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Yields, zoochemical profiles, and antioxidant activities of extracts from freshwater clam (*Corbicula fluminea*) using different solvents

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Abstract

Freshwater clam is among the many aquatic organisms that possessed many medical and biological effects. Several factors may affect the growth of phytoplankton, and so will likewise affect the secondary metabolites present. The study aimed to determine the crude extract yields, zoochemical profiles, and the antioxidant activities of the freshwater clam (*Corbicula fluminea*) using ethanol, ethyl acetate and hexane as extracting solvents. Established test procedures were used to test the presence of prominent groups of zoochemicals. The antioxidant activity was assayed using free radical scavenging activities on 2,2-diphenyl-1- picrylhydraziyl (DPPH˙) radical. The results showed that the crude extract yield using ethanol (120.49±0.35 mg/g) is significantly higher than ethyl acetate (98.09±0.43) and hexane (82.81±0.06). The following secondary metabolites were found, namely: terpenoids, phenols, tannins, saponins, steroids, and alkaloids. The presence of phenolic substances and maybe other substances account for the antioxidant activities. Results of the study revealed that the scavenging effects of the crude extracts were in a concentration-dependent manner. Ethyl acetate extract showed the highest scavenging activity expressed as percentage inhibition, 30.25% at the highest tested concentration (1000 ppm) and 24.47% for the lowest tested concentration (100 ppm) while hexane extract showed the lowest scavenging activity with 25.52% (1000 ppm), and 19.08% (100 ppm). The three crude extracts of the freshwater clam demonstrate considerable antioxidant effects making the clam a promising nutritional food. As a health-promoting food, it will not just provide proteins, lipids, and others, but also remedies for oxidative stress.

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Introduction

Despite the considerable number of bioactive compounds from plant sources, persistent interests to explore the most promising sources remain and have grown until today. Marine world has been known for its astonishing biodiversity, thus it has become a rich natural resource for many biologically active compounds of which some remained almost unexplored (Abad *et al.,* 2011).

Recently, the development of new drugs and specific health foods have considered freshwater and marine products as sources of nutraceutical and functional foods (Koyama *et al.,* 2006). Freshwater clam (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.

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.

Antioxidants are known for their action against free radicals. They are capable of stabilizing or even deactivating dangerous free radicals (Percival, 1998). They act as scavengers, preventing cells and tissue damage which resulted from the oxidation process. The occurrence of numerous illnesses such as atherosclerosis, diabetes, cancer, and aging are often related to free radicals. Fortunately, nature has provided a wide variety of naturally occurring antioxidants and has blessed each cell with adequate protective mechanisms against any harmful effects brought by free radicals (Devasagayam *et al.,* 2004).
In the study of antioxidant substances, 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 antioxidant substances that go along with the extract.
This study investigated the key secondary metabolites and antioxidant activities of the extracts from freshwater clam that are thriving in a locality in Cotabato, Philippines.This will provide baseline information on the zoochemicals and antioxidant activity of freshwater clam.

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 (Fig. 2). 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 the Crude Extract Concentration and Yield

Approximately 10 mL of concentrated crude extract solution resulting from the use of the rotary evaporator at reduced pressure was transferred using a pipet into a previously weighed empty dish. It was then placed in an oven at a temperature of 50°C for ethanol, ethyl acetate and hexane fractions for one hour. Cycles of drying, desiccating and weighing were repeated until constant weight was obtained. Then, the crude extract yield was determined by multiplying the crude extract concentration by the total volume divided by the total weight of the dried clam meat. The whole process was done in triplicate.

Calculation for Crude Extract Yield (mg crude extract/g fresh clam meat):

$$\text{Crude Extract Yield} = \frac{\text{CEC} \times \text{total volume of conc crude extract soln}}{\text{total weight, (g) of dried clam meat}}$$

where:
CEC – crude extract concentration

Zoochemical Analysis

Established test procedures were applied to the sample extracts to test the presence of prominent groups of zoochemicals.

Test for Terpenoids: A 2.5-mL amount of sample extract was added with 1 mL of chloroform and mixed; then, 1.5 mL of concentrated sulfuric acid was carefully added. A layer of reddish-brown color at the interface indicated a positive result.

