturated sodium carbonate (8% w/v in water) was added, followed by the addition of distilled water up to 3 mL in total. The mixture was kept in the dark at room temperature for 30 mins. After the reaction time, the mixture was centrifugated at 3,000 rpm for 30 mins and the supernatant was taken and then measured at 750 nm with a spectrophotometer. The TPC was expressed as mg gallic acid equivalent (GAE) per g of dry sample.

Whereas, the TFC was determined by the aluminium chloride colourimetric method. Briefly, the quercetin standard stock solution (5 mg/mL in methanol) was diluted to make serial concentrations. Then, 0.6 mL of standard or extract (10 mg/mL) solutions were mixed with 0.6 mL 2% aluminum chloride. The mixture was kept to react at room temperature for 60 mins. After the reaction time, the absorbance was measured at 420 nm. The TFC was expressed as mg quercetin equivalent (QE) per g of dry sample.

2.3 Antioxidant activities assay

The antioxidant activity of the extracts was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. DPPH assay was performed according to the previously described method (Chew *et al.*, 2011). Briefly, the extracts were dissolved in dH_2O to make a serial concentration (0 – 500 µg/mL). 1 mL of extract solutions were mixed with 1 mL of 0.2 mM DPPH. The mixture was incubated at 37°C for 30 mins. After incubation time, the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. The percentage of inhibition was calculated according to the following equation:

$$\text{Percentage (\%) of inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Whereas $A_{control}$ is the absorbance of negative control and A_{sample} is the absorbance of sample.

The FRAP method was performed according to the previously described method (Halvorsen *et al.*, 2002). Briefly, 0.1 mL of extract solution (20 mg/mL) was reacted with 3 mL of FRAP reagent (2.5 mL buffer acetate, pH 3.6; 2.6 mL of 10 mmol/L 2,4,6-tripyridyl-

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striazine [TPTZ]; 2.5 mL of 20 mmol/L $FeCl_3.6H_2O$ were mixed before used). The mixture solution was incubated at room temperature for 30 min. $FeSO_4 \cdot 7H_2O$ containing Fe^{2+} was used as a calibration standard. After incubation time, the absorbance was measured at 596 nm. The FRAP value was expressed as mM Fe^{2+} equivalent/g of dry sample.

3. Results and discussion

3.1 Yield of extraction

In this present study, we successfully extract the bioactive compounds from water lettuce (*P. stratiotes*) by maceration with stirring either with 70% ethanol (70EtOH) or distilled water (dH_2O) as extraction solvents. The yields of 70EtOH and dH_2O extracts have no significant difference (p>0.05) as shown in Figure 1. The yield of 70EtOH and dH_2O extracts were about 16.80±1.01% and 16.45±1.06%, respectively. The maceration method has widely been used to extract some bioactive compounds including polyphenols. Maceration is a traditional method and simple procedure for polyphenol extraction from natural products and is suitable for thermolabile compounds (Jovanović *et al*., 2017; Lezoul *et al*., 2020). Polyphenols are thermolabile (Maghsoudlou *et al*., 2019; Roselló-Soto *et al*., 2019). Therefore, maceration is a suitable method to extract polyphenol compounds from plant materials (Sharma *et al*., 2015; Jovanović *et al*., 2017). A previous study reported that the extraction yield of some leaves of the Pepino (*Solanum muricatum*) plant by using a maceration method with 70% ethanol and water as solvents were about 17.09% and 18.63%, respectively (Lezoul *et al.*, 2020). Additionally, Sun *et al*. (2015) also reported that the mixture of ethanol-water solvent shows a high extraction yield from *Apis mellifera* compared to water solvent.

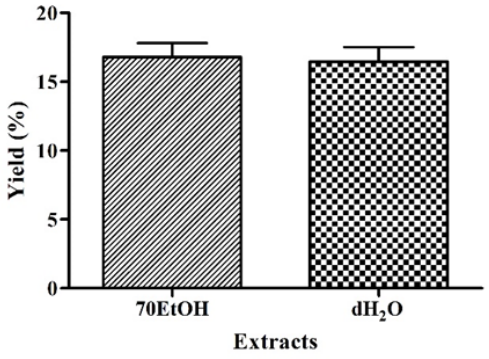

Figure 1. Extraction yield of water lettuce (*Pistia stratiotes*) extracts. 70EtOH: 70% ethanol, dH_2O: distilled water.

3.2 Total phenolic and flavonoids contents

The TPC of 70EtOH extract was significantly (p<0.05) higher than dH_2O extract as shown in Figure 2A. The TPC 70EtOH extract was about 238.36±28.51 mg GAE/g of dry sample. Similar to the TPC, the 70EtOH extract also contains significantly higher flavonoid content (p<0.05, 209.65±7.71 mg QE/g of dry sample) when compared to dH_2O extract as shown in Figure 2B. A previous study also reported that 70% ethanol is the best solvent for polyphenol extraction as indicated by the high yield in the extract (Hwang and Nhuan, 2014; Daud *et al.*, 2017; Oosthuizen *et al.*, 2018). The mixture of ethanol-water is suited to penetrate plant matrix including hydrophobic areas (Vongsak *et al.*, 2013). Additionally, 70% ethanol is a polar solvent although slightly less polar than water (Kim and Lee, 2003). Previous studies reported that 70% ethanol extracts also showed a high level of total polyphenolic and flavonoid contents compared to water extract in some medical plants (Haq *et al.*, 2019; Lezoul *et al.*, 2020).

