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Total phenolics and total flavonoids of extracts from freshwater Clam (*Corbicula fluminea*) using different solvents

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Abstract

The ethanol, ethyl acetate, and hexane extracts of the freshwater clam (*Corbicula fluminea*) were studied for the total phenolics and total flavonoids. Total phenolics and total flavonoids of the extracts were evaluated using Folin-Ciocalteau and Aluminum chloride colorimetric methods respectively. The findings showed that the total phenolics of the ethanol extract (1.67±0.28mg GAE/g of dried sample) were substantially higher than the total phenolics obtained from the ethyl acetate (0.70±0.00mg GAE/g) and hexane extracts (0.56±0.23mg GAE/g). While the total flavonoids in the ethyl acetate extract displayed a slightly higher total flavonoid (43.84±0.92mg QE/g of dried sample) relative to ethanol (30.41±1.34mg QE/g of dried sample) and hexane extracts (20.28±0.00mg QE/g of dried sample). Using ethanol, the highest yield for extraction was obtained. Ethanol is the best solvent among the three - ethanol, ethyl acetate, and hexane in terms of extraction yield and total phenolics. In addition, it can be inferred that the presence of significant amounts of phenolics and flavonoids suggests that freshwater clam is a promising source of antioxidants that provides nourishing proteins and oxidative stress remedies.

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Introduction

A part of the growing advanced technology is the daily exposure to various oxidants such as environmental pollutants, radiation, chemicals, several food ingredients and preservatives, pesticides and even physical stress which eventually cause depletion of immune system antioxidants resulting to cell damage and many serious ailments. Antioxidants hold a significant function in the prevention of cell and tissue damage. It has been widely proven that plants provide a long list of secondary metabolites to include flavonoids and phenolic compounds that act as chemical defense, yet, experts continue searching for another possible source of natural antioxidants which include aquatic species. Several studies have already been conducted exploring the presence of these compounds from freshwater and marine organisms. Freshwater clam is among the many aquatic plants that have attracted the attention of many researchers.

Recently, development of new drugs and specific health foods have considered freshwater and marine products as sources of nutraceutical and functional foods (Koyama *et al*., 2014).

Freshwater clam, *Corbicula fluminea* (Fig. 1) is a clam that belongs to class Bivalvia and family corbiculidea. Juvenile freshwater clam has entirely developed shell and has a tan to brown and sometimes yellow-green to brown or black, solid shells and are generally rounded to slightly triangular in shape.

Fig. 1. (a) The Freshwater clam and its (b) meat from Del Carmen, President Roxas, Cotabato.

This clam has been found to possess various medical and biological effects, including cholesterol-lowering, hepatoprotective agent (Chijimatsu, *et al*., 2008; Hsu, *et al*., 2010), antioxidant, anticancer, antihypertension, and hypocholesterolemic effects (Kong *et al*., 2011) but its active constituents have not been studied extensively, Kong *et al*., added.

Factors such as local environmental conditions and geographical location may affect the growth of phytoplankton, the primary food source of freshwater clams, and so will likewise affect the secondary metabolites present. In addition, the method of extraction, especially the kind of solvent used should be given consideration. The solvent's characteristics, polarities and the nature of the extractables can affect the yield of the crude extract. According to Tomsone *et al*., (2012), the solvent polarity is a very important parameter to consider to have higher extract yields – the higher the polarity of the solvent, the better the solubility. This, in turn, can affect the amount of the bioactive compounds that go along with the extract.

With these, freshwater clam from the Philippines, particularly in Del Carmen, Pres. Roxas can be a good source of secondary metabolites because the area has rich biodiversity. Also, no studies so far have been made on these freshwater clams that are endemic to that particular area. The purpose of the study is not only to determine the total phenolics and the total flavonoids of *Corbicula fluminea* but also to contribute to the validation of the claims of other researchers on its beneficial therapeutic effect.

Thus, the result of this study will provide baseline information to concerned agencies for the possibility of finding a new and potential source of natural antioxidants.