Test for Phenols: A 1-mL volume of sample extract was added with 2 mL of distilled ad a few drops of 10 % aqueous ferric chloride solution. A formation of blue or green color indicated a positive result.

Test for Glycosides: A 1-mL quantity of sample extract was added with 2 mL chloroform and 2 mL acetic acid, mixed, then, allowed to cool in an ice bath; to the cold mixture was carefully added concentrated sulfuric acid. A color change from violet to blue-green indicated the presence of glycosides.

Test for Quinones: A 1-mL volume of sample extract was treated with alcoholic potassium hydroxide solution. A coloration from red to blue indicated a positive result.

Test for alkaloids: A 1-mL amount of sample extract was added with 0.75 mL of 1% hydrochloric acid. The mixture was heated in a water bath for 15 minutes after which 3 drops of Wagner's reagent were added. The formation of orange precipitate confirmed the presence of alkaloids.

Test for flavonoids: A 2-mL quantity of sample extract was added with a few drops of concentrated hydrochloric acid followed by a piece of magnesium ribbon. The appearance of magenta red/pink color after 3 minutes indicated a positive result.

Test for tannins: A 1-mL portion of sample extract was added with 1 mL of 5 % ferric chloride solution. The formation of yellowish brown precipitate indicated a positive result.

Test for saponins: A 1-mL volume of sample extract was added with 10 mL of distilled water. The mixtures were then shaken for 30 seconds and the formation of a honeycomb/froth indicated a positive result.

Test for coumarins: A 1-mL amount of sample extract was dried for a couple of days in a water bath. The dried extract was then placed in a vial, covered with a filter paper that was pretreated with 1N sodium hydroxide solution. Then, it was heated and allowed to boil for 15 minutes. After boiling, the filter paper was taken and examined under the UV light. The presence of yellow fluorescence indicated a positive result.

Test for anthraquinone: A 1-mL volume of sample

extract was dried for a couple of days in a water bath. The dried extract was extracted with 5 mL chloroform, filtered, and the filtrate was added with 2 mL of 10 % ammonium hydroxide. The formation of a bright pink color indicated a positive result.

Total Antioxidant Activity: DPPH Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. This method measures the electron-donating activity of other compounds in the mixture, and, hence provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to a mixture will react with and bleach DPPH. DPPH is reduced from a purple compound to a light-yellow compound by electrons from oxidant compounds. The reaction of DPPH with hydroxyl groups involves a hemolytic substitution of one of the phenyl rings of DPPH yielding 2-(4-hydroxyphenyl)-2-phenyl-1- picryl hydrazine as a major product while 2-(4nitrophenyl)-2phenyl-1-picrylhydrazine is also formed via a series of secondary processes. The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged (Al Hafiz, 2010).

The DPPH solution (0.006%) was freshly prepared by dissolving 6 mg DPPH in 50 ml methanol. Freshly prepared DPPH solution was taken in the test tubes and extracts were added followed by a series of dilutions (100-1000 µg) to every test tube to attain a final volume of 2 mL. It was incubated for 30 minutes in the dark and the decrease in absorbance was measured at 517 nm using a spectrophotometer. The percentage inhibition of radicals was calculated using the following formula.

$$\% \text{ Inhibition } = \frac{(1 - \text{absorbance of the sample})}{\text{absorbance of the control}} \times 100$$

Statistical analysis

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

Results and discussion

In this study, the crude extracts from the local freshwater clam were produced using ethanol, ethyl acetate, and hexane. Table 1 shows the crude extract yield from the meat of freshwater clam expressed as mg crude extract/g of dried clam meat. Using analysis of variance, ethanol extract has given a significantly higher extraction yield when compared to ethyl acetate and hexane extracts @ $p < 0.05$.

Table 1. Crude extract yield from the meat of freshwater clam.

Extract	Crude extract Concentration (mg/mL)	Crude extract yield (mg of crude extract/ g of dried clam meat
Ethanol	11.22	120.49±0.35
Ethyl acetate	9.14	98.09±0.43
Hexane	7.71	82.81±0.06

Results are the means of triplicate measurements ± standard deviation.

