Secondary Metabolites as Indicators of Copper Mine Tailing Stress in Sonneratia Alba J. Smith in Marinduque Island, Philippines

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***Abstract*— Secondary metabolites of Sonneratia alba leaves and stems coming from areas exposed to copper mine tailing contaminated and uncontaminated areas were determined using phytochemical screening tests and thin layer chromatography. The number of secondary metabolites present in the samples from contaminated sites was more (8 to 9) compared to areas that were not contaminated or exposed to copper (5 to 7). Results of the thin layer chromatography for the antioxidant phenols showed that the contaminated site in Marinduque had more bands (2) compared to that only (1) for the other sites. Of interest are the phenols and flavonoids, as these are the secondary metabolites associated with heavy metal stress. Based on the results of the study, there are reasons to believe that the secondary metabolites screened from Sonneratia alba in particular the antioxidant phenols are indicators of heavy metal stress in the environment. The Sonneratia alba stands in Brgy. Ipil, Sta. Cruz, Marinduque continue to experience heavy metal stress even if mining have stopped a long time ago.**

***Keywords*— secondary metabolites, thin layer chromatography, heavy metals, Sonneratia alba.**

I. Introduction

Several studies have shown that the presence of toxic heavy metals in biological systems can cause oxidative stress promoting the formation of reactive oxygen species that damage cells [1]. The plants could scavenge and concentrate metals from the waters [2]. Plants that come in contact with elevated acid levels or metal-bearing waters could potentially suffer adverse health effects. Plants dispose of these reactive molecules by producing phytochemicals or antioxidants and activating defense system present in several sub-cellular components. Phytochemicals are non-nutritive chemicals that have protective or disease preventive properties in plants [3]. Plants have ascorbate, reduced glutathione, $\propto$-tocophenol and carotenoids as major types of antioxidant [1]. They also have polyamines and flavonoids that protect them from free radical injury.

It has been established that mangroves are able to tolerate high levels of heavy metals, however there are still a lot to be understood on effects of the accumulation of metals on the biological processes (e.g. biochemical changes at the sub-cellular level) on the mangrove plant. The accumulation of heavy metals on mangroves exposed to copper mine tailing in the past and an area far from copper mine activities in Negros Oriental, Philippines have shown a significant difference on the amount of copper concentration on the leaves of the *Sonneratia alba* collected from both study areas [4]. Copper concentration on the leaves from the copper mine tailing exposed mangrove ranged from 27.03 $\pm$ 8.65 to 61.16 $\pm$ 40.49 mg Cu Kg-1 dry weight while the copper concentration of leaves collected from the area far from copper mine activities ranged from 8.33 $\pm$ 1.23 to 17.35 $\pm$ 0.48 mg Cu Kg-1 dry weight.

Exposure of *Avicennia marina* in laboratory conditions to concentrations of Cu and Zn lower than those that induces visible toxicity yielded significant increases in peroxidase activity and decreases in photopigments establishing an inverse relationship between photosynthetic pigments and increasing leaf tissue metal concentrations with Cu and Zn [5]

Secondary metabolites polyphenols and tannin were determined in the leaves of 14 species of mangroves [6]. It appeared that the balance among micronutrients, such as Zn, Cu, and Mn, may influence a competition between the different classes of phenolic compounds [7]. It further suggested that flavonoids and tannins production depends of the amounts of foliar nutrients, Cu and Mn in particular, which are cofactors of enzymes involved in phenol degradation and lignin biosynthesis [7].

