Original Research Article

Brine Shrimp Lethality of *Atuna racemosa* Raf. (Chrysobalanaceae) Fruits Extracted with Varying Acetic Acid Concentrations

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Submission Date: 02-01-2019; Revision Date: 15-03-2019; Accepted Date: 12-04-2019

ABSTRACT

Introduction: *A racemosa*, commonly known as "Tabon-tabon" in the Philippines, is found to be native to some parts of Northern Mindanao and has been known for its ethnomedicinal uses. **Objectives:** In this study, preliminary investigation on the cytotoxic activity, i.e. brine shrimp lethality, of the fruit extracted with a safer solvent such as acetic acid was conducted. **Materials and Methods:** Extraction of *Atuna racemosa* Raf. halved fruits was carried out using 1, 3 and 5% aqueous acetic acid. The cytotoxic properties of the aqueous acetic acid extracts were determined using Brine Shrimp Lethality Assay (BSLA). **Results and Discussion:** Alkaloids, anthraquinones, flavonoids, phenols and saponins were all detected in the 1%, 3% and 5% acetic acid extracts of *A. racemosa* fruits. Percent mortality of brine shrimp nauplii is an indication of the cytotoxic effects of the aqueous acetic acid fruit extracts of *A. racemosa*. The percentage mortality of the nauplii is directly proportional to the concentration of the extracting solvent. Percent mortality of the brine shrimp nauplii exposed to 5% aqueous acetic acid extract was statistically higher as compared to that of 1 and 3%. The concentration of the acetic acid may have influenced the extractability of cytotoxic compounds from the fruits of *A. racemosa*. **Conclusion:** The present findings imply that the aqueous acetic acid extracts of *A. racemosa* fruits were cytotoxic against brine shrimp nauplii. This property may be accounted to the detected phytochemicals in the extracts.

Key words: Brine shrimp lethality, Acetic acid, *A. racemosa* fruit, Varying concentrations.

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INTRODUCTION

People have been using medicinal plants as mainstay of traditional herbal medicine since antiquity to date.[1] As of now, 60-80% of the world's population rely on plant based medicines which are being used as traditional health care system.[2] These medicinal plants contain chemically diverse classes of bioactive compounds such as tannins, alkaloids and flavonoids which exhibit various pharmacological properties.[3]

The search for drugs from plant origin to combat the menace of drug resistant pathogenic microorganisms and to discover new antitumor and anticancer agents has been receiving renewed interest among researchers throughout the world.[4] In cancer drug discovery, compound libraries are screened for cytotoxic activity. Cytotoxic agents with different mechanisms of action together are effective in killing tumor with fewer side effects.[5] Hence, it is significant to identify new medicinal plants and its cytotoxic activity for life threatening diseases like cancer.[6]

Atuna racemosa Raf. is among the promising plants worth investigating. *A racemosa* is commonly known as "Tabon-tabon" in the Philippines and is found to be native to some parts of Northern Mindanao. Its pulp and sap has been used in a local Filipino dish, "kinilaw" to create a unique kind of sour-sweetness. It has been reported that *A. racemosa* fruits have high phenolic content and exhibit antioxidant properties.[7] Accordingly, the plant shows effectivity in treating suppurating wounds.[8] In addition, it has been reported that the fruit

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DOI : 10.5530/ajbls.2019.8.4

extract is effective against methicillin-resistant *Staphylococcus aureus*.[9] In this study, preliminary investigation on the cytotoxic activity, i.e. brine shrimp lethality, of the fruit extracted with a safer solvent such as acetic acid was conducted.

MATERIALS AND METHODS

Sample Collection, Preparation and Extraction

Healthy and matured fruits were randomly collected by hand early in the morning from the growing *A. racemosa* trees. The collected fruits were placed in the polyethylene bags and brought to the laboratory. Plant samples including the leaves, fruits and flowers were submitted to the Botany Section of the Central Mindanao University (CMU) Museum, Musuan, Bukidnon, Philippines for and authentication.

The collected fruits of *A. racemosa* were washed thoroughly with tap and the fruits were cut halved using a stainless knife. The open surface that has been in contact with the knife was scraped out with a ceramic spoon and discarded. The remaining endosperm was scraped out and then mixed thoroughly for extraction.

A 100 gm portion was added with 100 mL of 5% aqueous acetic acid. The mixture was stirred using a magnetic stirrer for 1 hr and 30 mins. Separate sets of samples were extracted in the same manner but using lower concentrations of aqueous acetic acid, i.e., 1% and 3%. After extraction, the mixture was centrifuged for 15 mins at 3500 rpm and filtered through a Buchner funnel. The residue was discarded while the filtrate was set aside for freeze-drying. After freeze-drying, dried extracts were contained in a tightly closed container and was stored at -20°C.

