PHYTOCHEMICAL SCREENING AND CYTOTOXICITY LEVEL OF MACROALGAE IN LORETO, DINAGAT ISLAND, PHILIPPINES

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ABSTRACT

The use of macroalgae as a substitute for synthetic drugs is gaining popularity. Macroalgae is a simple and fascinating species that is a treasure chest of natural chemicals and beneficial ingredients and can be found in some areas of the marine environment. This study was undertaken to evaluate the toxicity effect through brine shrimp bioassay and presence of the secondary metabolites through phytochemical screening of ethanolic extract in selected macroalgae collected along the intertidal zone in selected areas of the Barangays in Loreto Dinagat Islands. A total of ten macroalgae were collected containing two samples for red algae, four samples for green algae and four samples brown algae. Screening of phytochemical constituents showed positive results for flavonoids, anthraquinones, saponins, tannins, steroids and alkaloids. The toxicity levels of each selected macroalgae were dose-dependent. Three of the samples of macroalgae were identified as highly toxic, and seven of the selected macroalgae were identified as moderately toxic. The result of the present study confirmed that some of the macroalgae present in Loreto Dinagat Islands were rich sources of phytoconstituents which can be isolated and further screened for various biological activities. This study also supports the use of the macroalgae in the treatment of diseases in traditional medicine and also recommends the physico-chemical characterization and further biological evaluation of the macroalgae.

Keywords: Macroalgae, bioactive components, lethal concentration.

1 INTRODUCTION

Macroalgae is a simple and fascinating plant that is a treasure chest of natural chemicals and beneficial ingredients. They are the oldest plants on Earth and do not produce roots, flowers or fruits. They absorb their nourishment from the sea and in the most part reproduce asexually through their spores. They can be microscopic in size or very large, and they can grow on the surface of the sea or at considerable depths. There are over 17,000 species recorded to date, which for simplicity can be divided into four classes by their color, namely green, red and brown varieties. Macroalgae are popular and abundant food in East Asia and also well known for their medicinal effect due to presence of active phenolic constituents. Phlorotannins and the alkaloids, the major phenolic group of brown algae and green algae, have extensively investigated for their vast array of bioactivities such as antioxidant, anti-inflammatory, anticancer and antidiabetic.

There has been no record of identification and determination of macroalgae species along the intertidal zone of Loreto, Dinagat Islands. Although macroalgae have been observed only through ocular inspection, there has been no record as to their identification and characterization which would present a problem in determining which of the species is useful, edible and harmful to mankind. These observations lead the researchers to conduct a

preliminary study to gather information regarding on the macroalgae present along the intertidal zones of Loreto Dinagat Islands. This research also will contribute to the worldwide research for reliable, effective and more affordable alternative medicinal plants, thus this study was also designed to evaluate the cytotoxicity through lethal concentration in the brine shrimp bioassay and bioactive component with presence of secondary metabolites of the selected macroalgae in terms of tannins, flavonoids, anthraquinones, steroids and alkaloids content using phytochemical screening from the powdered samples of macroalgae.

2 MATERIALS AND METHODS

Collection and Identification of Macroalgae

The collection of the fresh macroalgae was done through handpicking in selected areas during the low tide along intertidal zone of Loreto, Dinagat Islands. The macroalgae species were brought to Natural Science Department for further examinations and were identified through browsing from websites on macroalgae identifications on the Internet, and other related studies on macroalgae. There were ten species of macroalgae that were collected. The macroalgae were washed thoroughly with freshwater to remove salt, sand and epiphytes.

