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Phytochemical screening, DPPH Radical Scavenging Activity and Brine Shrimp Lethality of the Leaf Extracts of *Atuna racemosa*

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ABSTRACT
The decoction (ArD), hydroethanolic (ArHE) and ethanol (ArE) leaf extracts of Atuna racemosa were evaluated for their chemical components using phytochemical screening, assessed for toxicity using brine shrimp lethality test and investigated for their antioxidant properties using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. Phytochemical screening results indicate all extracts to contain steroids and tannins of the same degree and varying amounts of alkaloids, saponins and flavonoids. Anthraquinones and cyanogenic glycosides were negative in the three extracts. In the toxicity test against the brine shrimp Artemia salina, the three extracts exhibited strong activities with the highest activity shown by the hydroethanolic extract (ArHE) with acute and chronic LC_{50} values of 15.15 ppm and 12.18 ppm, respectively. The hydroethanolic extract (ArHE) also indicated the highest ability to scavenge the DPPH radical at the three concentrations tested. These results show that generally the three extracts of A. racemosa are good candidates for further investigation.
Keywords: *Atuna racemosa, toxicity, radical scavenging, phytochemicals, medicinal plant*

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INTRODUCTION

Most of medicinal plants have already been utilized for a large number of years to flavor and preserve food even before knowing their medicinal value[1]. It has been said that the health benefits of these plants lie in some chemical substances that produce a definite physiological action on the human body [2]. In the Philippines, ten medicinal plants have been already endorsed by the Philippine Department of Health (DOH), all of which have been thoroughly tested and have been clinically proven to have medicinal value in the relief and treatment of various ailments. However, other than the contemporary medicinal plants, there still lie a great portion of plants that remain unknown but may have potential medical benefits.

One particular subject of interest is *Atuna racemosa* or "Tabon-tabon" as it is locally called in Northern Mindanao, Philippines. It has been claimed that Filipinos were already using this plant to remove the foul smell of fish even before the Spaniards came as implicated by the "Tabon-Tabon" shells found near fish bones in an anthropological site in the south [3] (Market Manila RSS, 2008). In the Philippines, very little is still known about "Tabon-tabon" but is said to be native to Northern Mindanao and Camiguin Island. Information about it is not clear yet unlike other medicinal herbs but the pulp juice is often used in cuisine [4]. The study by Noreeen *et al.* [5] reported *A. racemosa* as a plant traditionally used for inflammatory conditions. An ethnobotanical study on the uses of *A. racemosa* subsp. *racemosa* (Chrysobalanaceae) in Samoa revealed that its extract is used as an anti-inflammatory massage oil and a putty to caulk boats. Minor medicinal uses were also reported and a survey of herbarium material shows that the fruit of *A. racemosa* is widely used throughout the Pacific region [6]. In a study by Buenz *et al.* [7] , alcoholic extracts of the leaves and kernels of *A. racemosa* collected in the Independent State of Samoa

were prepared and subjected to antimicrobial test against two Gram positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). The results show that the extracts from *A racemosa* to possess specific activity for the Gram positive bacteria tested.

In this study, leaf extracts of *A. racemosa* or "Tabon-tabon" were assessed for the presence of phytochemicals, evaluated for toxicity using brine shrimp lethality test, and investigated for their antioxidant properties using the DPPH radical scavenging method.

MATERIAL AND METHODS

Plant collection and Preparation of Extracts

Leaves of *A. racemosa* was collected from Extension, Wao, Lanao del Sur, Philippines. The decoction (ArD) was prepared by boiling around 1kg of fresh, clean and cut leaf samples in sufficient amount of distilled water (1:1 ratio). The mixture was then filtered and freeze-dried to give ArD. About 1kg of the plant's dried leaf sample were pulverized using a sterile electric blender, weighed and percolated with enough 95% ethanol; and another set-up percolated with 50% ethanol and 50% water after three days. Each solution was filtered, concentrated *in vacuo* using a rotary evaporator at temperatures not exceeding 40^0C, freeze-dried and weighed to give the ethanol extract (ArE) and hydroethanolic extract (ArHE), respectively.

Phytochemical Screening

The *A. racemosa* extracts (ArD, ArHE and ArE) were screened for the presence of alkaloids, saponins, flavonoids, steroids, tannins, anthraquinones and cyanogenic glycosides using the standard protocols as described by Aguinaldo et al. [8] with slight modifications.

Brine Shrimp Lethality Test

The three extracts were evaluated for lethality to the brine shrimp *A. salina* using standard protocol with a slight modification [9]. Three (3) concentrations of the extracts (1000-, 500-, 100-ppm) were prepared in three replicates. containers. Ten previously hatched brine shrimps were transferred to each test tube and sterilized sea water was added to the 5.00-mL mark. The number of dead and alive nauplii were counted after 6 hours and 24 hours corresponding to acute and chronic values, respectively. Using Reed-Meunch method [10], LC_{50} values for all the crude extracts were determined.