The higher extraction yield displayed by ethanol may show the superior ability of this solvent to recover a greater amount of extractable compounds from the freshwater clam. This is followed by ethyl acetate and, then, by hexane. There is an indication here that solvent polarity matters. The higher results using polar solvents indicate that most of the extractable substances are polar. Results of the study conformed to Khedher *et al.,* (2014) who investigated the solvents effect on Phenolic Contents and Antioxidant

Activities of the *Echinops Spinosus* and the *Limoniastrum monopetalum*. They found out that the more polar solvent gave the highest yield in extractions.

Zoochemical screening also revealed the presence of a number of secondary metabolites in the three crude extracts of freshwater clam meat as presented in Table 2. The selected secondary metabolites included terpenoids, phenols, glycosides, quinones, alkaloids, flavonoids, tannins, saponins, coumarins, anthraquinones, and steroids.

The results showed that terpenoids, phenols, tannins, saponins, and steroids were found in the three extracts except for alkaloids where it was only found in ethanol extract. Glycosides, quinones, flavonoids, coumarins, and anthraquinones were absent.

Table 2. Results for the zoochemical screening for the various extracts of Freshwater clam meat.

Zoochemical	Ethanol extracts	Ethyl acetate extracts	Hexane extracts
Terpenoids	+	+	+
Phenols	+	+	+
Glycosides	-	-	-
Quinones	-	-	-
Alkaloids	+	-	-
Flavonoids	-	-	-
Tannins	+	+	+
Saponins	+	+	+
Coumarins	-	-	-
Anthraquinones	-	-	-
Steroids	+	+	+

Legend:

(-) –the absence of the ring, color change, precipitate or froth

(+) – the positive appearance of the ring, color change, precipitate or froth.

The presence of terpenoids, phenols, alkaloids, saponins, and steroids in the present study is similar with the study of Eswar *et al.,* (2015) who evaluated the preliminary qualitative analysis of clam *Paphia malabarica* from Girgon Chowpatty Creek, Mumbai. These results were also analogous to the secondary metabolites present in marine sponges which were investigated by Thambidurai *et al.,* (2017).

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

According to Eswar *et al.,* (2015), a large number of terpenoids are produced by plants and animals. Terpenoids are known for its therapeutic purposes and some selected terpenoids have anti-inflammatory activity (de las Heras *et al.,* 2003). Terpenoids also exhibited cancer chemo preventive effects, antimicrobial, antifungal, anti-viral, anti-hyperglycemic, anti-inflammatory and antiparasitic activities (Eswar *et al.*, 2015).

Fig. 2. Map of the sampling site, Barangay Del Carmen, Pres. Roxas, Cotabato.

These biological activities are also known for the presence of saponins. Presence of phenols in the extracts of freshwater clam in the present study is also a very good indication of antioxidant activity, the capacity to scavenge free radicals and thus a potential therapeutic agent against diseases.

Phenols and tannins are both polyphenolic compounds. Tannins are considered a good astringent. As mentioned by Khanbabaee *et al.,* (2001), tannins possess antiviral, antibacterial and antitumor activities.

The ethanol extracts of freshwater clam showed the occurrence of alkaloids. As mentioned by Thambidurai *et al.,* (2017), alkaloids were carefully isolated from some marine mollusks organisms with aliphatic nitrogen holding elements. Its pharmacological applications include antimalarial, antiasthma and anticancer (Thambidurai *et al*., 2017).

Alkaloids are used for plant growth and protection (Babbar, 2015) hence when eaten by animals, it can cause death (Sharma, 2017).The three crude extracts of freshwater clam revealed the presence of steroids which is similar to the results obtained from clam *G. divaricatum* extract (Eswar *et al.,* 2016). Steroids are widely distributed among plants and animals and are biologically important on the brain and spinal tissues and sex hormones (Marwat *et al.,* 2005). Moreover, the antioxidant activity of the three crude extracts of freshwater clam was evaluated by their ability to scavenge free radicals. The scavenging effects of the three crude extracts on DPPH radical are shown in Fig.3.