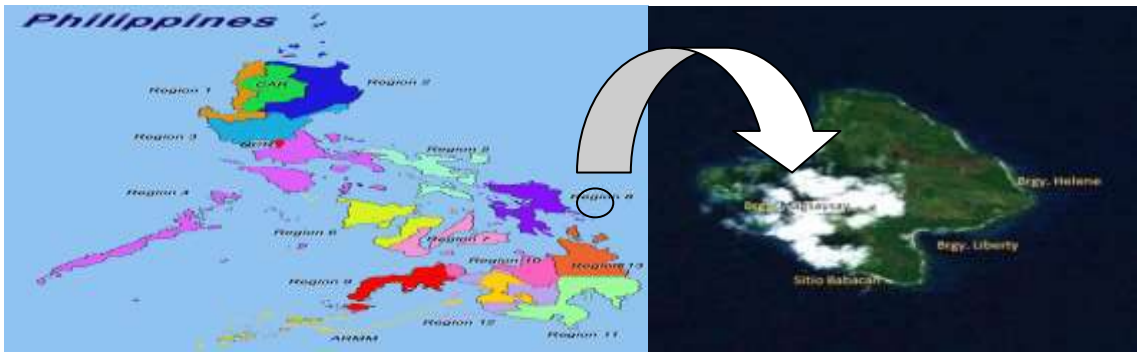


Figure 1: The map of the collection area showing Loreto, Dinagat Island

Figure 2: Selected areas of collection of macroalgae, showing the left, middle and right side of the intertidal zone in the Barangays in Loreto, Dinagat Island

Preparation of Macroalgae (Guevara *et. al.* 2005)

Each collected macroalgae species were weighed 10 grams and were placed in the evaporating dish and dried in the oven at 75^{o}C for about 4 to 6 hours until constant weight were obtained and already easy to be powdered. The macroalgae species were powdered using the mortar and pestle for the application in the phytochemical screening of secondary metabolites and for brine shrimp bio assay using the nauplii larvae and was labelled properly and serves as the stock samples.

Phytochemical Screening of Secondary Metabolites

To test for alkaloids, about 0.5 g of the extract will be stirred with 5 ml of 1% aqueous hydrochloric acid on a steam bath. A few drops of Dragendorff's reagent are used to treat 1 ml of the filtrate. Turbidity or precipitation with this reagent will be taken as evidence for the presence of alkaloids. Exact 0.5 g of the extract will be dissolved in distilled water in a test tube. Frothing which persisted on warming will be taken as preliminary evidence for saponins. Also, to test for presence of tannins, about 0.5 g of the extract will be dissolved in distilled water and about 10 ml of bromine water will be added. Decolourization of bromine water indicated the presence of tannins. Borntrager's test will be used for detecting the presence of anthraquinones. In this case 0.5 g of the plant extract will be shaken with benzene layer separated and half of its own volume of 10% ammonia solution added. Pink, red or violet coloration in the ammoniacal phase will be an indication for the presence of anthraquinones. The presence of cardiac glycosides will be confirmed by Lieberman's test, Salkowski test and Keller-Killani test (Culei 1982; Sofowora 1993; Trease & Evans 2002) and cyanogenic glycosides will be carried out according to the methods described by Harborne (1973) and Trease and Evans (1983).

Toxicity Test: The Brine Shrimp Bioassay

Preparation of the Macroalgae

The macroalgae collected were weighed on a top loading balance accurately down to 0.1 g. The macroalgae were then individually placed in a porcelain dish and dried in an oven at 50^{o}C for 6 hours. After 6 hours the macroalgae were weighed again, dried in the oven until a constant weight was obtained. It is not recommended that a higher drying temperature be used as this may cause some bioactive natural products present in the macroalgae to undergo decomposition. The dried macroalgae were powdered using the mortar and pestle. Each powdered macroalgae of 0.1g were introduced in three separate clean dry regular-sized (160mm X 15mm) test tubes.

Ten-Fold Serial Dilution of the Powdered Macroalgae in Artificial Seawater

To a tube containing 0.1g or 100 mg of powdered macroalgae, 10 ml of artificial seawater (3.8 grams of rock salt dissolved per 100 ml distilled water) was introduced and the test tubes was corked and manually shaken by inverting 5 times. The concentration of the macroalgae extract in this test tube was 10 mg/ml or 10, 000 μ/ml. using a permanent marker, this test tube was labelled # 1. Four other test tubes were labelled 2, 3, 4 and 5. The test tubes were placed in a rack side by side according to their number. To test tube # 2, 3, 4, 5 artificial seawater of 9ml volume was added using a 10 ml glass pipette. This process performed is a 10-fold serial dilution indicating that in each tube as one move from tubes # 1 to 5, the concentration of the macroalgae which is being reproduced by 10 times of the tube previous to it.

