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Phytochemical screening and toxicity testing of *Atuna racemosa* Rafin. chrysobalanaceae shell and seed extracts

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ABSTRACT

This research evaluated the phytochemical profile and toxicological properties of the aqueous, ethyl acetate, methanol, and decocted extracts of the shell and seed of *Atuna racemosa* Rafin. Chrysobalanaceae (tabon-tabon). The phytochemical screening was qualitatively tested while *Brine Shrimp Lethality Assay* (BSLA) was employed for toxicity testing of the extracts. Phytochemical screening of *A. racemosa* extracts resulted in the detection of the presence of alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins and terpenoids. The *A. racemosa* shell and seed extracts induced medium to highly toxic activity to brine shrimp nauplii at LC_{50} values of 268.605 µg/mL (aqueous shell) 165.195 µg/mL (aqueous seed), 277.9 µg/mL (ethyl acetate shell), 419.919 µg/mL (ethyl acetate seed), 116.032 µg/mL (methanol shell), 92.0427 µg/mL (methanol seed), 482.78 µg/mL (decoction shell), and 121.111 µg/mL (decoction seed), respectively. Ethyl acetate and methanol extracts of *A. racemosa* seed showed good toxicological properties. Further investigation is needed to determine the bioactive components present in these extracts.

1. Introduction

Medicinal plants have continued to attract attention in the global search for effective antimicrobial agents because of its cheapness, availability, accessibility and the current problems associated with the use of antibiotics. Such problems are the microbial infections that lead to the production of highly reactive molecules from the metabolism of oxygen that can cause extensive damage to cells and tissues (Dhalwal et al., 2007).

The *Atuna racemosa* Rafin. Chrysobalanaceae, locally known as "tabon-tabon", is a little known and exotic tropical fruit that can be found in Northern Mindanao in the Philippines. The tree of the fruit also grows in Papua, New Guinea, Indonesia, Malaysia, Thailand and other tropical Asian countries and Pacific Islands. In Northern Mindanao, tabon-tabon extract is mainly used as a main ingredient of a raw-fish dish however; no ethnobotanical studies have been conducted. Although few studies elsewhere of *A. racemosa* (Buenz, 2006; 2007; Buenz et al., 2007) had been conducted, however, limited studies has been made involving comparison between shell and the seed of the fruit. Thus, investigating its phytochemical profile in several extracts and toxicity testing as initial study can be important for future product development. This study tries to understand the properties of the shell and seed of *A. racemosa.*

2. Materials and method

2.1. Sample preparation

The fruit samples were washed thoroughly with running water, cut lengthwise into quarters, and the seeds were scraped. The shell and seed were washed thoroughly, cut into pieces and dried at room temperature for five days with proper air ventilation. It was ground to fine powder using a laboratory scale mill or blender. Fig. 1 shows the sample use in this study.

2.2. Preparation of extracts

This procedure is as follows:

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- Decocted Extracts: In a one liter beaker, 50 grams of sample was placed and added with 800 mL distilled water. The mixture was bought to boiling for 30 minutes. The decocted extract was filtered and stored in the refrigerator at 4°C until use.
- Aqueous Extracts: A one is to five (1:5) ratio of the weight of the sample to the volume of the extracting solvent was used in the extraction. About 100 grams *A. racemosa* shell/seed were soaked in 500 mL distilled water at room temperature for 48 hours. The *A. racemosa* shell and seed aqueous extracts were filtered and concentrated in rotary evaporator at 95°C. Then, it was stored in the refrigerator at 4°C until use.
- Ethyl acetate Extracts: About 100 grams *A. racemosa* shell/seed were soaked in 500 mL ethyl acetate at room temperature for 48 hours. The *A. racemosa* shell/seed ethyl acetate extracts were filtered and concentrated in rotary evaporator at 70°C. Then, it was stored in the refrigerator at 4°C until use.
- Methanolic Extracts: About 100 grams *A. racemosa* shell/seed were soaked in 500 mL methanol at room temperature for 48 hours. The *A. racemosa* shell/seed methanolic extracts were filtered and concentrated in rotary evaporator at 55°C. Then, it was stored in the refrigerator at 4°C until use. Figs. 2, 3, and 4 presents the schematic diagram of the study.