Marinduque island had experienced a major environmental disaster from one of the largest copper mines in the Philippines [8]. In March 1996, the first wave of a major mine tailing spill went into Makulapnit and Boac Rivers and continued until August of 1996 with an estimated volume of 2 to 3 million cubic meters [9]. Elevated concentrations of Cu (706-3,080 ppm), Mn (445-1,060 ppm), Pb (43-56 ppm), and Zn (131-276 ppm) were present in the tailings [8]. Although the event had occurred almost 19 years ago, the heavy metal contamination are still of considerable concern because they are not usually eliminated from aquatic ecosystems by natural processes. They are either accumulated in sediments or biota, or transported to other ecosystems (e.g. from land to streams by storm water runoff). Metals such as arsenic, cadmium, chromium, copper and mercury accumulated in river and lake sediments can be remobilized and incorporated into food webs [10].

In the interest of determining the long term effect of the spill on mangroves, this study assessed the effect of the accumulation

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of copper on the biological process of mangroves as may be manifested by the presence of secondary metabolites. It is hoped that by comparing the presence or absence of secondary metabolites (e.g. phenols, flavonoids and tannins) in the samples taken from different sites will establish the potential of secondary metabolites as indicators of heavy metal stress.

II. Materials and Methods

A. Study sites

Three (3) mangrove areas were covered in this study. Two areas are in Marinduque Island. The first area, Calancan Bay is located in Barangay Ipil, Sta. Cruz (13° 32'53.07" N and 121° 57'59.77" E). This was the disposal site for the tailings of the copper mining industry in Marinduque estimated to have reached a total volume of 200-300 million tonnes from 1975 to 1990 [2]. The chemical analysis of the pore waters from this area revealed elevated dissolved concentrations of selenium, arsenic, copper, aluminum, iron and molybdenum which exceeded the concentrations regular sea water and limits set by the U. S. Environmental Protection Agency (EPA) Recommended Chronic or Acute Water Quality Criteria for saltwater (Table 1). The mangrove soil 13 years after the mining operation has stopped but still yielded a high copper concentration of 51.94 ppm [11]. The normal level for copper in soil is 20 ppm.

TABLE I: Results of pore water analysis in Calancan Bay [2] and the standards set by .the U. S. EPA for acute (short-term exposure) and chronic (long-term exposure) water quality criteria for selected metals in salt water [2]. All concentrations are in parts-per-billion (ppb).

Metal	Calancan tailings pore water (filtered)	EPA Acute	EPA Chronic
Arsenic	44	69	36
Cadmium	3	42	9.3
Zinc	50	90	81
Copper	60	4.8	3.1
Lead	0.6	210	8.1
Selenium	150	290	71
Silver	4	1.9	
Nickel	<1	74	8.2

The second area in Marinduque Island is located in Barangay Kay Duke, Torrijos (13° 22'42.21" N and 122° 08'32.17" E). This is far from the copper mining site but sits in proximity to a rich copper deposit.

Climate pattern in Marinduque falls under the Climate Type III category. Historical data showed that Marinduque experienced less than 0.2°C rise in temperature and have experienced medium rainfall decrease but was frequented by more typhoons compared to the third site [12].

The third site is located in Barangay Rupagan, Bacolod Lanao del Norte, Philippines (08° 07'33.63" N and 124° 01'49.06" E). The area is not known to have experienced mining nor is it in proximity to metal deposits. In terms of climate, it also belongs to the Climate Type III category but are less frequented by typhoons compared to the Marinduque sites however have experienced an increase in temperature close to 0.5°C and a high decrease in rainfall [12].

B. Plant material

Sonneratia alba was the choice species to be studied because it is common in the 3study sites. The leaves and stem tissues of this mangrove plant were randomly collected from the study areas.

C. Preparation of samples

The collected leaf and stem tissue samples were air-dried for two weeks or until crispy then ground to power consistency using a blender. The powdered samples were then stored in plastic bags until ready for phytochemical screening and Thin Layer Chromatography (TLC) Analysis.

D. Determination of the secondary metabolites

1. Preparation of methanolic extract (leaves and bark)

100 grams of the powdered samples were soaked in 200 ml methanol in an Erlenmeyer flask for 24 hours. The extracts were then filtered to separate the debris using a Whatman no. 1 filter. The extracts were then allowed to evaporate in a well ventilated room to concentrate the samples to syrup consistency for 48 hours.