DPPH Radical Scavenging Activity

Using the method of Lee and Shibamoto [11], the DPPH radical scavenging activity of all extracts were examined by comparison with those of known antioxidants Ascorbic Acid (AA) and Butylatedhydroxytoluene (BHT). The extracts were prepared at concentrations of 1000- , 500- and 100-ppm. 1000 ppm stock solution was prepared by dissolving 3mg of the extract with 3.0mL methanol. Volumes of 1000µL, 750µL, 150µL from the 1000ppm stock solution were transferred in a 10mL test tube and were added with methanol to make 1.5mL solution. The mixture was shaken vigorously and allowed to stand at room temperature for few hours. The percent of DPPH decoloration of the samples was then calculated according to formula:

$$Antiradical\ activity = \frac{Acontrol - Asample}{Acontrol} X\ 100$$

Each sample were assayed in triplicate and mean values were calculated.

RESULTS

Phytochemical Screening

The results obtained for the phytochemicals found in each extract of *A. racemosa* is summarized in Table 1.

Table 1: Phytochemical screening results for *A. racemosa* extracts

Phytochemicals	*A. racemosa* extracts		
	ArD	**ArHE**	**ArE**
Alkaloids	+	++	+++
Saponins	+++	++	+
Flavonoids	+	++	+++
Steroids	+++	+++	+++
Tannins	+++	+++	+++
Anthraquinones	-	-	-
Cyanogenic glcosides	-	-	-

+ slight; ++ moderate; +++ heavy; - absence

ArD –decoction, **ArHE** – hydroethanolic extract, **ArE** - ethanol extract

Brine Shrimp Lethality Test

The results obtained for the mortality rate of the brine shrimp *A. salina* after 24-hour exposure and the LC_{50} values of the extracts of *A. racemosa* is summarized in Table 2.

Table 2: Brine shrimp mortality and LC_{50} values of the *A. racemosa* extracts

A. racemosa Extracts	Mortality after 6-h Exposure*, %			Acute LC_{50}, ppm	Mortality after 24-h Exposure*, %			Chronic LC_{50}, ppm
	100 ppm	500 ppm	1000 ppm		100 ppm	500 ppm	1000 ppm	
ArD	0.00	0.00	80.00	18.73	5.97	13.16	88.57	16.78
ArHE	0.00	52.38	76.92	14.15	0.00	69.44	89.09	12.18
ArE	0.00	27.50	75.61	16.61	0.00	28.21	78.05	16.28

*mean of triplicate analysis
ArD –decoction, **ArHE** – hydroethanolic extract, **ArE** - ethanol extract

DPPH Radical Scavenging Test

Table 3 summarizes the averaged DPPH-radical scavenging activities of the extracts of *A. racemosa*.

Table 3: DPPH antiradical activities of *A. racemosa* leaf extracts at various concentrations

Test Sample	Antiradical Activity*, %		
	100 ppm	500 ppm	1000 ppm
ArD	31.11	21.61	89.42
ArHE	46.03	93.34	93.72
ArE	18.07	90.22	91.08
AA**	93.65	96.70	96.74
BHT***	73.02	94.03	94.42

*mean of triplicate analysis
**Ascorbic Acid standard
***Butylated hydroxytoluene standard
ArD –decoction, **ArHE** – hydroethanolic extract, **ArE** - ethanol extract