Materials and methods

Sample Collection and Preparation

The specimen of the freshwater clam in the study were coming from a privately- owned rice field of Barangay Del Carmen, Pres. Roxas. President Roxas is one of the municipalities comprising Arakan Valley. It is a first-class municipality in the province of Cotabato. It is situated at geographical coordinates of

7° 9' 16" North, 125° 3' 21" East. Live freshwater clams were collected by hand and immediately washed with tap water to remove decomposed organic wastes of dead plants and leaves and other objects which were included during the collection. Approximately 1000 grams of freshwater clam meat was removed by blanching with a pre-boiled water. Then, the freshwater clam meat was subjected to atmospheric drying in an oven with nitrogen blanketing. The dried freshwater clam meat was divided equally into three (or about 300g each) and was transferred into a previously cleaned sample container. The first 300g of the meat was soaked with 95% ethanol, the second 300g with ethyl acetate, and the remaining 300g with hexane for 24 to 48 hours. Each mixture was filtered through a Buchner funnel with gentle suction. The flask and the freshwater clam extract were washed with a fresh portion of the solvent and the washings were transferred to the funnel to combine with the first filtrate taking note of the total volume of the solvent used. After the filtration process, the residue was discarded, and the filtrate was concentrated under *vacuum* at a temperature below 50°C using rotary evaporator. The extract was stored in a tightly stoppered bottle at 0 - 5°C until its analysis.

Determination of Total Phenolics

Total phenolics (TP) was determined according to the Folin-Ciocalteu reagent using gallic acid as the standard. The Folin – Ciocalteu reagent (FCR) or Folin's phenol reagent, or Folin – Denis reagent, also known as the gallic acid equivalence process (GAE), is a mixture of phosphomolybdate and phosphotungstate used for phenolic and polyphenolic antioxidants colorimetric in vitro assay. Chemically, the phosphomolybdotugstic acid is a hetero-polyacid which produces blue color with the phenolic group.

One (1)mL of the extract was added to a flask with 9mL distilled water. Then, 1mL of Folin-ciocalteu's phenol reagent was added and was mixed thoroughly. Ten (10)mL of 7% sodium carbonate was added to the mixture and finally diluted with 25mL distilled water and allowed to stand at room temperature for 90 minutes. The absorbance was measured using a spectrophotometer at 750nm. The standard calibration curve was prepared using 25 to 150µg/mL in 80% methanol of gallic acid. The total phenolic was expressed as µg gallic equivalents (GAE) / g of dried samples and reported as mean value ± SD.

Calculations:

$$TP = \frac{\frac{\text{mg GAE (gallic acid)}}{1000\ \text{mL}} \times \frac{1\ \text{mL}}{\text{mg CE}} \times \frac{\text{mg CE}}{\text{mL of CE. sol'n}} \times \text{Vol.}_{(\text{conc. CE}),\ \text{mL}}}{\text{Wt. of dried clam meat (g)}}$$

Determination of Total Flavonoids

The total flavonoids (TF) was determined using the aluminum chloride colorimetric method with some modifications. The principle involved in aluminum chloride ($AlCl_3$) colorimetric method is that $AlCl_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Also, it also forms labile acid complexes with the ortho hydroxyl groups in the A- or B-ring of flavonoids (Bag *et al.*, 2015).

About 1mL aliquot of the extract solution was added to a 10mL volumetric flask with 4mL distilled water. Then, 0.3mL of 5% $NaNO_2$ was added and allowed to stand for 5 minutes; 0.3mL 10% $AlCl_3$ was further added. After the 6th minute, a 2mL of 1M NaOH was added and diluted with distilled water up to the 10mL mark. The solution was shaken vigorously, and the absorbance was recorded at 510 nm wavelength. The same wavelength was used for the generation of a standard calibration curve using known concentrations of quercetin (50, 100, 150, 200 and 300mg/L). The concentrations of flavonoids in the sample was then calculated from the calibration curve and was expressed as µg quercetin equivalent (QE) per g of dried sample. Then, it was reported as mean ± SD.

Calculations

$$TF = \frac{\frac{\text{mg (quercetin)}}{1000\ \text{mL}} \times \frac{1\ \text{mL}}{\text{mg CE}} \times \frac{\text{mg CE}}{\text{mL of CE. sol'n}} \times \text{Vol.}_{(\text{conc. CE}),\ \text{mL}}}{\text{Wt. of dried clam meat (g)}}$$

where:

CE – crude extract

Statistical Analysis

All experimental results were reported as a mean ± standard deviation of triplicate parallel measurements. One – way analysis of variance was used for the statistical analyses and p- values < 0.05 were considered as significant.

Results and discussion

Total Phenolics from the Crude Extracts of Freshwater Clam

The total phenolics extracted from the freshwater clam, *Corbicula fluminea,* was determined according to the Folin-Ciocalteu spectrophotometric method. Total phenolics values in Table 1 were obtained from the calibration curve y = 0.0025x + 0.0291 with R^2 = 0.9814, where x is the absorbance and y is the concentration of the gallic acid solution (mg/mL) expressed asmg GAE/g of dried sample.

Table 1. Total Phenolics extracted from the Crude Extracts of Freshwater Clam Meat.

