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Antioxidant activity and toxicity of *Annona muricata* leaves extract

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Abstract: *Annona muricata* leaf is used in traditional medicine to treat many diseases and their complications. This study aimed to optimize the preparation of *A. muricata* water extract with a high antioxidant effect and determination of its toxicity on brine shrimp and rat. In this study, water extraction was performed at different temperature (40°C, 60°C, 80°C and 100°C) for different times (15 min, 30 min, 1 h, 2 h, and 3 h). Antioxidant *in-vitro* tests such as DPPH, FRAP and TPC were used for the optimization of extract. The results showed that the best parameter for *A. muricata* extract preparation was 100°C/30 min. The extract was again prepared by using the parameter of 100°C/30 min based on the antioxidant test. The brine shrimp lethality test (BSLT) and single-dose acute toxicity test on rat were carried out to study its toxicity. Based on BSLT results, the LC_{50} (median lethal concentration) values were not detected. Besides that, all rats that were given *A. muricata* extract were not died after 14 days of treatment. In a conclusion, the results showed that the optimized *A. muricata* extract might be not toxic.

Keywords: *Annona muricata*, water extract, antioxidant, phenolic compound, brine shrimp lethality test, acute toxicity test

INTRODUCTION

Oxidative stress refers to the surplus of reactive oxygen species (ROS) or oxidants over the ability of the cell to perform an effective antioxidant response (Ray *et al*., 2012). Oxidative stress affects macromolecular impairment and is associated with various disease states such as cancer atherosclerosis, neurodegeneration, diabetes, and ageing (Liguori *et al*., 2018). Reactive oxygen species are produced during mitochondrial oxidative metabolism or any cellular response to bacterial invasion cytokines and xenobiotics (He *et al*., 2017). An antioxidant can scavenge and remove ROS to protect tissues and organs from oxidative injury (Chan *et al*., 2008). Antioxidants can abolish free radicals and avoid chronic diseases caused by ROS (Carlsen *et al*., 2010). Medicinal herbs are usually associated with high antioxidant have the potential to avoid oxidative injury and provide health benefits to the human body.

Annona muricata (*A. muricata*) is a medicinal herb that has been using for centuries to treat many diseases. It is a tropical plant species belonging to the family Annonaceae

(Coria-Téllez *et al*., 2018). *A. muricata* is also known as soursop, Graviola or guanabana. It is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world (Moghadamtousi *et al*., 2015). Natives of Malaysia used *A. muricata* leaves to treat external and internal parasites (Badrie and Schauss, 2010). It was reported that *A. muricata* has medicinal uses and purposely grown for its fruit (Patel and Patel, 2016). Minari *et al*., (2014) reported that *A. muricata* leaves may have protective effects on the development of breast carcinogenesis. Besides that, different parts of *A. muricata* especially the leaves have been used for many ethnomedicinal purposes by traditional healers (Sun *et al*., 2016). However, the leaf of *A. muricata* is the most utilized parts used for a wide array of ethnomedicinal uses. Although, the preparation of *A. muricata* leaves extract to get the antioxidant efficacy is still not standardized and optimised. This study focuses on the optimization of the temperature and duration for *A. muricata* leaves extraction to get *A. muricata* leaves extract with high antioxidant efficacy. Besides that, the toxicity of the extract was determined using brine shrimp and single-dose acute toxicity by using rat.

MATERIAL AND METHODS

Water extraction of Annona muricata: The method of water extract preparation was imitated by Nik Hasan *et al*., (2020). First, the leaves of *A. muricata* was washed and dried. Then, *A. muricata* was put into the oven for the drying process at the temperature of 55°C for 48 h. After that, *A. muricata* was ground into powder. Then, 10 g of *A. muricata* was weighed by using electronic balance and put into glass tubes. After that, the glass was added with 100 mL of distilled water. The parameter were 100°C/30 min, 100°C/1 h, 100°C/2 h, 100°C/3 h, 80°C/30 min, 80°C/1 h, 80°C/2 h, 80°C/3 h, 60°C/30min, 60°C/1 h, 60°C/2 h, 60°C/3 h, 40°C/30 min, 40°C/1 h, 40°C/2 h, and 40°C/3 h. The extract was filtered and put into a labelled tube. The extract was freeze-dried and kept at 4°C until used.

