Comparison of Aqueous and Methanolic Extraction of *Annona muricata* Leaves by Freeze Drying Method and Study of Its Anti-Oxidant Activity

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ABSTRACT

Introdution: *Annona muricata*is a medicinal plant from Annonaceae family with a custard like texture. Each part of the plant contain various phytochemical constituents that contribute to medicinal activity. Some phytochemical constituents are acetogenins, coumarins, phenolic, alkaloids and flavonoids. **Objective**: This study aimed to identify the phytochemicals in *Annona muricata*leaves extract specifically phenolic and flavonoids and to evaluate the anti-oxidant activity by using two different types of solvents. **Methodologies**: Water and methanol were chosen as the solvents for extraction of the *Annonamuricata* leaves. This was to differentiate the yield of extraction of both solvents. Maceration is the extraction method that was chosen to extract the *Annona muricata*leaves. Freeze drying is the chosen method for drying purpose. Then, the extract was stored in the deep freezer to preserved it until it was ready to be used. **Results**: The yield of extracts by using both solvents were obtained and it showed methanolic extract produced a higher yield compare with aqueous extract. The results also revealed the presence of phenolic and flavonoid compound in *Annona muricata*leaves but the content varies in both type of extracts. However, there were no significant difference in phenolic and flavonoid content. The finding in this study proved that *Annona muricata*leaves did have anti-oxidant activity in methanolic and aqueous extract. **Conclusion**: Since there is no production of any product that are based on *Annona muricata*therefore, it is an opportunity in pharmaceutical area to incorporated *Annona muricata*in new pharmaceutical product such as tablets or creams

Keywords:*Annona muricata*; extraction; anti-oxidant; sour soup; flavonoid

INTRODUCTION

Before medicines were discovered, natural products were dominant in treating various type of ailments including *Annona muricata.* These products are extracted from living organisms around us such as animals, plants and microorganism. These natural resources has become a major source of active pharmaceutical ingredient that can be used as medicines. Natural products are also considered a safe treatment because they only exhibit very limited side effects to the patient compared to conventional drug[1]. Anti-oxidant is essential for living things especially human because it involves in metabolic processes and signaling mechanisms[2]. Anti-oxidant have the ability to treat various types of ailment because they have the anti-bacterial, anti-viral, anti-inflammatory, anti-allergy, anti-thrombotic and also vasodilator properties[3]. In recent years, many studies have reported that *Annona muricata* contained high anti-oxidant activity. However, there are limited number on the study of *Annona muricata* especially on the leaves using freeze drying method. Thus, this study aims to extract the *Annona muricata* leaves and evaluate the anti-oxidant activity.

MATERIALS AND METHODS

Materials

Dried *Annona muricata*leaves were obtained from a farm that are based in Johor, Malaysia. Methanol, gallic acid, aluminium chloride, potassium acetate, DPPH and beta hydroxy acid (BHA) were purchased from Techub, Malaysia. Folin-Ciocalteu reagent, sodium carbonate and butylated hydroxytoluene (BHT) were obtained from Pro Prima, Malaysia.

Preparation of Extract

The extraction was done by using maceration technique. This method was adopted from a journal of agriculture and food chemistry with a slight modification[4]. The dried leaves powder was dispersed in 150 mL of extraction solvent. Distilled water and methanol were used as the solvent in this extraction process. The mixture was mixed in an incubator shaker for 24 hours with 200 rpm at room temperature. After that, the mixture was filtered using a Whatman No.4 filter paper and washed with the solvent again. The mixture was washed repeatedly until the colour of the solution disappeared indicating that the active ingredient was fully extracted. Extraction with methanol were evaporated using rotatory evaporator before it was pre-freezed. Next, the mixture was transferred to a smaller vial and deep freeze at -60°C. The pre-freeze product were freeze dried at -50°C to -45°C for 24 hours. After being freeze dried, the extraction was weighed and sealed in a glass bottle and stored at -20°C prior to be used. Each extraction was conducted in triplicate.

Detection of Phenolic

0.05 g of extract was dissolved in 5 mL of distilled water. Few drops of Ferric (III) Chloride (FeCl3) solution was added. A dark green colour indicated phenolic compound presence in the extract.

Detection of Flavonoid

Few drops of Ferric Chloride ($FeCl_3$) solution was added to the small amount of extract. An intense green colour indicated flavonoid is presence in the extract.

Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteu colorimetric test as described in journal of food and drug analysis[5]. Gallic acid was used as standard because it has the highest content of anti-oxidant. 1.6 mL of *Annonamuricata* extract (50 μg/mL) or gallic acid was mixed with 0.2 mL of Folin-Ciocalteu Reagent (10%) and 0.2 mL of Sodium Carbonate Solution (10%). The mixture was stirred and kept at room temperature for 30 minutes. The absorbance was read using UviLine 9400 Spectrophotometer provided by AHS Laboratory Supplies. The wavelength used to get the absorbance reading was 760 nm. This test was conducted in triplicate. The phenol content was calculated as Gallic Acid Equivalent (GAE).

Total Flavonoid Content

Aluminium chloride colorimetric method was used to determine flavonoid content in *Annonamuricata* extract[6]. Quercetin was used as the standard because it has high content of flavonoid. Quercetin graph was drawn by using different concentration. 1 mL of extract (50 μg/mL) solution was mixed with 0.2 mL aluminium chloride 10% ($AlCl_3$) solution, 0.2 mL potassium acetate 10% and 5.6 mL distilled water. The mixture was kept at room temperature for 30 minutes. 415 nm wavelength was used to get the absorbance reading using UviLine 9400 Spectrophotometer provided by AHS Laboratory Supplies. The test was conducted in triplicate. Total flavonoid content was calculated as Quercetin Equivalent (QE).

DPPH Test

DPPH test was conducted based on the method reported in journal of pharmacognosy and phytochemical research[7]. The stock solution was prepared by dissolving

0.005 g of methanolic and aqueous extract in 5 mL of methanol solution. A two-fold dilution was carried out to dilute the concentration to 500, 250, 125 and 62.5 μg/mL. DPPH solution was prepared by dissolving 0.005 g of DPPH powder in 100mL of methanol solution. 1mL aliquot of *Annonamuricata* extract with different concentration was added to 4mL of DPPH solution. The mixture was shaked vigorously. Mixture was kept at room temperature and in the dark for 30 minutes. The absorbance was read using UviLine 9400 Spectrophotometer provided by AHS Laboratory Supplies by using wavelength of 517nm. Butylated Hydroxytoluene (BHT) was used as a standard and methanol with DPPH solution was used as a blank. The test was conducted in triplicate. The test was done in a dark room to preserved the DPPH solution. The anti-oxidant activity was calculated based on the formula:

$$\%\ \boldsymbol{of\ Scavenging\ Activity} = \frac{\boldsymbol{Acontrol - Asample}}{\boldsymbol{Acontrol}}$$

Statistical Analysis

The measurement were carried out in triplicates and data obtained were analyzed using the Statistical Package for the Social Sciences (SPSS) Version 20. Independent T-Test and One-Way analysis of variance (ANOVA) were used to determine the significant difference of all tests that were carried out. The significant difference was determined when P value <0.05. Results of this study were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Annona muricata Extracts

The extracted content was in a powder form and yield different colour based on the solvent used as shown in Figure 1. Aqueous extract produced a brown coloured powder and methanol extract produced a green coloured powder. The green colour is contributed by chlorophyll pigment that presence in the plant. Methanol able to extract chlorophyll pigment better than aqeuous thus producing a green coloured powdered extract instead of brown coloured powder. It was found that extraction by using methanol as the solvent produced a higher yield (5.97%) as compared to aqueous solvent (2.59%). The difference in both yield were due to polarity of the solvent and the compounds that were presence in the extract. It is known that aqueous is more polar than methanol because methanol have the presence of methyl that reduced its polarity. However, some compounds that are presence in the extract has a varying solubility in different polar solvents that caused different yield of extract powder. The findings showed that methanol and water were a good solvents to extract *Annona muricata* leaves.

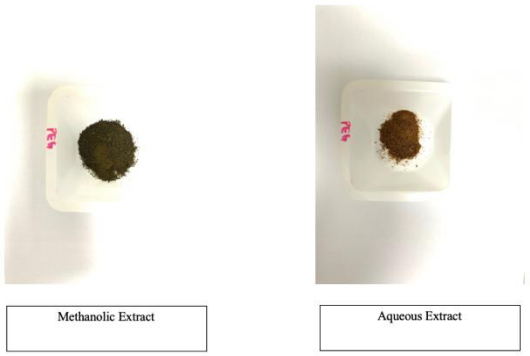

Figure 1. *Annona muricata* Extracts

Table 1. Extraction yield of *Annona muricata* Extracts.

