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Preparation of *Hemigraphis reptans* water extract with different parameters (temperatures and extraction times): antioxidant and toxicity study

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Abstract: *Hemigraphis reptans* (G. Forst.) belongs to the Acanthaceae family. This plant is traditionally used to treat centipede bites and facilitate the process at birth. However, scientific experiments on *H. reptans* are still few. This study is to determine the antioxidants activity and toxicity of *H. reptans* water extract that was prepared with different parameters. Powder of dried *H. reptans* was placed in tubes containing water and heated to different temperatures of 40°C, 60°C, 80°C, and 100°C. For each temperature, the extract was heated at different extraction times (30 min, 1 h, 2 hours, and 3 hours). All extracts were tested for antioxidant tests (DPPH and FRAP), phenolic content, and brine shrimp lethality test. The DPPH result showed *H. reptans* water extract that was prepared using the parameter of 80°C/30 min has the highest DPPH inhibition. Besides that, the FRAP test showed that *H. reptans* water extract prepared using a parameter 60°C/1 hour and 80°C/1 hour has the highest FRAP value. For the TPC test, preparation of *H. reptans* water extract using a parameter of 80°C/30 min has the highest phenolic content. For MDA test, it showed that 100°C/2 hours has the lowest malondialdehyde level. For the toxicity test, the brine shrimp lethality test showed that *H. reptans* is mildly toxic and only reduces the population of brine shrimp if the concentration is too high which was 5 mg/ml. This study showed that *H. reptans* has the potency to be used as a source of antioxidants. However, *in vivo* study using an animal laboratory must be performed to verify the toxicity of this plant.

Keywords: *Hemigraphis reptans*, temperature, duration time, antioxidant, toxicity

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INTRODUCTION

Oxidative stress is related to an increased risk of some diseases such as hypertension, diabetes, dyslipidemia, obesity, and inflammation (Čolak *et al*., 2021). Under normal circumstances, the rate and amplitude of oxidant formation are balanced by the rate of their removal. Besides that, oxidative stress can be overcome by antioxidants (Pizzino *et al*., 2017). Natural antioxidants existing in herbs are responsible for obstructing or avoiding the destructive consequences of oxidative stress (Dhalaria *et al*., 2020). Applications of antioxidants are growing due to their several roles in eradicating harmful effects of oxidative stress (Yavari *et al*., 2015). Herb with high content of compounds with antioxidant properties is able to scavenge free radicals and other reactive oxygen species (ROS) (Pham-Huy *et al*., 2008). Compounds exhibiting antioxidant activity are capable of neutralizing or eliminating free radicals and other ROS (Munteanu *et al*., 2021). The antioxidant compounds are recognized to have protective functions against oxidative damage and are associated with a reduced risk of chronic diseases. However, improper technique and not controlling all factors during extract preparation will be damaging the antioxidant content of the extract. Optimum temperature and extraction time are two important factors to prepare an extract (Che Sulaiman *et al*., 2017). Different temperatures and extraction times that are used will produce an extract with different levels of antioxidants (Yim *et al*., 2013). In order to avoid structural changes to the target compound that has antioxidant activity during extraction, the extraction temperature and extraction time for herbal extraction would be prudently selected (Zhang *et al*., 2018; Justine *et al*., 2019). Many studies have focused on the antioxidant activity of the herbal extract. However, limited antioxidant activity studies have been conducted on *Hemigraphis reptans*

Hemigraphis reptans (G. Forst.) belongs to the family of Acanthaceae (Sarma *et al*., 2021). It is habitually found in shaded areas on lawns. It can be recognized with the dark green leaves, crenate margins, and prominent dark purple veins. Moreover, the underside of the leaves is purple in colour (Sarma *et al*., 2021). *Hemigraphis reptans* was traditionally used to treat centipede bites in Papuan New Guinea (Koch *et al*., 2015). In Vanuatu, this herb was claimed to have an effect on delivery (Bourdy *et al*., 1996). Besides that, there was one experiment for anti-dengue activity using *H. reptans*, however, *H. reptans* showed low efficacy (Rothan *et al*., 2014). This results in antioxidants and the toxicity of *H. reptans* is still scant. Therefore, in the present study *H. reptans* was used for water extract preparation. The extract was subjected to antioxidant and brine shrimp lethality tests to determine its effect and toxicity.