Producing the Brine Shrimps from the Desiccated Cysts

Artemia salina was obtained from a local pet shop. Less than one gram brine shrimp cysts were suspended in 1,000 ml artificial seawater contained in a shallow oval-shape plastic container (35cm x 15cm x 10cm) and covered with plastic cellophane and punched with several holes and kept illuminated by a fluorescent lamp for 48 hours.An aquarium aerator was used for the *Artemia* suspension to provide an ample supply of oxygen in the medium which also prevents bacterial contamination. After 48 hours, the cyst would have hatched producing fast swimming nauplii. These nauplii were used in the bioassay. A wide mouth plastic medicine dropper will serve well to collect the nauplii from the suspension.

The Bioassay Proper

Approximately 15 shrimps were collected using a wide-mouth plastic medicine dropper. The

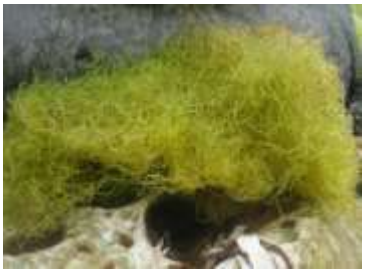
shrimps were added to each vial. A magnifying lens was used for checking the viability and weak being of the nauplii. The plastic container was closed with a plastic cellophane cover and kept under white light during the 24-hour period. The numbers of viable nauplii (number of surviving) were counted in each vial after 24 hours. A pipette sucked into the vials was used to count the surviving shrimps macroscopically, held against a well-lighted background. A scientific calculator was useful particularly for the logarithmic transformation of some data.

3 RESULTS AND DISCUSSION

A total of ten species of macroalgae were selected along the intertidal zone of Loreto, Dinagat Island. These species are the macroalgae samples:

Amphiroa foliacea (Red Algae)

Amphiroa sp. (Red Algae)



Chaetomorpha sp. (Green Algae)

Ulva reticulate (Green Algae)

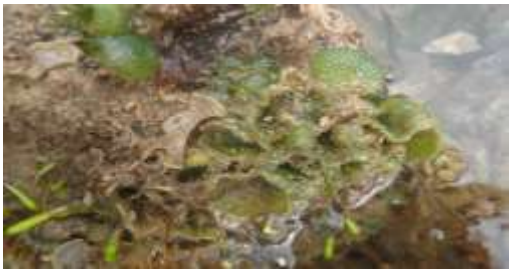
Hydroclathrus clathratus (Brown Algae)

Dictyota cervicornis (Brown Algae)

Pandina australis (Brown Algae)

Sargasum crassifolium (Brown Algae)

Boodlea composita (Green Algae)



Dictyosphaeria cavernosa (Green Algae)

Figure 3: The Macroalgae samples in Loreto, Dinagat Island

Qualitative Analysis for the Presence of Phytochemicals

Ten samples of macroalgae were able to test through phytochemical screening to show the presence of secondary metabolites and antioxidant properties. The preliminary phytochemical investigation showed the presence of phytoconstituents such as alkaloids, flavonoids, tannins, anthraquinones, steroids and saponins.

Table 1 shows the qualitative phytochemical analysis of crude ethanolic of ten seaweed sample for alkaloid test. Alkaloids are present in most of the macroalgae samples except in seaweed 1 (*Amphiroa foliacea*).

Table 1: Qualitative phytochemical analysis of crude ethanolic of ten macroalgae samples

Test for Alkaloids (Using Dragendorff's, Meyer Reagent)			**Alkaloids**		
Algae	**Scientific Name**	**Type of Algae**	**DR**	**MR**	**CT**
1	*Amphiroa foliacea*	Red	▪	▪	▪
2	*Amphiroa sp.*	Red	▪	▪	▪
3	*Chaetomorpha sp.*	Green	▪	▪	▪
4	*Ulva reticulata*	Green	▪	▪	▪
5	*Hydroclathrus clathratus*	Brown	▪	▪	▪
6	*Dictyota cervicornis*	Brown	▪	▪	▪
7	*Padina australis*	Brown	▪	▪	▪
8	*Sargasum crassifolium*	Brown	▪	▪	▪
9	*Boodlea composita*	Green	▪	▪	▪
10	*Dictyosphaeria cavernosa*	Green	▪	▪	▪

Legend: DR- Dragendroff's reagent; MR: Mayers reagent; (-) Absence; (+): Less presence; (++): Moderate presence; (+++): High presence.