Fig. 1: *A. racemosa* Rafin. Chrysobalanaceae sample tested for the presence of phytochemicals and its toxicological effects

2.3. Phytochemical screening for secondary plant metabolites

This procedure is as follows:

- Screening for Alkaloids (Wagner's Test): A 5 mL of extract was stirred with 5 mL of 2 M hydrochloric acid and was filtered. Then, the filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). The formation of brown/reddish precipitate indicated the presence of alkaloids in the extracts.
- Screening for Anthraquinones: A 1 mL of each extracts was added with few drops of 10% ammonia solution. The formation of pink precipitate indicated the presence of anthraquinones.
- Screening for Coumarins: A 1 mL of each extracts was treated with 3 mL of 10% NaOH. The presence of coumarins was indicated by the formation of yellow color.
- Screening for Flavonoids (Alkaline Reagent Test): A 1 ml of each extracts was treated with 5 drops of 1 M sodium hydroxide solution. The formation of an intense yellow color indicated the presence of flavonoids.
- Screening for Saponins (Foam Test): A 0.5 mL each extracts was treated with 5 mL of distilled water. It was shaken vigorously for 15 minutes. The formation of foam that persists for about ten minutes indicated the presence of saponins.
- Screening for Tannins: A 1 mL of each extracts was treated with 2 mL of 5% ferric chloride solution. The formation of a dark blue or greenish black color indicates the presence of tannins.
- Screening for Terpenoids: A 0.5 mL of each extracts was treated with 2 mL of chloroform. The solution was carefully added with 1 mL of concentrated sulfuric acid. The formation of red brown color at the interface indicates the presence of terpenoids.

2.4. Toxicity test: Brine shrimp lethality assay

This procedure is as follows:

- Constant Weighing: Five milliliters of *A. racemosa* shell/seed extracts were added to the pre-weighed aluminum dish and dried in an oven to evaporate its solvent. The samples were put in a desiccator and weighed. The procedure was repeated until constant weight to determine its concentration.

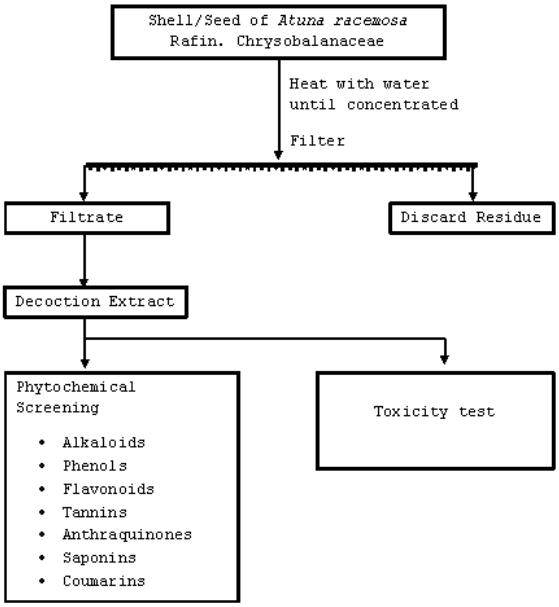

Fig. 2: Schematic diagram for the decoction extract of *A. racemosa* testing

- hrimp Hatching: Brine shrimp eggs were obtained from the New Aqua Laboratory in Naawan, Misamis Oriental, as a gift sample for the research work. Seawater was collected at Luz Banzon, Jasaan Misamis Oriental, and was filtered. One liter of

seawater was used to fill a small plastic container and added with one gram of brine shrimp eggs. The controlled aerator was put inside the container. Two days were allowed for the shrimp to hatch and mature as nauplii (larva).

- Test Extracts: The concentrations of each extract calculated were used to calculate the required volume needed for stock solutions. The equation below shows how to calculate the volume of extract required for stock solutions (1)

$$Vol\ of\ extract\ (mL) = Css \times Vss / Cext \quad (1)$$

where Css is the concentration of the stock solution (ppm); Vss is the volume of the stock solution (mL); and Cext is the concentration of the extract (ppm).