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A) Leave of *H. reptans*, B) *H. reptans* plant, C) Underside of Leave of *H. reptans*

METHODS

Collection of plant and authentication: The plant was collected at Forest Research Institute Malaysia (FRIM). The sample was submitted to Natural Product Division for authentication. The plant was identified by a certified botanist and kept at Kepong Herbarium (KEP). The SBID code was SBID 001/22.

Preparation of water extract: The plant was collected and washed. The plant was dried using an oven with a temperature of 55ºC for 48 hours. After that, the plant was ground into powder. Five milligrams of *H. reptans* were weighed using an electronic balance and placed in falcon tubes. One milliliter of distilled water was added. The tubes were placed in a water bath and heated at different temperatures (40ºC, 60ºC, 80ºC, and 100ºC) and at different times for each temperature (15 min, 30 min, 1h, 2hrs, and 3hrs). The extract was then centrifuged for 15 min at 2000 rpm. The suspension was kept in a freezer at -20ºC until used.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Assay: The DPPH scavenging assay was performed according to Tiveron *et al.*, (2012) with slight modifications. A DPPH reagent was prepared by mixing 1.77g of DPPH powder in 10 mL methanol. Previously prepared *H. reptans* extracts (50 µL) were mixed with 100 µL of DPPH reagent into a 96-well microplate. The microplate was incubated for 30 min in the dark. The absorbance of the solution was measured at a 540nm wavelength. The experiments were performed in triplicates.

Ferric Reducing Antioxidant Power (FRAP) Assay: The FRAP assessment followed the procedure described by Benzie and Strain (1996). The FRAP reagent contained 2.5 mM of 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM FeCl3 • 6H2O, and 25 mM of 300 mM acetate buffer (pH = 3.6). A standard curve was prepared using a concentration of 0.1–2 mmol / l FeSO4 • 7H2O. The 30 µL of the *H. reptans* extract was placed in a 96-well plate. Then 200 µL of FRAP reagent was added to the microplate and mixed using a microplate mixer for a few minutes. The reaction

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mixture was stirred at 37°C for 30 min and the absorbance was measured at 593 nm using the spectrophotometer. The experiments were performed in triplicates.

Total Phenolic Content (TPC): The TPC of *H. reptans* extracts was determined by the Folin-Ciocalteu method following Singleton and Rossi (1965) with some modifications. In short, 20 µL of the *H. reptans* extract was mixed with 10 µL of Folin-Ciocalteu reagent in each microplate well. The mixture was kept for 5 min at 37°C. Then, 40 µL of 7.6% sodium carbonate was added. The microplate was then placed for 2 hours in a dark condition. Subsequently, 765 nm was used to measure the absorbance against the blank. The experiments were performed in triplicates.

Thiobarbituric Acid Reactive Species (TBARS) test: The modified TBARS test was used to measure the lipid peroxide formed using egg yolk homogeneity as the lipid-rich medium (Upadhyay *et al*., 2014). Two hundred and fifty microliters of 10% egg homogeneity were placed into a test tube. Then, 50 µL of *H. reptans* extract was then mixed in the test tube. Distilled water was added to the test tube to make 500 µL. Finally, 25 µL Ferrous sulfate ($FeSO_4$) (0.07 M) was added to the above mixture and incubated for 30 min to induce the lipid peroxidation process. Subsequently, 750 µL of 0.8% TBA, 750 µL of 20% acetic acid, and 25 µL of 20% TCA were added to the test tube. The test tube was heated in a boiling water bath for 60 minutes and cooled under tap water. Each test tube was added with 3.0 ml of 1-butane and centrifuged at 3000 rpm for 10 min. The top layer was measured at 532 nm. The experiments were performed in triplicates.