Table 2 shows the qualitative phytochemical analysis of ethanolic of ten macroalgae for anthraquinones and steroids test. It showed that anthraquinones and unsaturated steroids were both present in all brown macroalgae samples. Further, the abundant steroids present were unsaturated and two samples of macroalgae present with two deoxysugars.

Table 2: Qualitative phytochemical analysis of crude ethanolic of ten macroalgae

Test for Anthraquinones & Steroids			**Anthraquinones**		**Steroids**	
Algae	**Scientific Name**	**Type of Algae**	**BT**	**MBT**	**KKT 2-DS**	**LBT US**
1	*Amphiroa foliacea*	Red	▪	▪	▪	▪
2	*Amphiroa sp.*	Red	▪	▪	▪	▪
3	*Chaetomorpha sp.*	Green	+	+	▪	▪
4	*Ulva reticulata*	Green	▪	▪	▪	▪
5	*Hydroclathrus clathratus*	Brown	+	+	▪	▪
6	*Dictyota cervicornis*	Brown	+	+	▪	▪
7	*Padina australis*	Brown	+	+	▪	▪
8	*Sargasum crassifolium*	Brown	+	+	▪	▪
9	*Boodlea composita*	Green	+	+	▪	▪
10	*Dictyosphaeria cavernosa*	Green	▪	▪	▪	▪

Legend: BT: Borntrager's Test; MBT: Modified Borntrager's Test; KKT: Keller-Killani Test; 2-DS: 2-deoxysugar; LBT: Lieberman-Burchard Test; US: Unsaturated Steroid; (-): Absence; (+): Present

Table 3 shows that all of the ten macroalgae samples were all present with tannins. Saponins and flavonoids were not present in *Amphiroa foliacea and Amphiroa sp* which are classified as red algae. Saponins were not present in *Padina australis,* brown algae and

Dictyosphaeria cavernosa, green algae.

Table 3: Qualitative phytochemical analysis of crude ethanolic of ten macroalgae test for tannins, saponins & flavonoids

Test for Tannins, Saponins & Flavonoids			**Tannins**		**Saponins**	**Flavonoids**
Local Name	**Scientific Name**	**Type of Algae**	**CT**	**HT**	**FT**	**BMM**
1	*Amphiroa foliacea*	Red	+	+	·	·
2	*Amphiroa sp.*	Red	·	·	·	·
3	*Chaetomorpha sp.*	Green	+	+	+	+
4	*Ulva reticulata*	Green	+	+	+	+
5	*Hydroclathrus clathratus*	Brown	+	+	+	+
6	*Dictyota cervicornis*	Brown	+	+	+	+
7	*Padina australis*	Brown	+	+	·	+
8	*Sargasum crassifolium*	Brown	+	+	+	+
9	*Boodlea composita*	Green	+	+	+	+
10	*Dictyosphaeria cavernosa*	Green	+	+	·	+

Legend: CT: Condensed Tannins; HT: Hydrolysable Tannins; FT: Froth Test; BMM: Bate Smith & Metcalf Method; (-): Absent; (+): Present

The table 4 shows the summary of the preliminary phytochemical analysis of the ten macroalgae samples that was used in the study. The phytochemical screening of the all selected macroalgae showed that it contained phytoconstituents such as alkaloids, flavonoids, tannins, anthraquinones, steroids and saponins. Tannins and steroids were all present in all selected macroalgae. The macro algae samples that was abundant in its secondary metabolites were the macroalgae 3 (*Chaetomorpha sp.*), a green algae, macroalgae 5 (*Hydroclathrus clathratus),* a brown macroalgae, algae 8 (*Sargasum crassifolium*), a brown algae and algae 9 (*Boodlea composita*) a green algae which contains all the secondary metabolites tested. And the less abundant of the secondary metabolite was the macroalage 1 (*Amphiroa foliacea*), a red algae which contains only tannins and two deoxysugars.