Diphenyl picrylhydrazyl (DPPH) assay: The antioxidant activity (AC) of extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging radical method as reported by Fatiha and Abdulkader (2019). In short, the *A. muricata* extracts were prepared in water at a concentration of 5.0 mg/mL. After that, 50 µL of the samples were added to 150 µL DPPH (0.2 mM) in a 96-well microtiter plate. DPPH solution and methanol served as blank. Absorbance was determined at 540 nm using a microtiter plate reader (Bio-Rad Elx 800), and the percentage of DPPH radical scavenging activity (% RSA) was calculated according to the following equation:

$$\%RSA = 100 \times ([\text{absorbance of control-absorbance of sample}]/\text{absorbance of control}).$$

Ferric reducing antioxidant power (FRAP) assay: The ferric reducing ability of the plant materials was assessed using the method described by Benzie and Strain (1996). The absorbance was measured spectrophotometrically at 595 nm. The final results were expressed as mol Fe (II) equivalent/g sample in a fresh sample.

Total phenolic content (TPC) assay: The determination of the phenolic compounds was performed with the Folin–Ciocalteu method using gallic acid as standard a reported by Siddiqui *et al.*, (2017). Briefly, 20 µL of each 5mg/mL *A. muricata* extract was mixed with 100 µL of the Folin–Ciocalteu (Sigma-Aldrich, USA) reagent in a microplate and shaken for 4 min followed by the addition of 75 µL of sodium carbonate (100 g/L). After 2 hours of incubation in the dark at room temperature, the absorbance was measured at 750 nm on the microplate reader. The distilled water was used as a reaction blank. All assays were performed in triplicate.

Experimental animals: A. muricata extraction with high antioxidant activity for animal study. In this experimental animal, a new *A. muricata* extract was prepared based on the results of DPPH, FRAP and TPC. The preparation of *A. muricata* water extract was performed with the temperature of 100°C for 1 h to get the finest extract with optimum antioxidant activity. First, 100 g of *A. muricata* was added into a beaker containing 1000 mL of distilled water. Then, it was heated at a temperature of 100°C for 30 min. The extract was filtered and put into a tube. The extract was freeze-dried and kept at 4°C until used.

Brine shrimp lethality test (BSLT): The brine shrimp lethality bioassay was carried out on the *A. muricata* extract using the standard procedure from Hamidi *et al.*, (2014) with slight modification. Briefly, brine shrimp (*Artemia salina*) eggs were hatched in artificial seawater prepared from commercial sea salt with a concentration of 25 g/L. After 24 hours, the matured shrimps were ready for the assay. Then, the extract was dissolved in water to get an extract concentration of 100 mg/mL. A two-fold serial dilution was carried out with salt water to obtain a test solution in the range of 0.09 – 100 mg/mL. One 96-well plate was used for the experiment. Sea saltwater was added to the plate in the amount of 90µL for each well. Then, suspension of larvae (10 µL) containing about 10 – 20 larvae each was added into the well. After that, 100 µL of each sample were put into a 96-well plate and incubated for 24 h. A column of the well containing 200 µL of saltwater and larvae was used as the normal control. After 24 h, the test tubes were examined. The number of dead larvae in each well was counted. The survival percentage and lethal concentration (LC_{50}) were determined.

In vivo acute toxicity evaluation: The studies were approved by the Institutional Animal Use and Care Committee (IACUC), Forest Research Institute Malaysia (FRIM). The animals were acclimatized in cages under standard environmental conditions of light/dark cycles (12 h/12 h) and temperature (23 ± 1°C). The animals had free access to tap water and a standard pellet diet.

Acute oral toxicity: The test was performed according to a method that was reported by Saleem *et al.*, (2017) with slight modification. The sighting study was started by treating one rat with 300 mg/kg *A. muricata* extract. The rat was observed for one hour after treatment, and then intermittently for 4 h and 24 h. After 24 h, because the first rat was not died, one new rat was treated with a higher concentration of 2000 mg/kg *A. muricata* extract and observed for 24 h. After 24 h, after observing the second rat was not die, the acute single oral test was started with the other four rats were treated with 2000 mg/kg of the extract and observed for one hour after treatment, and then intermittently for four hours, and thereafter the rat were further observed for up to 14 days following treatment. Subsequently, the animals were sacrificed.