Brine Shrimp Lethality Test: For the preparation of sea salt water, 2.5 g of sodium chloride was weighed, dissolved in 100 ml distilled water, and filtered as reported by Omeke *et al*., (2018). This simulated seawater was put into a 100 ml beaker and added with brine shrimp eggs. The shrimp was allowed for 24 hours to hatch. For the test, the brine shrimp was put into a 96-well plate. Every one well was added with 20 µl of sea salt water containing 10 to 15 brine shrimp. Then, 100 µl of sea salt water was added to the well. For the treatment, 80 µl of sample solution was added to the well until the final volume in one well became 200 µl. For the control, only 80 µl of sea salt water was added to replace the samples. In this experiment, the *H. reptans* sample solution was added to get final concentrations which are 5 mg/ml, 2.5 mg/ml, 0.625 mg/ml, and 0.3125 mg/ml. The 96-well plate was left for 24 hours. After that, the dead brine shrimp was calculated. Then, methanol was added to the 96-well plate to kill all the brine shrimps. All brine shrimps were calculated to get their total number.
Percentage of death (%) = (dead brine shrimp / total brine shrimp) x 100
Percentage of living brine shrimps (%) = 100 – (percentage of death)

Statistical analysis: Each sample analysis was performed in triplicate. All of the presented results are the means (±standard deviation) of at least three independent experiments. Statistical analysis was performed by SPSS 16 for Windows. Statistical significance was observed using an independent samples t-test ($p < 0.05$).

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RESULTS

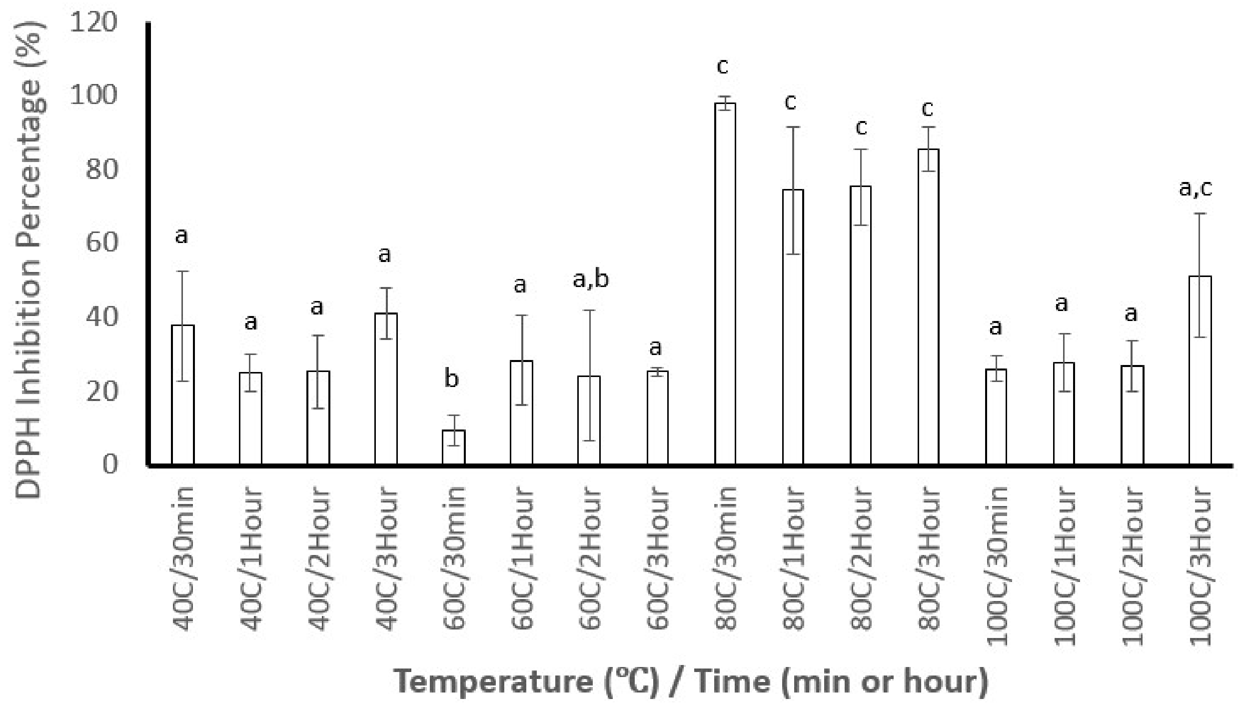

Figure 1: DPPH free radical scavenging activity of *H. reptans* extract with different temperatures and times. Values are mean ± standard deviation (n = 8). Different superscript letters indicate a significant difference at $p < 0.05$.