Table 4: Summary on the presence of the active phytocompounds in the ten macroalgae found in Loreto, Dinagat Island

Present Active Phytochemical Compounds							Total no. Phytocompounds
Macroalgae	Alkaloid	Anthraquinones	Steroid	Tannins	Saponins	Flavonoid	
Amphiroa foliacea	·	·	·	·	·	·	2
Amphiroa sp.	·	·	·	·	·	·	3
Chaetomorpha sp.	·	·	·	·	·	·	6
Ulva reticulata	·	·	·	·	·	·	5
Hydroclathrus clathratus	·	·	·	·	·	·	6
Dictyota cervicornis	·	·	·	·	·	·	6
Padina australis	·	·	·	·	·	·	5
Sargasum crassifolium	·	·	·	·	·	·	6
Boodlea composita	·	·	·	·	·	·	6
Dictyosphaeria cavernosa	·	·	·	·	·	·	4

Legend: (+) : present; (-) : absent

Brine Shrimp Bio Assay

Shown in the Table 5, the percentage mortality of the red macroalgae using the brine shrimp assay was dose-dependent. There were two red algae samples that were collected, the *Amphiroa foliacea* and *Amphiroa sp.* In Figure 4, shows the graph of the percentage mortality of the red algae. The *Amphiroa foliacea* (in blue) has the higher percentage of mortality compared to *Amphiroa sp* (in red).

Table 5: Percentage mortality rate of red macroalgae using the brine shrimp bio assay

Concentration µg/ml	**% Mortality Rate of Red Macroalgae Using the Brine Shrimp Assay**	
	Algae 1 (*Amphiroa foliacea*)	Algae 2 (Amphiroa *sp.*)
10,000	89	84
1,000	73	64
100	49	49
10	33	31
1	16	16
0	7	4

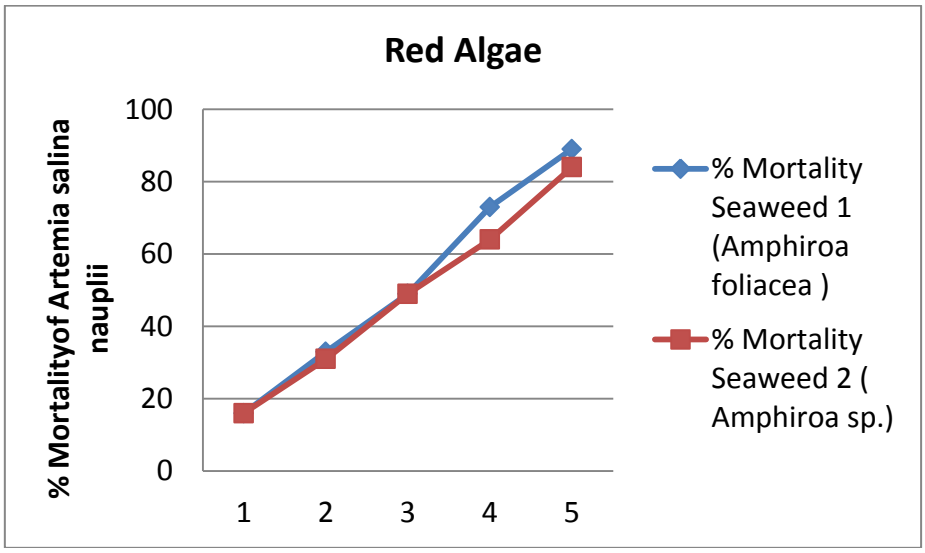


Figure 4: Graph of the percentage mortality of red macroalgae

The percentage mortality rate of green macroalgae is shown in Table 6. There were four green algae samples that were collected, the *Chaetomorpha sp, Ulva reticulate, Boodlea composita,* and *Dictyosphaeria cavernosa*. It shows that the percentage mortality of the green macroalgae were dose-dependent. Shown in Figure 5 is the graph of the percentage mortality of the green algae. It shows that *Boodlea composita* (in green) has the highest percentage of mortality rate and *Chaetomorpha sp.* (in blue) has least percentage of mortality rates of all the green macroalgae samples.