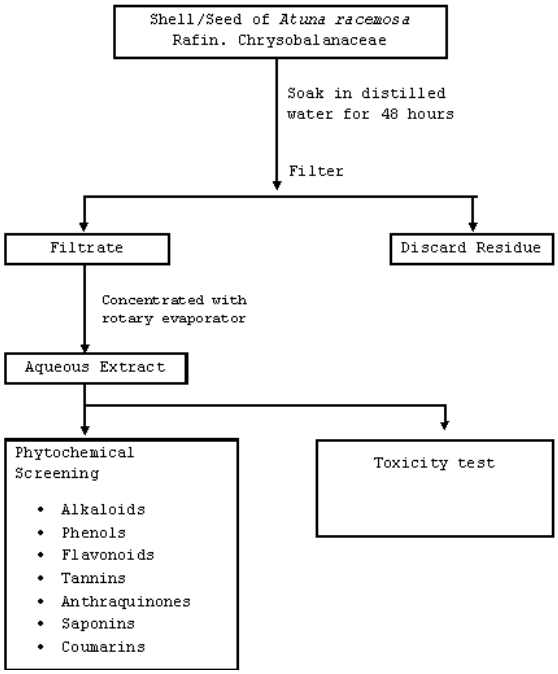

Fig. 3: Schematic diagram for the aqueous extract of *A. racemosa* testing

The volume of extract calculated for each concentration of stock solution (10000 ppm or 5000 ppm) was diluted to 5 mL of methanol. The final concentrations used were 1000 ppm, 500 ppm, and 100 ppm. The test tubes for each prepared solution of the different concentrations were labeled and air-dried until the solvent thoroughly evaporated. Five replicates were performed for each concentration level.

- Bioassay: Into each test tube containing the air-dried extracts, 5mL of seawater was added and stirred. A control sample was prepared consisting only of 5 mL of seawater. Ten brine shrimp nauplii were transferred into each test tube. Thus, there were a total of 50 brine shrimps per dilution. Using a magnifying glass or naked eye, the number of surviving shrimps were counted and recorded after 24 hours. The percent mortality (%M) was calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death of the brine shrimp nauplii is attributed to the bioactive compounds present in plant extracts. Using probit analysis in minitab software, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. Toxicity profile of extracts was classified according to Clarkson's criteria for the toxicity assessment of plant extracts shown in Table 1.

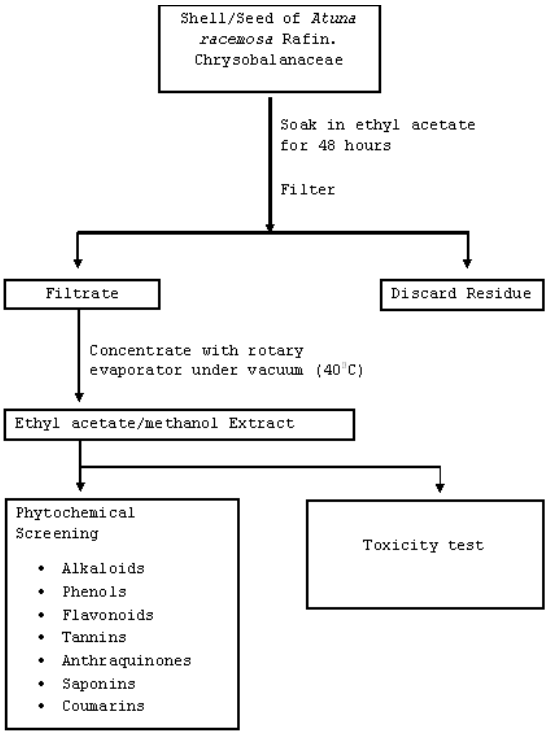

Fig. 4: Schematic diagram for the ethyl acetate and methanol extracts of *A. racemosa* testing

Table 1: Clarkson's Toxicity Assessment of Plant Extracts (Clarkson et al., 2004)

LC_{50} (µg/mL) concentration	Toxicity Profile
0 - 100	Highly Toxic
100 – 500	Medium Toxic
500 – 1000	Low Toxic
1000 – above	Non-toxic

3. Results and discussion

3.1. Phytochemical screening

The phytochemical characteristics of eight extracts tested were summarized in Table 2. The results revealed that the methanol extracts contain a greater number of different phytochemicals that were screened than the aqueous, ethyl acetate and decocted extracts. The extracts from *A. racemosa* shell and seed obtained different phytochemicals because of the different chemical characteristics and polarities present in the plant parts that were soluble to a particular solvent. Ethyl acetate is the least polar, then methanol and water as the most polar. Water is more polar than methanol but the higher activity of the methanolic extracts can be attributed to the presence of higher amounts of polyphenols as compared to other extracts (Tiwari et al., 2011). It means that they are more efficient in cell

walls and seeds degradation which have unpolar character and cause polyphenols to be released from cells. An enzyme polyphenol oxidase degrades polyphenols in water extracts, whereas in methanol they are inactive. Since nearly all of the identified components from plant active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through methanolic extraction (Tiwari et al., 2011).