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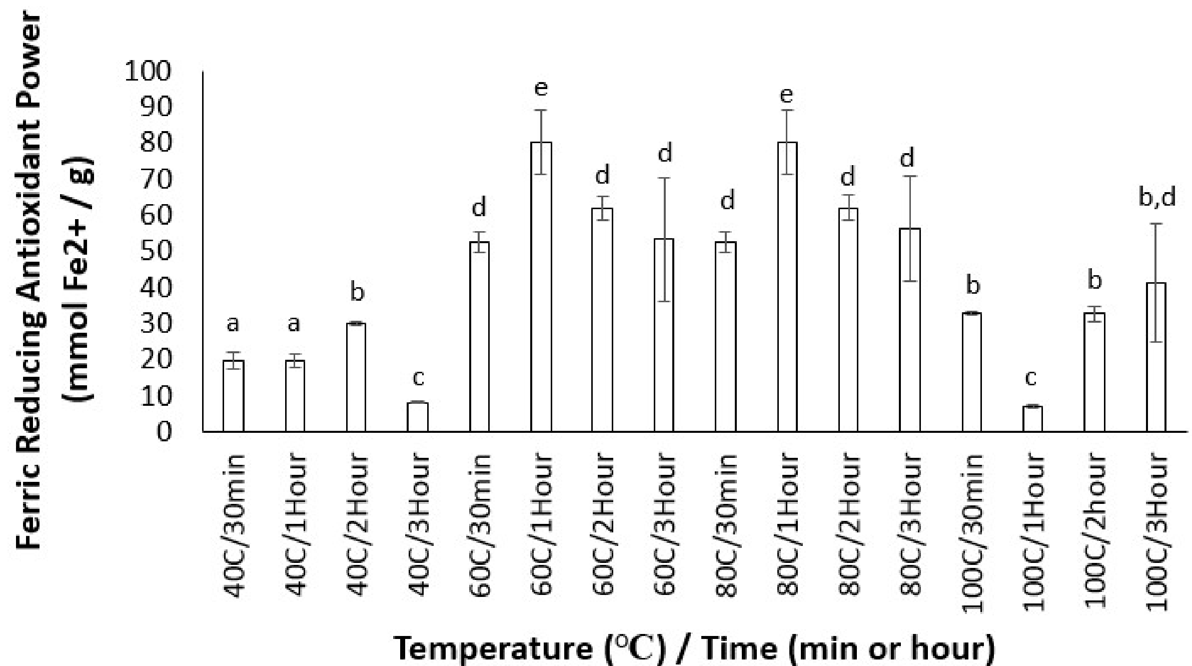

Figure 2: FRAP value of *H. reptans* extract with different temperatures and times. Values are mean ± standard deviation (n = 8). Different superscript letters indicate a significant difference at $p < 0.05$.