DISCUSSION

Phytochemical screening

Phytochemical screening results (Table 1) showed that the three extracts of *A. racemosa* contain alkaloids, saponins, flavonoids, steroids, and tannins but do not have anthraquinones and cyanogenic glycosides. Tannins are present in all the extracts of *A. racemosa*. Cowan [12] reported that tannins are polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. They are used in pharmaceutical preparations because of their astringent action. Tannins are known to possess general antimicrobial and antioxidant activities [13] (Riviere et al., 2009). Adekunle and Ikumapayi [14] reported that at low concentration, tannins can inhibit the growth of microorganisms, and act as an antifungal agent at higher concentration by coagulating the protoplasm of the microorganism. Other reports show that tannins may have potential value as cytotoxic and/or antineoplastic agents [8]. Aside from the use of tannins as antimicrobial agents or prevention of dental caries, they are also being used in the manufacture of plastics, paints, ceramics and water softening agents [15]. The presence of tannins in all of the crude extracts examined may justify the antioxidant activities of *A. racemosa*. Flavonoids, (a large group of naturally occurring plant phenolic compounds including flavones, flavonols, isoflavones, flavonones and chalcones), also known as nature's tender drugs, possess numerous biological/ pharmacological activities. Recent reports of antiviral, anti-fungal, antioxidant, anti-inflammatory, antiallergenic, antithrombic, anticarcenogenic, hepatoprotective, and cytotoxic activities of flavonoids have generated interest in studies of flavonoid-containing plants. Of these biological activities, the anti-inflammatory capacity of flavonoids have long been utilized in Chinese medicine and the cosmetic industry as a form of crude plant extracts [16-18]. The presence of flavonoids in all crude plant extracts may confirm the antioxidant activity of *A. racemosa*. Triterpenoids are also present in the crude extracts of *A. racemosa*. These are studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic effects. They are used in the prevention and treatment of hepatitis, parasitic and protozoal infections and for their cytostatic effects. The disadvantage of using triterpenoids is the toxicity associated with their hemolytic and cytostatic properties. Hand in hand with ongoing extraction and isolation of natural products therefore, is the development of synthetic derivatives with lower toxic and higher therapeutic potential [19] (Dzubak et al., 2006). Quantitative analyses of these phytochemicals may also be done to guide the researchers on

which particular bioactive class of compounds may be subjected to subsequent target isolation [20].

Brine shrimp lethality test

Results showed that the effects of the crude extracts of *A. racemosa* on the mortality of brine shrimp *A. salina* is highly dependent on the varying concentrations of *A.racemosa* as well as its type of crude extracts, with a notable pattern wherein percent mortality increases along with concentration, palpably starting on 500 ppm except on decoction (ArD), and that of all three extracts, 50:50 ethanol-water extract (ArHE) was observed to be the most toxic (active) based on LC_{50} values, 14.15 after six (6) hours of exposure (acute) and 12.18 after twenty-four hours (24) of exposure (chronic). The results in Table 2 indicated that all crude extracts of *A. racemosa* are highly active or toxic, having values less than 1000 ppm, among which ArHE was the most active, followed by crude ethanol extract (ArE) and the least active was ArD. Moreover, lethality of extracts on brine shrimp depended on the dosage. The extracts exhibiting the most chronic toxic effect is ArHE. It is important to note however that though ArD did not exhibit acute effects (0% mortality) for the first two concentrations, chronic effects were observed for 100 ppm, 500 ppm and 1000 ppm, respectively.

The brine shrimp lethality test (BSLT) has been used routinely in the primary screening of the crude extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials. Brine shrimp nauplii have been previously utilized in various bioassay systems. The variation in BSLT results (Table 2) may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids, triterpenoids) present in the crude extracts. According to Meyer *et. al.* [21], a crude plant extract can be considered toxic (active) if it has an LC_{50} value less than 1000ppm while non-toxic (inactive) if the value is greater than 1000ppm. BSLT results may be used to guide the researchers on which crude plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later [20].

DPPH radical scavenging test

The hydroethanolic extract of *A. racemosa* (ArHE) exhibited the highest antiradical activity in all of the concentrations tested. It is followed by the ethanolic extract (ArE). The activities of ArHE and ArE were relatively close to the standard (Ascorbic acid and BHT) at the 500- and 1000-ppm concentrations. Meanwhile, the decoction (ArD) hast the lowest antiradical activity among all the crude extracts. The results indicate that ArHE and ArE have the greater ability to scavenge the radical DPPH than the ArD extract in high concentrations (500- and 1000-ppm) (Table 3).

Antioxidants that scavenge free radicals play an important role in cardiovascular disease, aging, cancer, and inflammatory disorders [22]. In addition, these naturally occurring antioxidants can be formulated to give nutraceuticals, which can help to prevent oxidative damage from occurring in the body. One way of estimating antioxidant activity is by the use of the stable free radical DPPH [23-25]. The results indicate that among the crude extracts of *A. racemosa*, the hydroethanolic extract and the decoction exhibited the highest and lowest antioxidant capacity respectively in terms of both Ascorbic Acid and Butylated Hydroxytoluene equivalence.

CONCLUSION

Results of the study have shown that all the crude extracts of *A. racemosa* exhibited bioactivities in terms of toxicity to brine shrimp and antioxidant properties. The hydroethanolic extract (ArHE) was observed to be the most active (toxic) in the brine shrimp lethality test. Moreover, the ArHE also had the most palpable acute and chronic effect among all three extracts. The other extracts have acute and chronic effects as well and are highly active. These results warrant further investigations on the isolation and identification of the bioactive components present in the crude extracts. Over-all evaluation of the results of the various *in vitro* antioxidant property methods on the extracts of *A. racemosa* indicate such extracts as good candidates for further investigation.

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