Table 6: Percentage mortality rate of green macroalgae using brine shrimp assay

Concentration µg/ml	**% Mortality Rate of Green Macroalgae Using Brine Shrimp Assay**			
	Algae 3 (*Chaetomorpha sp.*)	Algae 4 (*Ulva reticulate*)	Algae 9 (*Boodlea composita*)	Algae 10 (*Dictyosphaeria cavernosa*)
10,000	76	91	100	91
1,000	62	80	84	76
100	49	53	53	58
10	29	33	27	33
1	16	20	11	16
0	2	2	7	2

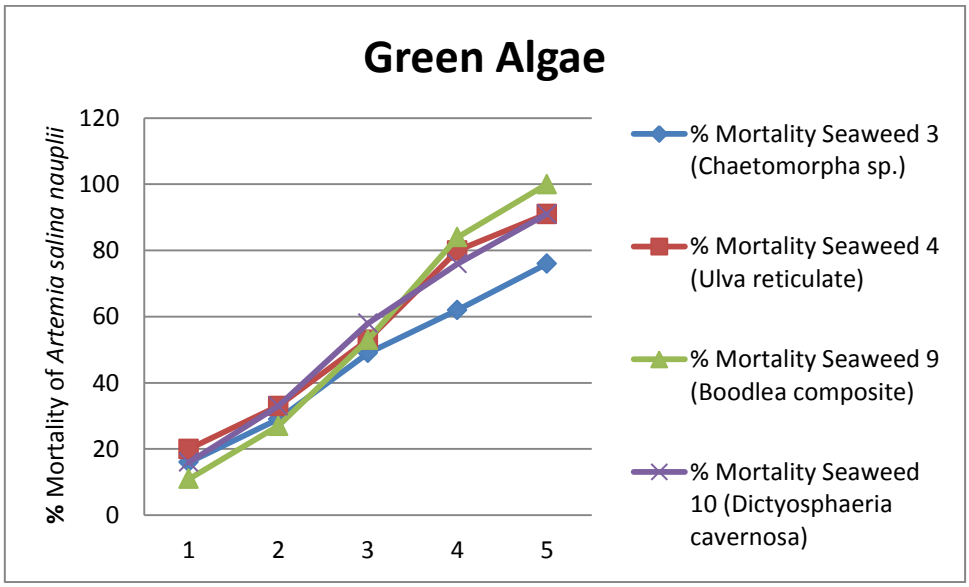


Figure 5: Graph of the percentage mortality of red macroalgae

Table 7 shows the percentage mortality rate of the brown macroalgae. There were four brown algae samples were collected, the *Hydroclathrus clathratus, Dictyota cervicornis, Padina australis* and *Sargasum crassifolium*. It shows that the percentage mortality rate of the brown algae were dose-dependent. The graph of the percentage mortality rate of brown macroalgae was shown in Figure 6. It shows that *Dictyota cervicornis* (in red) has the highest percentage of mortality rate and *Hydroclathrus clathratus* (in blue) has the lowest percentage of mortality rate among the brown algae.

Table 7: Percentage mortality rate of brown macroalgae using brine shrimp assay

Concentration µg/ml	**% Mortality Rate of Brown Macroalgae Using Brine Shrimp Assay**			
	Algae 5 (*Hydroclathrus clathratus*)	Algae 6 (*Dictyota cervicornis*)	Algae 7 (*Padina australis*)	Algae 8 (*Sargasum crassifolium*)
10,000	82	98	87	93
1,000	78	76	69	80
100	71	58	47	56
10	60	36	29	38
1	36	22	18	24
0	20	7	2	11