Extract	TP (mg GAE/g of dried sample
Ethanol	1.67±0.28
Ethyl acetate	0.70±0.00
Hexane	0.56±0.23

Results are the means of triplicate measurements ± standard deviation

As shown in the table, the amount of the total phenolics varies depending on the kind of solvent used. It ranged from 0.56± 0.23 to 1.67 ± 0.28mg GAE/g of dried sample. Among the three fractions used, ethanol extracts gave a significantly higher total phenolics compared to ethyl acetate, and hexane extracts and the latter two extracts are not significantly different from each other (p < 0.05). The different polarity of the solvents could explain the variation and that most of the phenolic compounds present in the freshwater clam might be polar in nature. According to Michiels *et al.*, 2012, the properties of extracting solvents significantly affected the measured total phenolics content (±25% variation) and antioxidant capacity (up to 30% variation) in fruits and vegetables, and it is said that solvent polarity is the most important parameter. Hence, the higher the polarity of the solvent used the better the solubility of phenolic compounds.

Findings of the present study agree with the total phenolics evaluated from *Limoniastrum monopetalum* and *Echinops spinosus* (Khedler *et al.*, 2014). Ethanol extract showed the highest amount of total phenolics than ethyl acetate, hexane, and chloroform extracts. A similar result was obtained from *Limnophila aromatica*, where the highest phenolic content was exhibited by ethanol extract (Do *et al.*, 2014). Furthermore, Settharaksa *et al.*, (2014) added that the high total phenolic and flavonoid contents in *Syzygium gratum* (Wight) s.n. were present in the water extract than in ethanol and methanol extracts.

Several studies reported the presence of phenolic compounds in clams. In the study of Ramasamy *et al.*, 2012, twelve bioactive compounds were detected in the marine clam, *Anadara granusa*, which include furan compound, palmitic acid, fatty acid esters, stearic acid, ketone, a phenol compound, and plasticizer compound. Phenolic compounds have a significant impact on humans due to its antioxidant activity. The redox properties of phenolics enable them to act as antioxidants which depend on the hydroxyl groups present in the molecular structure.

Total Flavonoids from the Crude Extracts of Freshwater Clam

The total flavonoid content was calculated from the calibration curve y = 0.0005x - 0.0027 with R^2 = 0.9947 using quercetin as a standard, expressed asmg quercetin equivalent (QE) per g of dried sample (Table 2). The content of total flavonoids in the freshwater clam ranged from 20.28±0 to 43.84±0.92mg QE/g of dried sample.

In contrary to the total phenolics, ethyl acetate extract displayed significantly higher total flavonoids when compared to ethanol and hexane extracts @ p < 0.05. Variations of the results may not only be due to the different polarities of the compound which were selectively more soluble in various solvents as confirmed by Ngo *et al.* (2017) but also to the effectiveness of the solvent to dissolve a particular compound (Adaramola *et al.*, 2016). The contrasting

result was obtained in the study of Khedler *et al*., 2014, ethanol extract showed higher flavonoids content compared to ethyl acetate, hexane, and chloroform extracts.

Table 2. Total Flavonoids extracted from the Freshwater Clam Meat.

Extract	TF (mg QE/g of dried sample)
Ethanol	30.41±1.34
Ethyl acetate	43.84±0.92
Hexane	20.28±0.00

Results are the means of triplicate measurements ± standard deviation

In the preliminary identification of the chemical components in the three extracts of clam *Paphia malabarica,* it was reported that it contains alkaloids, flavonoids, phenolics, saponin and sterols (Eswar *et al*., 2015). The presence of these compounds especially flavonoids made the clam a potent antioxidant. Flavonoids belonged to naturally occurring compounds which can act as antioxidants and believed to have positive effects on human health. This group of compounds is commonly found in a variety of fruits, tea (Beecher, 2003) and even in marine bivalve such as *P. malabarica* (Eswar *et al*., 2015).

Thus, based on the results, it was also implied that freshwater clam meat can be a source of bioactive compounds which exhibits antioxidant activity.

Conclusion

The extracting solvent significantly affected the total phenolics and total flavonoids of the freshwater clam meat from Del Carmen, Pres. Roxas, Cotabato, Philippines. The study has shown that as far as total phenolics, ethanol served as the best solvent among the three—ethanol, ethyl acetate, and hexane. However, the ethyl acetate extract maximized the number of total flavonoids. Variations could be the effect of the polarity and effectiveness of the solvent to dissolve a particular compound.

Furthermore, it can be inferred that the presence of considerable amount of phenolics and flavonoids suggests that freshwater clam is a promising source of antioxidants which provides nourishing proteins and remedies for oxidative stress.

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