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Statistical analysis: The results were expressed as the mean value ± standard deviation. Comparisons were performed within groups by the analysis of variance, using the ANOVA test. Significant differences between control and experimental groups were assessed by the SPSS version 21 (SPSS Inc, USA). A probability level $P < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

The assay of DPPH is based on the reduction of DPPH in the existence of hydrogen donating antioxidant. Extracts decrease the colour of DPPH due to the power of donating ability. DPPH is one of the compounds that have a proton free radical scavenging. Figure 1 clearly showed that BHT has the highest DPPH scavenging activity. The percentage of BHT scavenging activity was 66.41 %. The positive control vitamin C showed a lower percentage which was 60.25 % compared to BHT. The *A. muricata* extract that was prepared using parameter 60°C/15 min showed the highest scavenging activity compared to other parameters. The other parameter that showed better scavenging activity was 80°C/3 hours. Besides that, *A. muricata* extract those showed high antioxidant activity were *A. muricata* extract those were prepared by using the parameter of 100°C/15 min, 100°C/30 min, 100°C/1 h, 100°C/2 h, 80°C/2 h, 60°C/30 min and 40°C/30 min, 40°C/1 h, and 40°C/3 h. Lastly, the other extracts showed lower scavenging activity which was below 40%. The DPPH radical scavenging activity is correlated to the nature of phenolics contributing to their electron transfer and hydrogen donating ability (Rahman *et al.*, 2015). Therefore, the use of the parameter that preserves phenolic content is a constructive step to get the extract with the highest antioxidant activity. This result is supported by the previous study that was done by Andres *et al.*, (2020).

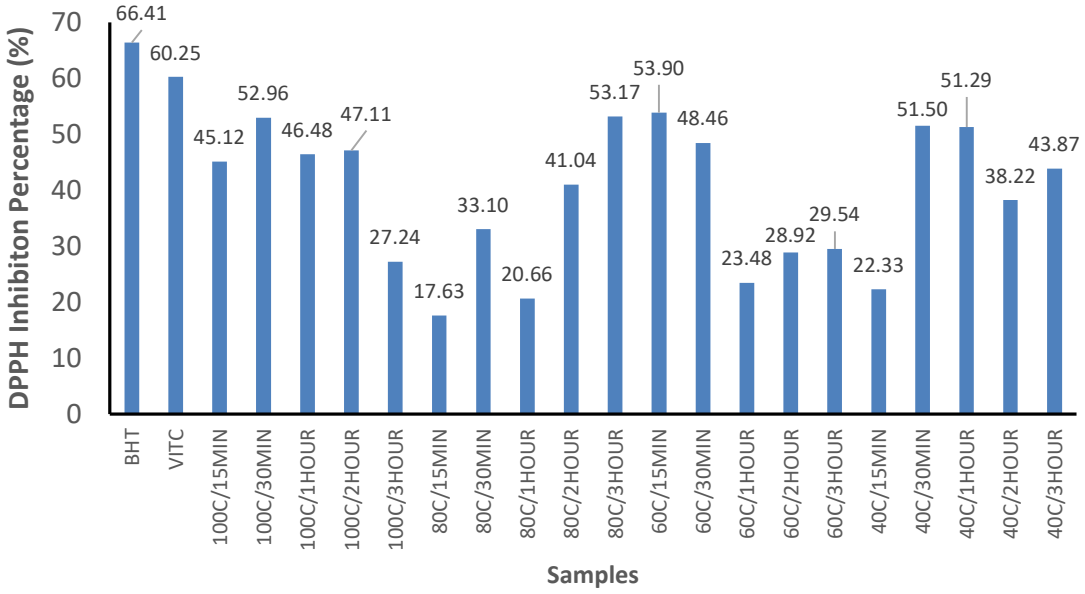

Figure 1: Percentage of DPPH inhibition of *A. muricata* extracts that were prepared using various temperature and duration

The FRAP assay is based on the reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form which has an intense blue colour. The change in absorbance is

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directly related to the total reducing power of the electron-donating antioxidants present in the extract. Figure 2 clearly showed that Vitamin C has the highest FRAP value compared to the others. The *A. muricata* extract that was prepared by using the parameter of 80°C/15 min showed the highest FRAP value compared to other *A. muricata* samples.

This was followed by *A. muricata* extract with a parameter of 40°C/1 h. The results clarify the ability of the extracts to function as good electron and hydrogen donors and therefore should be able to sack radical chain reaction by converting free radicals to more stable products. From Figure 2, the reducing power of the samples was in the following order: 80C/15 min > 40C/1 h > 100C/30 min. The data presented here indicated that the marked reducing activity of the extracts seems to be due to the presence of antioxidant in the extract that used lower temperature, which may preserve the compound's structure. Finding from this study was supported by the findings reported by Niazmand *et al.*, (2021).