Phytochemical analysis conducted on the shell and seed of *A. racemosa* extracts revealed the presence of secondary metabolites which are known to exhibit medicinal as well as physiological activities. From the table, it could be seen that saponins were present in all the *A. racemosa* shell and seed extracts. Saponins are rich in pharmaceutical properties such as the ability to increase immune responses and possession of antibacterial, antioxidant, anticancer, antidiabetic and anti-obesity properties (Abioye et al., 2013). It has the property of precipitating and coagulating red blood cells, forming foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness (Okwu, 2005).

Table 2: Phytochemical profile of the seed and shell extracts

Phytochemicals	*racemosa* extracts							
	Distilled Water (aqueous)		Ethyl Acetate		Methanol		Distilled Water(decoction)	
	Seed	Shell	Seed	Shell	Seed	Shell	Seed	Shell
Alkaloids	+	-	+	+	+	-	+	-
Anthraquinones	-	-	-	-	+	-	+	-
Coumarins	+	-	-	-	+	-	-	-
Flavonoids	-	-	+	+	+	+	-	-
Saponins	+	+	+	+	+	+	+	+
Tannins	+	+	-	-	+	+	+	+
Terpenoids	+	+	-	-	+	+	+	+

Legend: Present (+); Absent (-)

Ethyl acetate extracts of *A. racemosa* shell and seed contain alkaloids, flavonoids and saponins. Alkaloids have been reported to exert analgesic, antispasmodic and antibacterial activities (Amirkia and Heinrich, 2014). Flavonoids also possess diverse biological activities, for instance, antiulcer, anti-inflammatory, antioxidant, cytotoxic and antitumor, antispasmodic, antidepressant, and antimicrobial activities (Ghasemzadeh and Ghasemzadeh, 2011).

Aqueous and decocted extracts of *A. racemosa* shell contain the same phytochemicals which are saponins, tannins, and terpenoids. Tannins bind to proline rich protein, interfere with protein synthesis, and have antioxidant effects (Okuda and Ito, 2011). Tannins and terpenoids have both exhibit antimicrobial and antidiarrheal activities (Zwenger, 2008).

Aqueous and decocted seed extracts contain alkaloids, saponins, tannins and terpenoids except that coumarins are present in aqueous extract and anthraquinones are detected in decocted extract. Coumarins found to have bacteriostatic, anti-tumor, anticoagulant, and anti-inflammatory activities (Jain and Joshi, 2012). Plant extracts containing anthraquinones are increasingly used for cosmetics, food, dye and pharmaceuticals due to their wide therapeutic and pharmacological properties such as antioxidant, antifungal, antiviral and antimicrobial (Dave and Ledwani, 2012).

Methanol extracts of the seed contain all the phytochemicals tested, alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, and terpenoids. While the methanol shell extracts contain flavonoids, saponins, tannins and terpenoids.

3.2. Toxicity test: Brine shrimp lethality assay

The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties (Krishnaraju et al., 2005). Brine shrimp lethality assay of *A. racemosa* shell and seed extracts are shown in Table 3 and Table 4. Percent mortality was obtained from 5 replicates of each concentration and used to determine the 50% lethal concentration. The LC_{50} at 95% confidence interval was calculated using probit analysis in minitab software.

The percent mortality (%M) was calculated to ensure that the death of the brine shrimp nauplii is attributed to the bioactive compounds present in plant extracts. The degree of lethality was directly proportional to the concentration of the extract. As the concentration of tabon-tabon shell and seed extracts increases, the percent mortality also increases. In the Table 3, maximum mortalities (100%) were observed at a concentration of 1000 ppm in both ethyl acetate and methanol extract. While in Table 4, 100% mortality was observed at 500 and 1000 ppm of methanol and decocted extracts, and 1000 ppm in aqueous extracts.

According to Clarkson's toxicity criterion for the toxicity assessment of plant extracts, most of the *A. racemosa* extracts were medium toxic against brine shrimp nauplii except the highly toxic methanol seed.

The result shows that all of the tabon-tabon shell and seed extracts tested are considered active or toxic where LC_{50} values are less than 1000 µg/mL (Olowa and Nuñeza, 2013). Methanol extract of tabon-tabon seed was the most toxic extract in this study with a highly toxic LC_{50} of 92.0427 µg/mL. Thus, it means that approximately 50% of the brine shrimp nauplii will not survive when exposed to a concentration of methanolic extract of *A. racemosa* seed at 92.0427 µg/mL. While Methanol shell extract ranks as the second potent or active extract against

brine shrimps at 116.032 µg/mL LC_{50}. The toxicity of methanol shell and seed extracts may be due to the observed phytochemicals present as shown in Table 2, the extracts with the most number of phytochemicals tested. A positive correlation in the toxicity of methanol extract is observed with its antibacterial potential. A similar result from the study of Buenz et al. (2007) which suggest that there is likely more than one active compound in the methanol extract of tabon-tabon seed: certainly an antibiotic, however, also a compound effective at killing brine shrimp nauplii. However, the active components remain unidentified.