2. Phytochemical screening

A total of nine (9) phytochemicals were screened for this study. Unless otherwise specified, the phytochemical screening method as described by B.Q. Guevara was used [13].

2.1. Alkaloid

The Wagner's test was used to detect alkaloids [14]. The extracts were treated Wagner's reagent. If a brown/reddish precipitate formed, it was an indicative of the presence of alkaloids. The Wagner's reagent was Iodine in Potassium Iodide.

2.2. Tannin/Phenols

For the tannin and phenols, the Ferric Chloride test was used. The extracts were treated with ferric chloride. A blue to black color change indicated the presence of hydrolyzable tannins, while a brownish to green color indicated the presence of condensed tannins. If polyphenols were present, colored products with ferric chloride were produced.

2.3. Saponin

To determine if saponin was present, the Froth Test was used. Saponin is present if a "honeycomb" froth forms, and stood for 10 minutes after adding distilled water to the sample and vigorously shaking it for 30 seconds.

2.4. Steroids (unsaturated) and Terpenoids

To determine the presence of unsaturated steroids and terpenoids, the Liebermann-Burchard Test was used. The positive results gave color changes ranging from blue to green, red, pink, purple or violet when compared to the control.

2.5. Flavonoids

For the flavonoids, two tests were employed. One was to test for leucoanthocyanins using the Bate-Smith and Metcalf method and the other one was to test for the Y-benzopyrone nucleus using the Wilstatter "cyanidin" test.

Positive result for the Bate-Smith and Metcalf method showed a strong red or violet color when compared to the control. For the Wilstatter "cyanidin" test, a positive result showed color change ranging from orange to red, to crimson and magenta, and occasionally to green or blue when compared to the control.

2.6. Glycosides

The Keller-Kiliani test for 2-deoxysugars was used to determine the presence of glycosides [15]. A brown ring on the interface of the acid and the aqueous layers that form in the extract indicated a deoxyribose sugar which was a characteristic cardenolides.

3. Thin Layer Chromatography (TLC)

The TLC initiated for this study focused on phenolic antioxidant as these secondary metabolites were more associated with the adaptation of plants heavy metal conditions.

Two (2) stationary phases (TLC plates) were used for this study. Pre-coated TLC Silica Gel 60 and TLC Cellulose both produced by Merk®. These were trimmed to strips and the position of origin marked by a straight line. For the mobile phase, the solvent used was n-butanol: acetic acid: water with a ratio of 12:1:1 [16].

The methanolic extract of *Sonneratia alba* was placed on the origin line established in the TLC plates and was placed on a glass chamber and leaded. The solvent was allowed to travel for two hours on the TLC plates. The TLC plates were then dried and sprayed with Ferric Chloride and Potassium Ferricyanide aqueous solution (1:1) for visualization of the phenolic antioxidant spots [13].

E. Statistical Analysis

To compare the secondary metabolite assemblage that was observed in the leaves and stem tissues of *Sonneratia alba*, cluster analysis was used in this study. The clustering method used was Unweighted Pair Group Average (UPGA), the output of which was a Dendogram that showed the sites clustered or linked based on the presence or absence of a particular secondary metabolites in the sites.

III. Results and Discussion

A. Phytochemical screening

Table 2 shows the result of the phytochemical screening. Samples from Marinduque relatively had a higher number of secondary metabolites (ranging from 8 to 9) compared to that of the area in Lanao del Norte for both leaf and bark samples (ranging from 5 to 7). The results are indicative that *Sonneratia alba* in Marinduque may be experiencing more stress compared to that of the mangroves sampled in Bacolod, Lanao del Norte due the availability of copper in the soil in combination with climatic conditions. The production of secondary metabolites are affected by climate [17]. Seasonal production of flavonoids and hydrolysable tannins in some mangrove leaves depends on various biotic and abiotic factors, such as temperature, rainfall and humidity [7].