Phytochemical Screening

The phytochemical screening was conducted employing Thin Layer Chromatography (TLC).[10,11] The crude aqueous acetic acid extracts of *A. racemosa* fruit were screened for the presence of alkaloids, anthraquinones, flavonoids, phenols, saponins and tannins.

Brine Shrimp Lethality Assay (BSLA)

The brine shrimp lethality test was conducted to make preliminary assessment on the cytotoxicity of the extracts. Brine shrimp (*Artemia salina*) eggs were obtained from MSU, Naawan, Misamis Oriental. The sea water was filtered and sterilized using autoclave. A 10,000 mg/L stock solutions of freeze-dried extracts in sea water were prepared. From the stock solution, appropriate volumes (500, 250, 50, 5 and 2.5 µL to prepare 1000, 500, 100, 10 and 5 ppm test solutions, respectively) were placed into separate test tubes. Ten brine shrimps were transferred to each of the test tube and sterilized sea water was added up to 5 mL mark.[12,13] A negative and positive control were prepared using sterilized sea water and aqueous solutions of potassium dichromate, respectively. The test tubes were placed under illumination for 24 hrs and survivors were counted. The percentage mortality of nauplii was calculated for each concentration of the sample using Equation 1.

$$\text{mortality}, \% = \left[\frac{\text{Number of dead nauplii}}{10}\right] \times 100\% \quad \text{Eq.1}$$

The 0 and 100% mortalities were corrected before the determination of Probit values.[14] Probit analysis was used to determine the LC_{50}.[15] Plant extracts with LC_{50} values less than 1000 mg/L were considered cytotoxic.[16]

Statistical Analysis

The data obtained were subjected to one-way ANOVA in Completely Randomized Design at 0.05 level of significance. Significant differences among the means were determined using Tukey's Test.

RESULTS

Phytochemical Screening

Phytochemical screening may be used to detect presence of various secondary metabolites which are perhaps responsible for the plant's medicinal value.[17] The results of the phytochemical screening are summarized in Table 1.

Brine Shrimp Lethality

The percent mortality of the brine shrimp and the LC_{50} of the aqueous acetic acid extract of *A. racemosa* fruits are presented in Table 2.

Table 1: Results of the phytochemical screening of *A. racemosa* Raf. fruits extracted with 1,3 and 5% acetic acid concentrations.

Phytochemical	Acetic acid concentration, %		
	1	2	3
Alkaloids	+	+	+
Anthraquinones	+	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Saponins	+	+	+
Tannins	-	-	-

Legend: (+) – present; (-) - absent

Table 2: Percent Mortality of the varying aqueous acetic acid extracts of *A. racemosa* Raf. Fruits.

Acetic Acid Concentration	Mean Mortality,%				Mean LC_{50}, mg/L	Inference**
	5 mg/L	10 mg/L	100 mg/L	500 mg/L		
1%	0.83*[a]	8.34[a]	96.67[a]	99.17*[a]	40.00	cytotoxic
3%	2.08[a,b]	20.00[a]	99.17*[a]	99.17*[a]	20.21	cytotoxic
5%	8.34[b]	25.00[a]	99.17*[a]	99.17*[a]	15.77	cytotoxic
$K_2Cr_2O_7$	26.67	56.67	99.17*	99.17*	8.32	cytotoxic

* - corrected values for 0 and 100% mortality[14]
** - LC_{50} < 1000 mg/L plant extract is cytotoxic[16]
[a,b] – means of the same letter superscript within a column are not significantly different at 0.05 level of significance by Tukey's test

DISCUSSION

Phytochemical Screening

Phytochemical screening may be used to detect presence of various secondary metabolites which are perhaps responsible for the plant's medicinal value.[17]

As presented in Table 1, alkaloids, anthraquinones, flavonoids, phenols and saponins were all detected in the 1%, 3% and 5% acetic acid extracts of *A. racemosa* fruits except for tannins. The positive results for phenolics and flavonoids in the fruit extracts are in agreement with previously reported studies. High phenolic content in the methanolic extracts of *A. racemosa* fruits has been reported in the literature.[7] The present findings may suggest that *A. racemosa* fruits contain bioactive compounds which may be responsible for its anti-inflammatory,[18] analgesic,[19] antibacterial,[20,21] antioxidant[7] and antifungal[19] activities. Moreover, a number of researches have shown that phenolics, flavonoids, saponins, alkaloids and anthraquinones have cardioprotective, anti-inflammatory, antioxidative, free radical scavenging, antimicrobial, anticancer and analgesic activities.[17,22-24]

Brine Shrimp Lethality

Brine shrimp lethality assay is a rapid, inexpensive and simple bioassay to test the bioactivity of plant extracts which in most cases correlates reasonably well with cytotoxic and anti-tumor properties.[25] Percent mortality results of brine shrimp lethality assay as summarized in Table 2 clearly indicated the toxic effects of the extracts. For the four varying concentration levels, the percentage mortality is directly proportional to the concentration of the extraction solvent. It can be clearly seen that an increase in the concentration of the acetic acid used in extraction results to an increase in the number of brine shrimp nauplii that were killed.