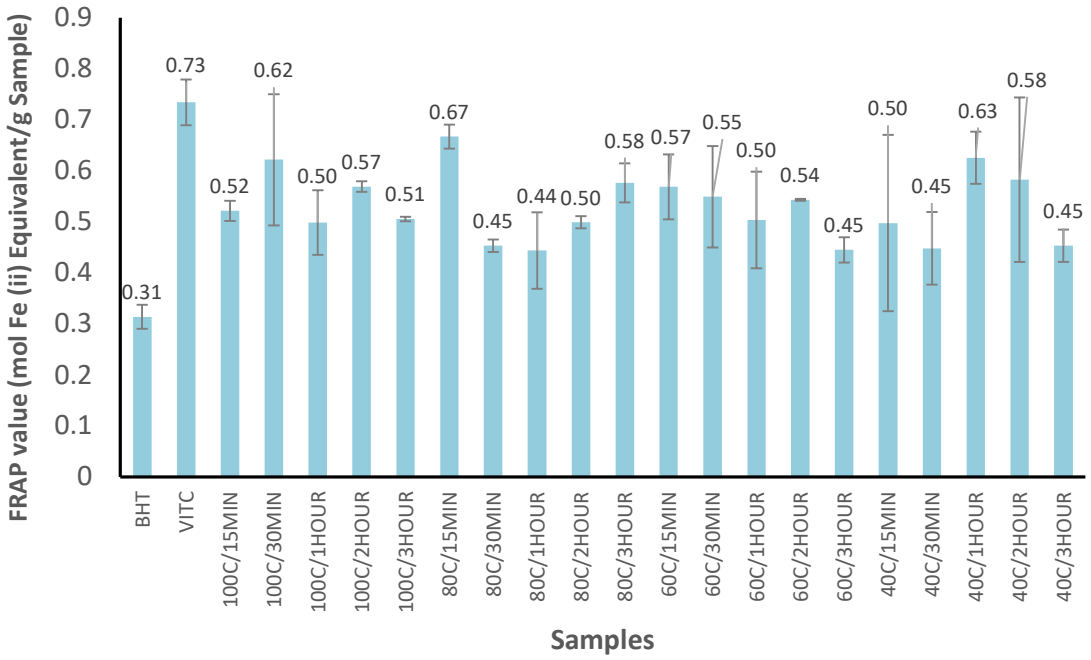

Figure 2: FRAP value of *A. muricata* extracts that was prepared using various temperature and duration

The total phenolic content of extracts was determined using the Folin-Ciocalteu assay. Total phenolic content determination is the process to measure the amount of phenolic content in the samples and might relate to the antioxidant activity (Aryal *et al.*, 2019). Figure 3 showed that the *A. muricata* extract that was prepared using the parameter of 40°C/30 min has the highest total phenols content. This was followed by the extract that was prepared with a parameter of 80°C/30 min. The third-highest TPC value was 100°C/30 min. Phenolic compounds contained in the plants have redox properties that allow them to acts as antioxidants (Liang *et al.*, 2010).

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High TPC showed by the temperature of 40°C has a positive relationship with antioxidant activity as shown in Figure 1 and Figure 2. It can be seen that high TPC gives a high antioxidant activity. Previous studies have shown that the capacity of the antioxidant is highly associated with the total phenolic compounds of the herb (Ulewicz-Magulska *et al.*, 2019). The BSLT is a simple and economical bioassay used for testing the toxicity of phytochemical present in the herbal extracts (Anderson *et al.*, 2018). Based on Figure 4, all concentrations of *A. muricata* extract displayed above 50% of brine shrimp survival.