As shown in the results of the cluster analysis based on the phytochemical assemblage (Figures 1 and 2), the sites in Marinduque were clustered together. Mangroves in Lanao del Norte, although they had experienced less rainfall, may not be significantly affected by less rain as earlier stated. *Sonneratia alba* can grow in salinities ranging from freshwater to seawater with growth maximized at 5 to 50% sea water [18].

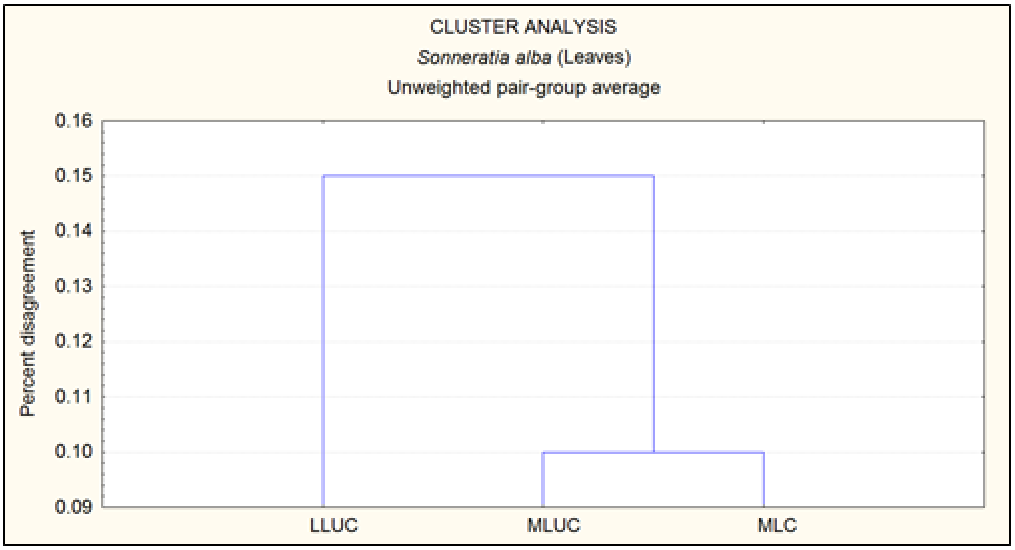
Of interest among the phytochemicals was the group of the antioxidant phenolic compounds which includes the flavonoids. Phenolic compounds have a role in protecting plants from heavy metals. The phenolic contents were copper concentration dependent [19]. The phenolic compounds can act as metal chelators and can directly scavenge molecular species of active oxygen as its mechanism in the antioxidant activity in plants under heavy metal stress [20].

Although all of the samples from the different sites tested positive for phenolic compounds, results of the TLC specific for antioxidant phenols showed a different picture for the leaf samples taken from copper mine tailing contaminated site in Marinduque.