3.3 Antioxidant activities of water lettuce extracts

The antioxidant activity of the extracts was determined by DPPH and FRAP methods as shown in Table 1. The 70EtOH extract possessed more effective antioxidant activity as indicated by low half-maximal inhibitory concentration (IC_{50}) and this value was significantly different (p<0.05) when compared to dH_2O extract. As a positive control, ascorbic acid showed

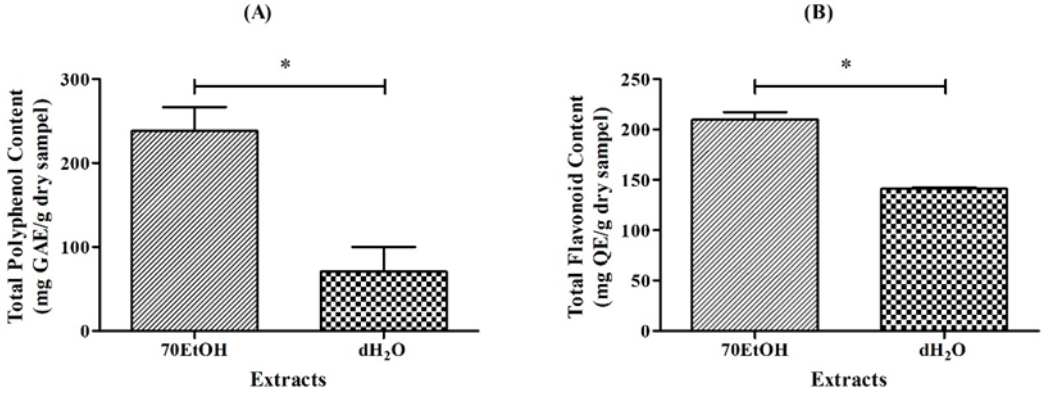

Figure 2. Total polyphenol (A) and flavonoid (B) contents of water lettuce (*Pistia stratiotes*) extracts. Statistically significance at *p<0.05 versus 70% ethanol extract. 70EtOH: 70% ethanol, dH_2O: distilled water.

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Table 1. The antioxidant activity by DPPH assay of 70% ethanol and distilled water extracts of water lettuce (*Pistia stratiotes*)

Antioxidant assay	70EtOH extract (µg/mL)	dH_2O extract (µg/mL)	Ascorbic acid (µg/mL)
DPPH (IC_{50})	459.08±25.38	1086.44±186.53*	10.85±0.52*

Statistically significance at *p<0.05 versus ethanol extract. DPPH: 2,2-diphenyl-1-picrylhydrazyl, 70EtOH: 70% ethanol, dH_2O: distilled water.

lower IC_{50} when compared to these extracts. The antioxidant activity of 70EtOH extract was also significantly (p<0.05) higher than the antioxidant potential of dH_2O extract according to the FRAP assay (Table 2). A previous study also reported that 70% ethanol exhibited high antioxidant activity in the DPPH method compared to water extract (Hwang and Nhuan, 2014; Haq *et al.*, 2019). Sun *et al*. (2015) also reported that ethanol extracts show strong antioxidant activity compared to water extracts.

Table 2. The antioxidant activity by FRAP assay of 70% ethanol and distilled water extracts of water lettuce (*Pistia stratiotes*)

Antioxidant assay	70EtOH extract (mM Fe^{2+} eq/g of dry sample)	dH_2O extract (mM Fe^{2+} eq/g of dry sample)
FRAP	118.43±2.17	71.76±0.45*

Statistically significance at *p<0.05 versus ethanol extract. FRAP: Ferric reducing antioxidant power, 70EtOH: 70% ethanol, dH_2O: distilled water.

The majority of plant compounds such as polyphenol compounds act as antioxidants by hydrogen atom transfer (HAT) or single electron transfer (SET) mechanism to neutralize the free radicals and resulted in the reduction of the harmfulness of free radicals (Lee *et al.*, 2015). The hydrogen donating mechanism of these compounds is involved in the prevention of free radical production (Ajila *et al.*, 2007). Polyphenol antioxidants can provide a hydrogen atom to free radical substrate and generate a non-radical substrate (Zeb, 2020). Flavonoids can be acts as chelating metal ions and prevent their participation in free radical production (Cano *et al.*, 2008; Lee *et al.*, 2015).

4. Conclusion

The bioactive compounds from water lettuce (*P. stratiotes*) have been successfully extracted by the maceration method at room temperature with 70% ethanol and distilled water solvents. The 70% ethanol extract possessed a higher yield of total phenolic and flavonoid compounds and showed great antioxidant activities. Therefore, 70% ethanol extract can be used as a source material for developing a food supplement, especially as an antioxidant agent.

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