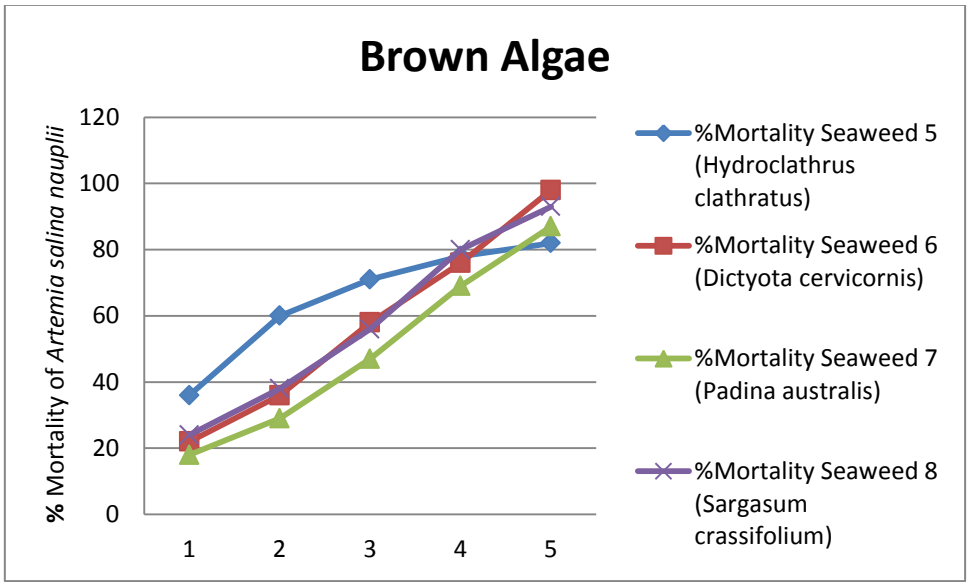


Figure 6: Graph of the percentage mortality of brown macroalgae

Table 8 shows the level of toxicity of each selected macroalgae. In the study, there were three macroalgae that were identified as highly toxic, the *Ulva reticulate,* a green algae, with the lethal concentration of 66.1 µg /mL, *Dictyota cervicornis*, a brown algae, with the lethal concentration of 88.7 µg /mL and *Dictyosphaeria cavernosa* which is a green algae, with the lethal concentration of 67.7 µg /mL. And seven of the selected macroalgae were moderately toxic.

Table 8: Lethal concentration of ten selected macroalgae

Macroalgae	Lethal Concentration (LC_{50})	Interpretation
Algae 1 (red) *Amphiroa foliacea*	185.7 µg/mL	Moderately Toxic
Algae 2 (red) *Amphiroa sp.*	203.1 µg /mL	Moderately Toxic
Algae 3 (green) *Chaetomorpha sp.*	232.3 µg /mL	Moderately Toxic
Seaweed 4 (green)*Ulva reticulata*	66.1 µg /mL	HighlyToxic
Algae 5 (brown)*Hydroclathrus clathratus*	261.9 µg /mL	Moderately Toxic
Algae 6 (brown) *Dictyotacervicornis*	88.7 µg /mL	HighlyToxic
Algae 7 (brown) *Padina australis*	129.5 µg /mL	Moderately Toxic
Algae 8 (brown) *Sargasum crassifolium*	143.1 µg /mL	Moderately Toxic
Algae 9 (green) *Boodlea composita*	121.6 µg /mL	Moderately Toxic
Algae 10 (green) *Dictyosphaeria cavernosa*	67.7 µg /mL	Highly Toxic

Legend (1-100; **Highly Toxic**: 101-500; **Moderately Toxic**: 501- 1000; **Slightly Toxic** and 1001 and above **Not Toxic)**

4 CONCLUSION

The result of the present study confirmed that some of the macroalgae present in Loreto, Dinagat Island may be rich sources of phytoconstituents which can be isolated and further screened for various biological activities. Macroalgae has been a source of a variety of major metabolites such as polysaccharides, alkaloids, tannins, steroids, flavonoids, and many other fine chemicals. Macroalgae could be collected and utilized effectively in product preparation for the beneficial of mankind. Further research studies should be carried out on the other species of macroalgae from the same habitat in order to provide complete data on the nutritive and other component of macroalgae.

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