Test Tubes	Amount of Extracts (g)	Yield of Extracts (%)
Methanolic Extract	4.775	5.97%
Aqueous Extract	2.073	2.59%

Preliminary Phytochemical Constituents Screening

Preliminary phytochemical screening revealed the presence of flavonoids and phenols compound in the *Annona muricata* extract. It is confirmed by the formation of dark green colour for phenolic and intense green colour for flavonoids. Further testing was done to estimate the actual content of phenolic and flavonoids in the extract. It is important to identify the content of the chemical constituents as it may affect the activity of *Annona muricata* extract.

Total Phenolic Content

The principle of Folin-Ciocalteu reagent is redox reaction. These reaction contributes to the anti-oxidant properties where it can act as reducing agents, hydrogen donators and singlet oxygen quenchers. The results were calculated based on the calibration curve ($y = 0.0075x + 0.3854$, $R^2 = 0.9862$) of gallic acid with the concentration ranging from 62.5 to 500 μg/mL and it were expressed as gallic acid equivalent (GAE) per gram of dry extract weight as shown in Table 2. Phenolic content depends on its solubility in different types of solvents and also the polarity of the solvents. High polar solvents able to produce a higher content of phenolic compound because it has a faster recovery rate. The higher the polarity of solvent, the faster the recovery of phenolic compound thus, producing higher yield of phenolic. It was found that aqueous extract contained higher phenolic content (56.51%) compared to methanolic extract (8.52%) because aqueous is more polar than methanol. However, the difference were not statistically difference ($P > 0.05$).

Table 2. Total Phenolic Content

Extraction	%Content (QE)	Mean (%) ± SD	Independent T-Test
Methanolic Extract	6.56	8.52 ± 4.35	P > 0.05
	13.5		
	5.50		
Aqueous Extract A	57.76	56.51 ± 11.78	
	44.16		
	67.62		

Total Flavonoid Content

Aluminium chloride colorimetric method was used to determine the flavonoid content in *Annona muricata* extract. The mechanisms of this method are production of acid stable and acid labile complexes by interaction of aluminium chloride. The flavonoids content in the extract were calculated based on the quercetin calibration curve ($y = 0.0048x + 0.5759$, $R^2 = 0.9072$) with the concentration ranging from 62.5 to 500 μg/mL. Quercetin was used as the standard for calibration graph because it contained abundant of flavonoids. Flavonoid content also depends on the polarity of the solvents used. In addition, flavonoid has the similar effects of solvent with phenolic compound. Therefore, there is a correlation between total phenolic content and total flavonoid content. Total flavonoid content was observed higher in aqeuous extract (40.43%) compared to methanolic extract (33.83%) due to high polarity of aqueous but the difference were not statistically difference ($P > 0.05$) as shown in Table 3.

Table 3. Total Flavonoid Content

Extraction	%Content (QE)	Mean (%) ± SD	Independent T-Test
Methanolic Extract	34.17	33.83 ± 0.99	$P > 0.05$
	32.72		
	34.60		
Aqueous Extract A	40.53	40.43 ± 0.18	
	40.53		
	40.22		

DPPH Radical Scavenging Test

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was used to determined the anti-oxidant activity of *Annona muricata* extract. The reaction occur when stable DPPH was scavenged by anti-oxidant compound through proton donation thus producing a reduced DPPH compound. Four different concentrations were selected based on some established journals on *Annona muricata* to evaluate the radical scavenging activity. It was observed that, there was an increase in trend of anti-oxidant activity with increasing concentration of the extract. The anti-oxidant activity of the extracts were influenced by the solvents used. Radical scavenging activity should have a correlation with flavonoid and phenolic content. However based on previous study, solvents used for extraction also play a role in determine the radical scavenging activity. It was stated that extraction that was done using organic solvents able to produce a stronger radical scavenging activity compared to inorganic solvents. Based on Table 4, it was observed that methanolic extract have the highest anti-oxidant activity which was 67.26% compared to aqueous extract which was 53.32% although aqueous contained higher phenolic and flavonoid content. The findings may due to type of solvent used during extraction because methanol is an organic solvent meanwhile aqeuous is inorganic solvent. Therefore, methanolic extract showed the better scavenging activity. This finding proved that *Annona muricata* leaf extract contained a significant level of anti-oxidant activity.