The results of the Analysis of Variance (ANOVA) at 0.05 level of significance show significantly different percent mortality at 5 mg/L extract only among 1, 3 and 5% aqueous acetic acid. Subsequent Post Hoc Tukey's Test indicate that at 5 mg/L, percent mortality of the brine shrimp nauplii exposed to 5% aqueous acetic acid extract was statistically higher as compared to that of 1 and 3%. The concentration of the acetic acid influenced the extractability of cytotoxic compounds from the fruits of *A. racemosa*. Similarly, improved extractability of cytotoxic compounds from the fruits of *A. racemosa* parallels increase in the concentration of acetic acid.

The relatively high cytotoxicity of 5% aqueous acetic acid can be related to the polarity of the acetic acid. Most of all, the detected phytochemicals such as phenolics, flavonoids, alkaloids and saponins are polar in nature and are therefore better extracted in highly polar solvents.[26] The 5% aqueous acetic acid being more polar is more capable of extracting greater concentration of bioactive polar compounds than the less polar 1% and 3%. Moreover, it has been found that 5% aqueous acetic acid offers a faster and more efficient approach for the extraction of phenolic compounds.[27]

In the determination of the median lethal concentration (LC_{50}), the LC_{50} of the $K_2Cr_2O_7$ and 1, 3 and 5% aqueous acetic acid extracts of *A. racemosa* fruit were 8.32 and 40.00, 20.21 and 15.77 mg/L, respectively. Since plant extracts with LC_{50} values less than 1000 mg/L can be considered cytotoxic,[16] then all fruit extracts (1, 2 and 3% aqueous acetic acid) are cytotoxic.

The presence of alkaloids, anthraquinones, flavonoids, phenolics and saponins in the aqueous acetic acid fruit extracts may justify the cytotoxic activity of *A. racemosa*.[22,24,28,29] Furthermore, present results support previous studies on the medicinal value of *A. racemosa* fruit. As have been reported, *A. racemosa* fruit exhibited anti-inflammatory,[18] antibacterial,[20,21] antifungal and analgesic properties.[19] The kernels (fruit) of *A. racemosa* have been also used in treating dysentery, liver problems and in preventing scabies and itching.[19]

CONCLUSION

The phytochemicals, i.e. alkaloid, anthraquinones, flavonoid, phenolics and saponins, detected in the aqueous acetic acid extracts of the *A. racemosa* fruits may account for the cytotoxicity of the extracts against the brine shrimp nauplii. Results warrants further investigation on the isolation of compounds responsible for their bioactivity. Lastly, development of nutraceuticals and other natural products from *A. racemosa* fruits is promising.

ACKNOWLEDGEMENT

The authors wish to thank the Chemistry Department of Central Mindanao University, Musuan, Bukidnon for the assistance during the conduct of the study.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in the subject matter or materials discussed in this manuscript.

ABBREVIATIONS

BSLA: Brine Shrimp Lethality Assay; **LC_{50}:** Median Lethal Concentration; **ANOVA:** Analysis of Variance.

SUMMARY

This study highlights the cytotoxic activity of the 1, 3 and 5% aqueous acetic acid extracts of *A. racemosa* based on the results of the BSLA. The percentage mortality of the nauplii is directly proportional to the concentration of the extracting solvent. Percent mortality of the brine shrimp nauplii exposed to 5% aqueous acetic acid extract was higher as compared to that of 1 and 3%. The concentration of the acetic acid may have influenced the extractability of cytotoxic compounds from the fruits of *A. racemosa*. The exhibited cytotoxicity may be also due to the alkaloids, anthraquinones, flavonoids, phenols and saponins that were detected in the extracts of *A. racemosa* fruits. Development of nutraceuticals and other natural products from *A. racemosa* fruits is promising.

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Cite this article: Ang AMG, Reyes FGE. Brine Shrimp Lethality of *Atuna racemosa* Raf. (Chrysobalanaceae) Fruits Extracted with Varying Acetic Acid Concentrations. AJBLS. 2019;8(1):25-9.