TABLE II: RESULTS OF THE PHYTOCHEMICAL SCREENING

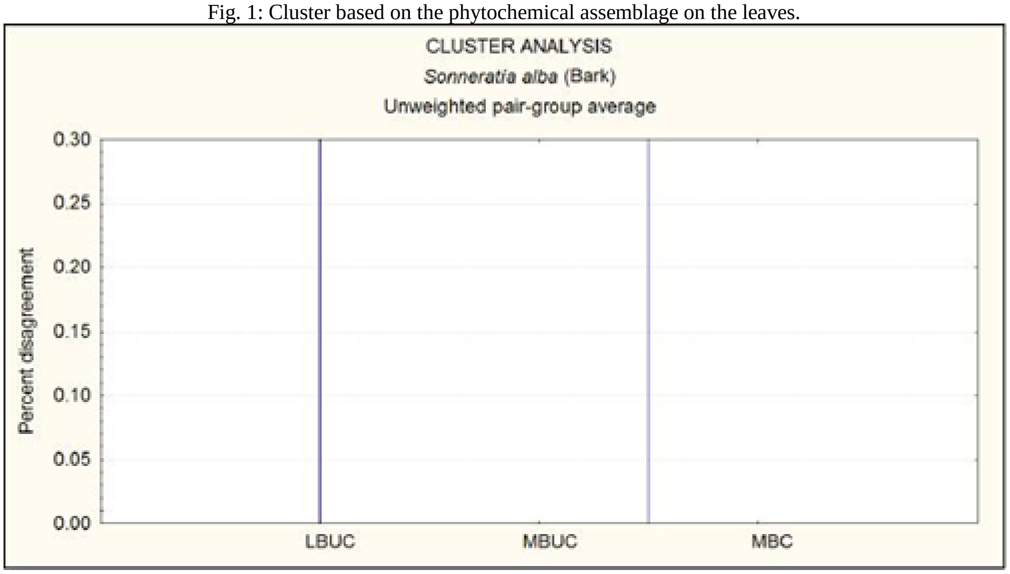
Phytochemical	Marinduque				Lanao del Norte	
	Leaves		Bark		Leaves	Bark
	Con	UnCon	Con	UnCon	UnCon	UnCon
Alkaloid	+	+	+	+	+	+
Tannin	+	+	+	+	+	+
Saponin	+	+	+	+	-	-
Steroid (Unsaturated)	+	+	+	+	+	-
Phenolic compounds	+	+	+	+	+	+
Flavonoid						
Leucoanthocyanin	+	+	+	+	+	+
Compounds with y-benzopyrone nucleus	-	+	-	-	-	-
Terpenoid	+	+	+	+	+	-
Glycosides	+	+	+	+	+	+
Number of Phytochemicals Present	**8**	**9**	**8**	**8**	**7**	**5**

Con = contaminated UnCon = uncontaminated



LLUC – Lanao Del Norte (leaves, uncontaminated)
MLUC – Marinduque (leaves, uncontaminated)
MLC – Marinduque (leaves, contaminated)

Fig. 1: Cluster based on the phytochemical assemblage on the leaves.



LBUC – Lanao Del Norte (bark, uncontaminated)
MBUC – Marinduque (bark, uncontaminated)
MBC – Marinduque (bark, contaminated)

Fig. 2: Cluster based on the phytochemical assemblage on the bark.

B. *Thin Layer Chromatography (TLC)*

Results of the TLC with visualization specific to antioxidant phenols show a different picture for the samples coming from the different sites and different tissues of the *Sonneratia alba*. The leaf methanolic extract coming from the contaminated site in Marinduque had manifested 2 bands compared to only 1 band in samples from uncontaminated sites, possibly indicating more types of antioxidant phenols present to address the heavy metal contamination. Further, the results also showed that the leaves coming from the contaminated area had more bands compared to that of the bark. The antioxidant phenols present in leaves was an indication that *Sonneratia alba* in Brgy. Ipil, Sta. Cruz in Marinduque continues to experience heavy metal stress.

Important antioxidant phenols in plants growing in heavy metal stress include flavonoids, phenylopropanoids, and phenolic acids [20].

IV. Conclusion and Recommendation

Based on the results of the study, there are reasons to believe that the secondary metabolites screened from Sonneratia alba in particular the antioxidant phenols are indicators of heavy metal stress in the environment. The *Sonneratia alba* stands in Brgy. Ipil, Sta. Cruz, Marinduque continues to experience heavy metal stress even if mining have stopped a long time ago.

To further substantiate the result of this study that suggests that antioxidant phenols present in *Sonneratia alba* are good indicators of heavy metal stress in the environment, it is recommended that a comparative study of the total phenolic content of samples between heavy metal contaminated and uncontaminated areas be conducted using the Folin-Ciocalteu's method with gallic acid as a calibration standard [19]..

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