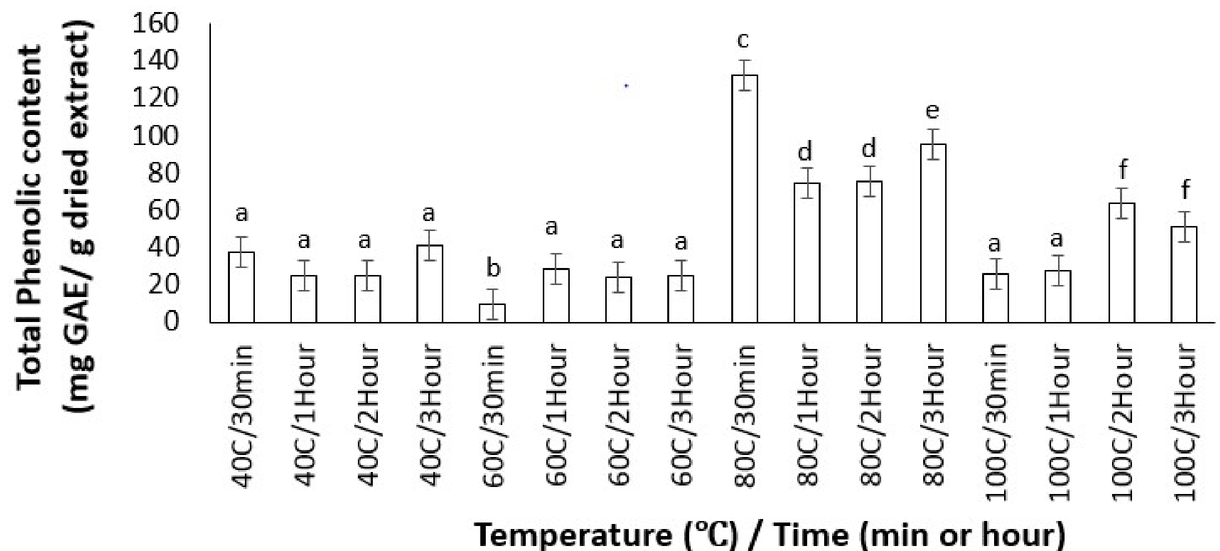

Figure 3: TPC level of *H. reptans* extract with different temperatures and times. Values are mean ± standard deviation (n = 8). Different superscript letters indicate a significant difference at $p < 0.05$.

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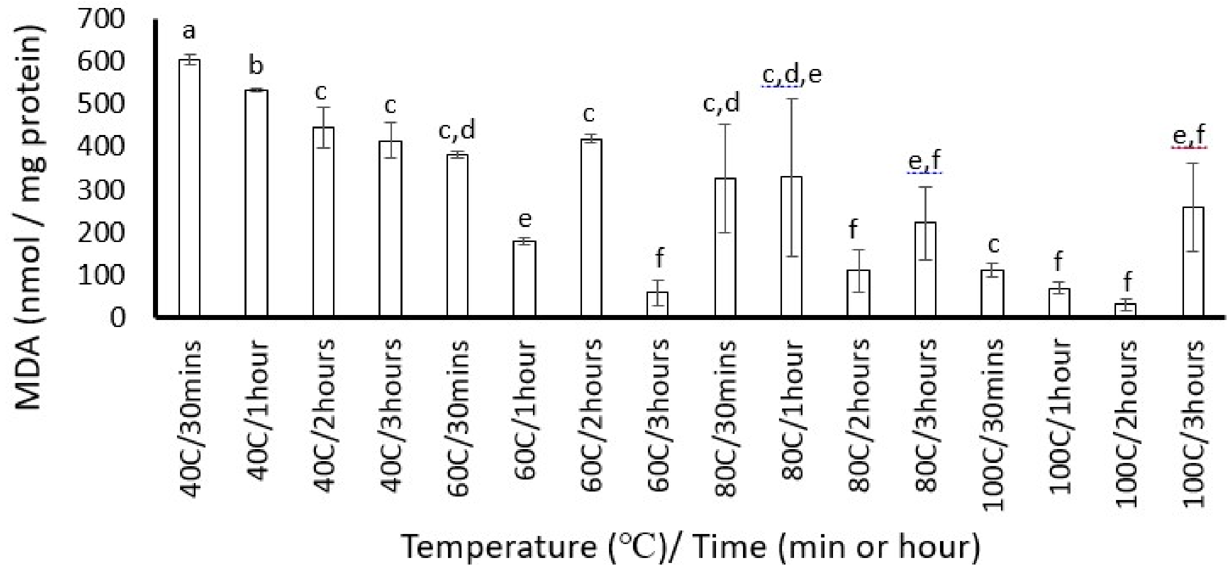

Figure 4: MDA level after treatment of *H. reptans* extract with different temperatures and times. Values are mean ± standard deviation (n = 8). Different superscript letters indicate a significant difference at $p < 0.05$.