In *A. racemosa* shell, decocted extract shows medium toxicity against brine shrimp with the highest LC_{50} of 482.78 µg/mL. While in the *A. racemosa* seed, ethyl acetate extract shows a medium toxic effect against nauplii having the highest LC_{50} value of all seed extracts of 419.919 µg/mL. According to its phytochemical profile, ethyl acetate extracts had the least presence of phytochemicals tested but showed the highest antibacterial activity against a gram negative bacteria. According to McLaughlin et al. (1998) pharmacology is simply toxicity at a lower dose which indicates that these findings about ethyl acetate extracts have implications for the use of this natural product as an antibiotic agent.

A. racemosa is toxic to brine shrimp nauplii which may also be toxic to humans. Hence, the use of tabon-tabon as spices for *kinilaw* and as folk medicine in treating ulcer should be minimized in concentration as it may cause negative effects to humans at high dosages.

4. Conclusion

Overall, phytochemical screening of *A. racemosa* seed methanolic extract revealed the presence of all phytochemicals tested, alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, and terpenoids. The *A. racemosa* shell methanolic extract contains flavonoids, saponins, tannins and terpenoids only. For ethyl acetate extracts, both *A. racemosa* shell and seed contain alkaloids, flavonoids and saponins.

For aqueous extract, the *A. racemosa* seed shows the presence of alkaloids, coumarins, saponins, tannins and terpenoids while *A. racemosa* shell has saponins, tannins and terpenoids only. For decoction extracts, *A. racemosa* seed has the presence of alkaloids, anthraquinones, saponins, tannins and terpenoids while *A. racemosa* shell contains saponins, tannins and terpenoids only.

Table 3: Toxicity of *A. racemosa* Shell Extracts with Varying Concentrations on *Artemia salina*

Concen-tration	% Mortality	LC_{50} (µg/mL)	95% Confidence Interval	Toxicity Profile
		Aqueous extract		
100	42			
500	48	268.605	44.7643 –637.082	Medium Toxic
1000	70			
		Ethyl acetate extract		
100	15.3			
500	62	277.9	220.404 –345.060	Medium Toxic
1000	100			
		Methanol extract		
100	48			
500	80	116.032	68.0558 –163.502	Medium Toxic
1000	100			
		Decoction extract		
100	32			
500	44	482.78	244.208 –1356.57	Medium Toxic
1000	64			

Table 4: Toxicity of *A. racemosa* Seed Extracts with Varying Concentrations on *Artemia salina*

Concen-tration	% Mortality	LC_{50} (µg/mL)	95% Confidence Interval	Toxicity Profile
		Aqueous extract		
100	32			
500	82	165.195	120.843 –212.713	Medium Toxic
1000	100			
		Ethyl acetate extract		
100	19.61			
500	49.02	419.919	295.155 –606.467	Medium Toxic
1000	74.51			
		Methanol extract		
100	62			
500	100	92.043	Significant at 0.05 %	Medium Toxic
1000	100			
		Decoction extract		
100	20			
500	100	121.111	Significant at 0.05 %	Medium Toxic
1000	100			

All of the extracts showed positive towards toxicity testing, indicating that the test samples are biologically active. Approximately 50% of the brine shrimp nauplii will not survive when exposed to aqueous extracts of *A. racemosa* shell and seed at a concentration of 268.605 µg/mL and 165.195 µg/mL

respectively. For ethyl acetate extracts, the *A. racemosa* shell and seed showed a 50% lethal concentration at 267.209 µg/mL and 419.919 µg/mL, respectively. The methanol extracts of *A. racemosa* shell and seed are potent against brine shrimps nauplii with LC_{50} values of 116.032 µg/mL and 92.0427 µg/mL. And for decoction extracts, approximately 50% of nauplii will not survive at *A. racemosa* shell and seed concentrations of 482.78 µg/mL and 121.111 µg/mL, respectively. According to Clarkson's criterion, methanol seed extract was highly toxic towards brine shrimp nauplii while the other *A. racemosa* shell and seed extracts were medium toxic towards brine shrimp nauplii.

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