Results of the study revealed that the scavenging effects of the crude extracts were in a concentration-dependent manner. As shown in the figure, ethyl acetate extracts of freshwater clam exhibited the highest DPPH radical scavenging activity expressed as percentage inhibition followed by ethanol extracts. For the highest concentration tested (1000 ppm), the percent inhibition ranged from 25.82% to 30.25%, and for the lowest concentration tested (100 ppm), it ranged from 19.08% to 24.47%.

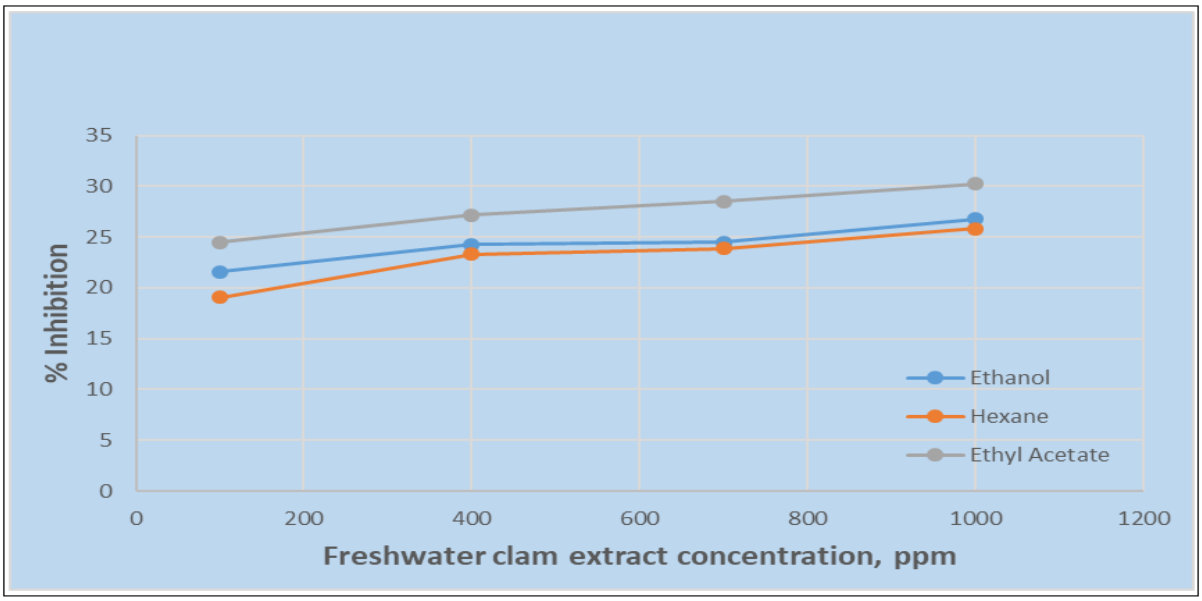

Fig. 3. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of Freshwater clam extracts in different solvents.

The differences were found significant at $p < 0.05$. These showed that varying the concentration of the extracts and the kind of solvent affects the radical scavenging activity.In addition, the differences in the antioxidant activities of the extracts may be attributed to the kind of solvent used in the extraction which gives different composition and antioxidant activities (Do *et al.,* 2013) and sample matrix (Tomsone *et al.,* 2012).

In the study of Odeleye *et al.*, (2016), the antioxidant potentials of the ethanolic extracts from three New Zealand Surf Clam namely, Diamond shell (*Crassula aequilatera)*, Storm shell (*Mactra murchisoni*) and Tua tua (*Paphies donacina*) were evaluated. The ethanolic extracts were further fractioned into petroleum ether, ethyl acetate, n-butanol, and an aqueous fraction. From the tested fractions, it was found out that the ethyl acetate fraction of *P. donacina* showed the highest scavenging activity of 76.14% at a concentration of 20 µg/mL.

Conclusion

The study has shown that as far as extraction yield, ethanol is the best solvent among the three—ethanol, ethyl acetate, and hexane. In fact, it also extracts a greater number of secondary metabolites. However, when compared according to antioxidant activity, it is the ethyl acetate extract that exhibits the highest activity. Overall, freshwater clam has shown interesting properties as a food item.

Its relatively high antioxidant activity measured using DPPH is indicative of its health-promoting power on top of the usual proteins and other primary metabolites that are essential nutrients.

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