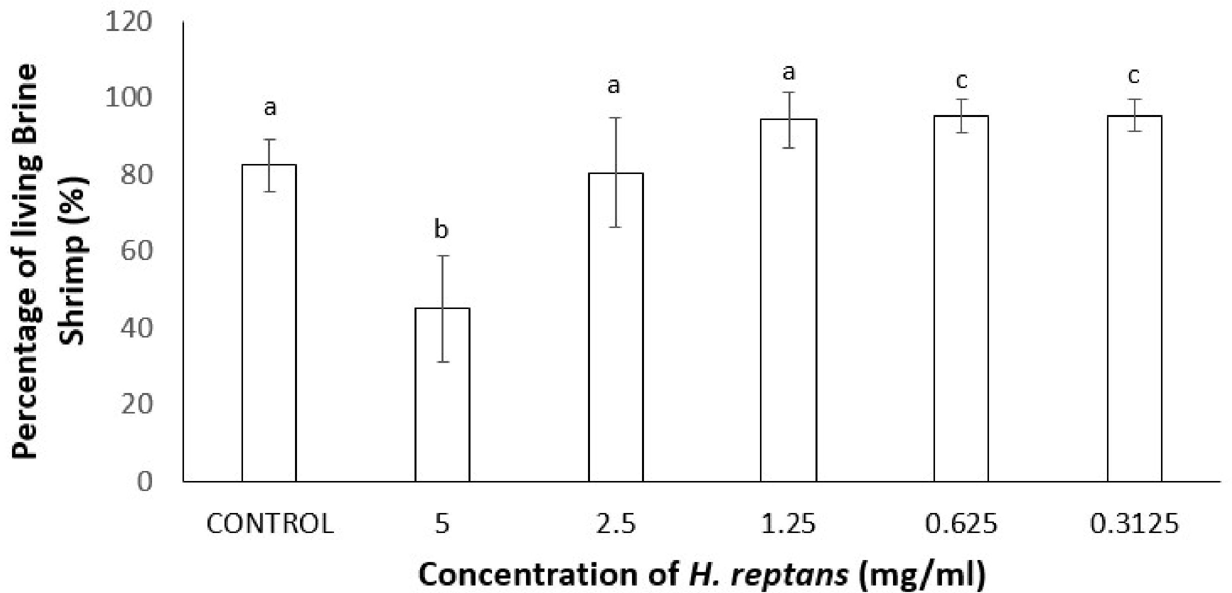

Figure 5: Percentage of living brine shrimp after exposure to *H. reptans* extract with different concentrations. Values are mean ± standard deviation (n = 8). Different superscript letters indicate a significant difference at $p < 0.05$.

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DISCUSSION

Extraction conditions are important to maximize extraction yields and enrich the antioxidant content (Ling *et al*., 2020). Extraction temperatures and extraction times are two factors that needed to be considered when employing extraction techniques on herbal samples to ensure complete extraction of the active compounds and avoid chemical modification (Che Sulaiman *et al*., 2017). A long extraction time may intensify active compound extraction; however, extremely high temperatures that are used in the extraction process cause decreased antioxidant efficacy (Dai and Mumper, 2010). Therefore, the DPPH test was used to determine the antioxidant activity of every extraction condition.

In Figure 1, antioxidant activities are expressed as the percentage of DPPH scavenging activity. The higher bar indicates the higher inhibition activity of oxidation. This experiment showed that the *H. reptans* extract that was heated at 80°C possesses the highest antioxidant capacity as compared to the other temperatures. The other temperature showed lower DPPH inhibition which 60°C/30 min having the lowest activity. A previous study reported that plant crude extracts containing compounds that have hydroxyl groups can act as hydrogen-donating antioxidants (Kumarappan *et al*., 2012). The presence of antioxidant substances containing hydrogen-donating groups such as flavonoids and phenols (Phuyal *et al*., 2020). From Figure 1, it was suggested that extraction used a parameter of 80°C/30min produced an *H. reptans* extract with high DPPH inhibition.

In the FRAP method, antioxidants reduce Fe (III) to Fe (II), which produces a complex with TPTZ in acetate buffer and is detected at 593 nm (Ruslan *et al*., 2018). In Figure 2, the temperature of 60°C and 80°C showed higher FRAP activity. This indicates that many compounds that were extracted by using this temperature have the capability to reduce Fe (III) to Fe (II). The highest activity of Fe(III) reduction was shown by the extract that was produced using the parameters of 60°C/1 hour and 80°C/ 1 hour.