Table 4. Radical Scavenging Activity

	Mean ± SD	**One-Way ANOVA**	**Post-hoc (Tukey)**
Butylated Hydroxytoluene 500 μg/ml	65.78 ± 1.69		ME 500 μg/ml AE 500 μg/ml
Butylated Hydroxytoluene 250 μg/ml	55.05 ± 0.81		ME 250 μg/ml AE 250 μg/ml
Butylated Hydroxytoluene 125 μg/ml	53.96 ± 1.45		ME 125 μg/ml AE 125 μg/ml
Butylated Hydroxytoluene 62.5 μg/ml	47.99 ± 4.83	P < 0.05	ME 62.5 μg/ml AE 62.5 μg/ml
Methanol Extract 500 μg/ml	73.45 ± 0.00		BHT 500 μg/ml AE 500 μg/ml
Methanol Extract 250 μg/ml	64.21 ± 0.99		BHT 250 μg/ml

		AE 250 μg/ml
Methanol Extract 125 μg/ml	47.06 ± 0.36	BHT 125 μg/ml AE 125 μg/ml
Methanol Extract 62.5 μg/ml	8.13 ± 0.38	BHT 62.5 μg/ml AE 62.5 μg/ml
Aqueous Extract 500 μg/ml	50.96 ± 3.89	BHT 500 μg/ml ME 500 μg/ml
Aqueous Extract 250 μg/ml	13.59 ± 2.45	BHT 250 μg/ml ME 250 μg/ml
Aqueous Extract 125 μg/ml	4.36 ± 0.69	BHT 125 μg/ml ME 125 μg/ml
Aqueous Extract 62.5 μg/ml	0.73 ± 0.34	BHT 62.5 μg/ml ME 62.5 μg/ml

CONCLUSION

In this study, the yield of extraction have been determined by using different type of solvents and methanol solvent showed a higher yield compared to aqueous solvent. Furthermore, it is proven that *Annona muricata* leaves has a significant anti-oxidant activity. It is contributed by the phytochemicals that are present in it such as phenolic and flavonoid. However, there are many phytochemicals that contribute to anti-oxidant activity which have not been study yet. The findings of this study become a new opportunity in pharmaceutical area because it can lead to development of new pharmaceutical product since there is no pharmaceutical product produced by using *Annona muricata* yet. The findings also essential for new pharmaceutical product development to use the freeze drying method thus producing a more stable and last longer product.

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REFERENCES

[1] Yahaya, G., Faten, A.E., Fred, W., Hany, A.S. (2014). Phytochemical screening , anti-oxidant activity and in vitro anticancer potential of ethanolic and water leaves extracts of Annona muricata (Graviola).*Asian Pac. J. Trop. Med., 7(1),* doi: 10.1016/S1995-7645(14)60258-3.

[2] Douglas, W.W., Paul, N., Harpal, S.B., Keith, G., Ram, S., Fabien, D.M., Rie, H., Toru, T. (2017). The role of food antioxidants, benefits of functional foods, and influence of feeding habits on the health of the older person: An overview.*Antioxidants*. *6(4)*: 1–20, 2017.

[3] Velioglu, Y.S., Mazza G., Gao, L., Oomah, B.D. Antioxidant Activity and Total Phenolics in Selected Fruits , Vegetables , and Grain Products. (1998). *Journal of Agriculture and Food Chemistry. 46(10)*: 4113–4117.

[4] Tao, W., Rosa, J., Haiyan, L., Liwei, G., Hordur, G.K., Sivakumar, R., Gudrun, O. (2012). Antioxidant capacities of phlorotannins extracted from the brown algae Fucus vesiculosus. *J. Agric. Food Chem.60(23)*: 5874–5883.

[5] Quy, D.D., Artik, E.A., Phuong, L.T.N., Lien, H.H., Felycia, E.S., Suryadi, I., Yi, H.J. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica.*J. Food Drug Anal., 22(3)*: 296–302.

[6] Sushant, A., Manoj, K.B., Krisha, D., Puspa, K., Roshani, G., Niranjan, K. (2019). Total Phenolic Content, Flavonoid Content, and Anti-Oxidant Potential of Wild Vegetables, from Western Nepal.*Plants*, *8(4)*: 96.

[7] Patil, M.P., Patil, K.T., Ngabire, D., Seo, Y.B., Kim, G.D. (2016). Phytochemical , Antioxidant and Antibacterial Activity of Black Tea (Camellia Sinensis), *International Journal of Pharmacognosy and Physicochemical Research. 8(2)*: 341-346.