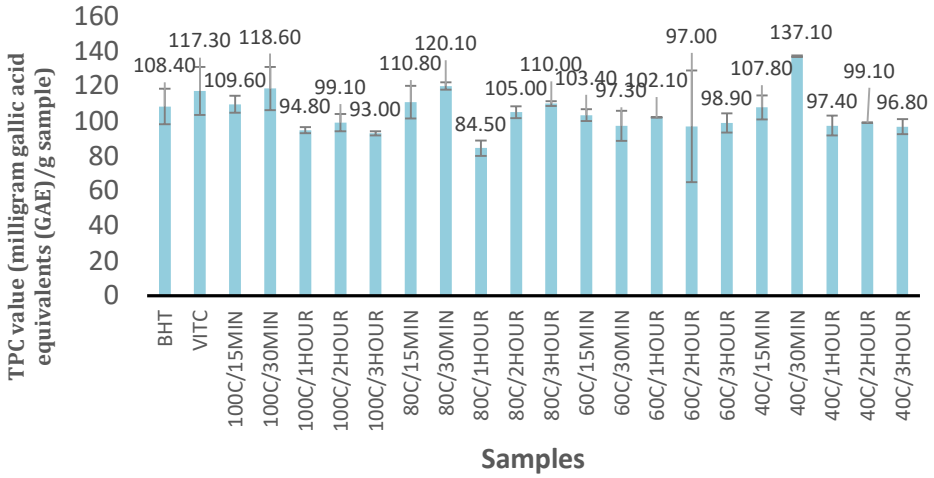

Figure 3: TPC value of *A. muricata* extracts that was prepared using various temperature and duration.

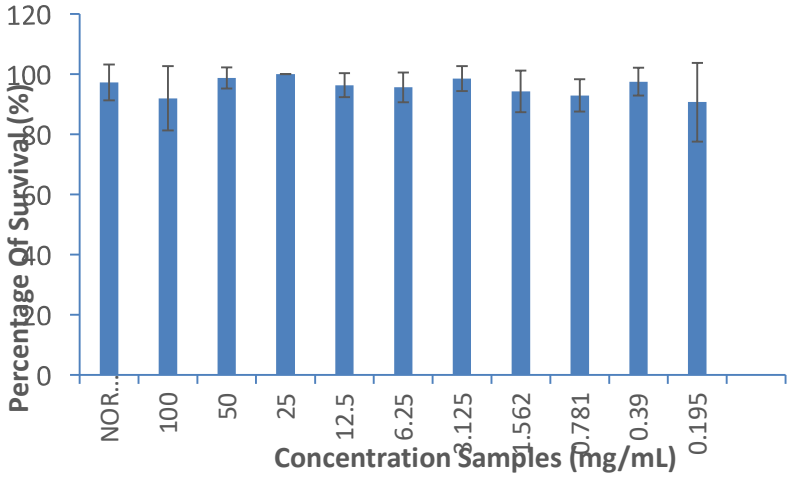

Figure 4: Brine shrimp lethality test of *A. muricata* extract that was prepared by using the parameter of 100°C/30 min

Acute toxicity test evaluates the toxicity effects that happen within a short time after administration of a single dose of herbal extract (Parasuraman, 2011). This testing is performed principally in rodents and is usually done early in the development of a new herbal product to provide information on its potential toxicity. Based on Table 1, no morbidity or mortality was observed in the treated groups at both doses during the acute toxicity study. As a result, the LD_{50} of the extract could be greater than 2000 mg/kg body weight. Besides that, there was a gradual increase in the body weight of the treated mice.

Study	OBSERVATION	REMARKS
Sighting study	Mice 1 – Active	Given dose: 300mg/mL Mice weight: Mice 0 – 27.3g
Sighting study	Mice 1 – Active	Given dose: 2000mg/mL Mice weight: Mice 1 – 27.3g
An acute oral single dose Day 1	 Mice 1 – Active Mice 2 – Active Mice 3 – Active Mice 4 – Active Mice 5 – Active	Given dose: 2000mg/mL Mice weight: Mice 1 – 27.3g Mice 2 – 31.5g Mice 3 – 24.2g Mice 4 – 25.1g Mice 5 – 29.2g
Day 14	 Mice 1 – Active Mice 2 – Active Mice 3 – Active Mice 4 – Active Mice 5 – Active	Mice weight: Mice 1 – 30.5g Mice 2 – 37.4 g Mice 3 – 24.2g Mice 4 – 25.1g Mice 5 – 29.2g

Table 1: Acute oral single dose toxicity of *A. muricata* that was prepared by using the parameter of 100°C/30 min.

CONCLUSION

As a conclusion, based on antioxidant tests it was shown that the best parameter to produce *A. muricata* extract was 100°C/30 min. The toxicity results showed *A. muricata* might be not toxic. However, further toxicological studies need to be done to ensure the safe usage of *A. muricata* leaves extract in human.

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DECLARATION OF CONFLICT OF INTEREST

The author has stated that there is no conflict of interest associated with the publication and no financial support, which could have influenced the outcome.

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