Moreover, phenolic compounds are important herbal components with redox properties responsible for antioxidant activity (Johari and Khong, 2019). The hydroxyl groups in herbal extracts are answerable for radical scavenging activity (Aryal *et al*., 2019). TPC value of the extract that used 40°C was below 60 mg GAE/g and the extract that was prepared used a temperature of 60°C was 40 mg GAE/g. Similarly, the value was below 40 mg GAE/g for the temperature of 100°C. However, the TPC value was increased to 60 mg GAE/g for 100°C/ 2 hours and 50 mg GAE/g for 100°C/ 3 hours. This showed that extraction time also has an effect on the extraction of the phenolic compound from the herbal sample. As shown in Figure 3, TPC values were higher in the extract that was produced using the parameter of 80°C/ 30 min. The highest TPC value was observed in the group of extracts that used a temperature of 80°C. This graph also displayed a similar pattern compared to the DPPH graph in Figure 1. This proves that the phenolic compound from *H. reptans* has the ability to scavenge the DPPH.

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The limitations of the present study exposed that the lower TPC in *H. reptans* extracts did not always correlate with the lower antioxidant activity by FRAP methods. Although, Figure 3 showed that the use of the temperature of 60°C produced an extract with lower TPC but in Figure 2, the temperature of 60°C showed higher FRAP reading. This might be due to the extraction of different compounds for each temperature. It is possible that the use of 60°C releases a compound that can act to reduce the Fe(III) causing it to show more of a higher FRAP reading along with the same temperature of 80°C. Therefore, a future study must be performed to recognize phenolic and flavonoid compounds in *H. reptans* extract that have antioxidant activity by DPPH and FRAP methods.

The antioxidant effect of *H. reptans* extract also was tested on egg yolk which was oxidized by $FeSO_4$. Figure 4 showed that treatment of *H. reptans* extract that was produced using the parameter of 100°C/ 2 hours can significantly reduce malondialdehyde (MDA). Malondialdehyde is a stable end product of lipid peroxidation and therefore can be used as an indirect measure of the cumulative lipid peroxidation (Mao *et al*., 2019). The level of MDA can be used to determine the effectiveness of herbal extract to reduce the oxidation process (Nguyen *et al*., 2019). The lower MDA level indicates a lower oxidation process. Almost all extract that was produced by using 100°C can reduce MDA below 100 nmol/ mg protein except 100°C/ 3 hours. This might be due to prolonged extraction time causing a reduction of active compounds. The temperature of 60°C and 80°C showed lower MDA production compared to the temperature of 40°C.

Moreover, the determination of *H. reptans* extract toxicity was performed by using brine shrimp lethality assay (BSLA). It is a simple and cost-effective bioassay used for testing the efficacy of plant extracts (Waghulde *et al*., 2019). The lethality of the test sample in the brine shrimp has been applied by many researchers and has proven to be a valuable tool in screening various herbal extracts (Wu, 2014). In Figure 5, it was shown that higher concentration causes more death to the brine shrimp. The concentration of 5 mg/ml causes death to 60% of brine shrimp in the 96-well plate. However, when the concentration was decreased to 2.5 mg/ml, the living percentage of brine shrimp was increased to 80%. This indicates that the extent of lethality was directly proportional to the concentration of the extract. The *H. reptans* extract might be mildly toxic if used in high concentrations.

CONCLUSION

The *H. reptans* water extracts that were prepared at different temperatures and times can be categorized as very strong antioxidants using the DPPH assay. The DPPH and FRAP result showed that *H. reptans* water extract prepared using a temperature of 80°C has the highest DPPH inhibition and FRAP value. Preparation of *H. reptans* extracts used parameter of 80°C/30 min produced the highest phenolic compound. *H. reptans* is also considered mildly toxic and only dangerous if used at a

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high concentration. *H. reptans* plant has the potential to be developed as a source of natural antioxidants.

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