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PHYTOCHEMICAL, BRINE SHRIMP LETHALITY ASSAY, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY SCREENING OF *NYPAFRUTICANS* ("NIPA") LEAF EXTRACTS

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ABSTRACT

The present study was designed to evaluate the phytochemicals, cytotoxicity and antioxidant activities of Nypafruticans extracts. Phytochemical screening revealed the presence of flavonoids (23.61%), alkaloids (2.64%), phlobatannins, saponins, and steroids. The ethyl acetate extract at 1000 ppm showed bioactivity (92% mortality) against naupliiArtemiasalina. The extracts showed no inhibition against *B. subtilis* and *E. coli*. Furthermore, the antioxidant activity of the extracts were tested by its ability to reduce Mo (VI) to Mo (V) through subsequent formation of green phosphate Mo(V)-complex at acidic pH and positive results were observed in ethyl acetate extracts and fractions as indicated by their ascorbic acid equivalents.

Key words: Antioxidant, Antibacterial, Cytotoxic, Phytochemicals, Nypafruticans.

INTRODUCTION

The development of microbial resistance towards antibiotics has heightened the importance of the search for new potential effective plants and plant constituents against pathogenic microorganisms[1]. The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and protective/disease preventive properties (phytochemicals). These phytochemicals, often secondary metabolites present in smaller quantities in higher plants, include the alkaloids, steroids, flavonoids, terpenoids, tannins, and many others[2]. The Philippines is now gaining popularity and getting recognition as a source of plants having medicinal values. *Nypafruticans* locally known as

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"Nipa" belongs to Arecaceae family and is found along tidal streams, brackish swamps and muddy banks throughout the Philippines. "Nipa" is a monoecious palm, with stout, trunkless and thornless rootstock. Leaves are at the ends of the rootstocks, large, rosette and compound, 5 to 10 meters long, arising from the stout underground stem. Leaflets are numerous and rigid up to 1 meter long and 2 to 7 cm centimeters wide. Fruit is globose, nodding, up to 30 centimeters in diameter. Carpels are numerous, dark-brown, striate, smooth, 10 to 14 centimeters long, compressed, obovate. Seeds are hard, white, and as large as a hen's egg. *N. fruticans* is one of the most important economic Philippine crops and is folklorically known to cure herpes, headache, toothaches, conjunctival inflammations and stimulant for debility[3]. A study reported that methanol extract of leaves and stems of *N. fruticans* were found to show significant anti-hyperglycemic and antinociceptive activity and thus have great potential as a source of natural products[4]. Another study also reported that the immature fruits of *N. fruticans* showed a high DPPH radical scavenging activity and antioxidant capacity[5]. Hence, the present study was aimed at evaluating the phytochemicals and screening the potentials of *N. fruticans* using the Brine Shrimp Lethality Assay[6] and its antioxidant capacity employing the Phosphomolydenum method[7].

EXPERIMENTAL

Sampling and sample preparation

The medicinal plant studied was collected and obtained from Brgy. Santiago, Iligan City, Philippines. The samples were then air-dried in shed for one month, cut into small pieces, after which it was grinded to a uniform powder and stored in polyethylene bags at room temperature.

Phytochemical evaluation and plant extraction

Qualitative phytochemical evaluation of the crude ethanol extract for the presence of phlobatannins, flavonoids, tannins, saponins, alkaloids, steroids and anthraquinones was carried out as per the standard of Guevarra[8] and the quantitative analysis for flavonoids and alkaloids was based on the method described by Edioga[9].

The ground sample (600.00 g) of the air-dried leaves was soaked in 95% ethanol for a week in the refrigerator at 5^{o}C. The extracts were concentrated using a rotary evaporator with the hot water bath set at 40°C to yield the crude ethanol extract, (35.9189 g). The crude ethanol extract was partitioned between water and hexane in 1:1 ratio to give the aqueous extract, and hexane extract (0.3414 g). The aqueous extract was subsequently partitioned

with dichloromethane and ethyl acetate in equal ratio to give the dichloromethane extract (0.3764 g) and the ethyl acetate extract (0.2632 g). Based on the preliminary data on cytotoxicity evaluation, the ethyl acetate fraction being the most bioactive was subjected to two rounds of gravity column chromatography using silica gel 60 (Merck, 70230 mesh, ASTM) as an adsorbent with gradient mixtures of hexane-ethyl acetate and ethyl acetate-methanol at 10% increment to yield ethyl acetate sub-fractions pooled by means of Thin Layer Chromatography (TLC).

Cytotoxicity evaluation (Brine shrimp lethality assay)

The crude extracts were subjected to Brine Shrimp Lethality Test at single concentration of 1000 ppm. Natural seawater was used as the culture medium. Sea water was boiled for 30 min and then filtered to obtain a sterile medium. The medium was poured into the hatching tank and about 100 to 200 mg cysts were introduced. A 100-watt lamp was positioned to provide direct light and warmth throughout the embryogenesis. After 36 hrs, the *Artemiasalina*nauplii were collected using Pasteur pipette from the hatching tank and were transferred to a Petri dish with 5.0 mL of seawater. The assay was carried out according to the principle and protocol previously described by Meyer et al.[10] with slight modifications. Ten nauplii were transferred to a sample test tube and sterilized seawater was then added to make a volume of 5.0 mL. A parallel series of test with a blank solution was also conducted. There were five replicates in each treatment. Number of survivors and percentage lethality were evaluated after 24 hrs.

Antioxidant activity (Phosphomolybdenum method) determination

The antioxidant activity of the plant extracts was examined on the basis of the sample solution to reduce Mo(VI) to Mo(V) through subsequent formation of green phosphate Mo(V)- complex. Four test concentrations in ppm (500, 100, 50, and 25) of extracts as test solutions and three concentrations also in ppm (200, 100, and 10) of ascorbic acid as standard solutions were employed with methanol used as control. In a capped test tube, 3.0 mL of the freshly prepared reagent (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate mixture)) was added to 0.30 mL of the test solution. The resulting mixture was then incubated in boiling water bath at for 90 min and allowed to cool at room temperature. The absorbance of each solution was read spectrophotometrically at 695 nm (Fischer Scientific Spectromaster, model 145). All observations were performed in triplicate and values are expressed as equivalents of ascorbic acid (AAE) in µg per mL of extract.

Antibacterial screening

Two bacterial species strains were tested (*gram-positive Bacillus subtilis* and *gram-negative Escherichia coli*). The Paper Disc Diffusion method[5] was used. Nutrient Agar (NA) were seeded with the bacterial inoculum. Sterile filter paper discs (6mm) were soaked in each of the extract (1000 ppm). The discs were then dried so as to remove the residual solvent. Each extract was tested in triplicate for three trials with ampicillin and extracting solvent as positive and negative control, respectively. The plates were kept at 4^{o}C for 4 hours for diffusion of extract. Thereafter were incubated at 38^{o}C for 24 hrs. Activity index for each extract was then determined by measuring the zone of inhibition.

RESULTS AND DISCUSSION

The phytochemical screening showed positive results for flavonoids, alkaloids, phlobatannins, steroids, and saponins. Total contents of 23.61% of flavonoids and 2.64% of alkaloids were measured from the crude ethanol extract of *N. fruticans.* At 1000 ppm, the crude extracts were observed to be bioactive against the nauplii*Artemiasalina*. Except for dichloromethane extract, all have more than 90% mortality out of 50 exposed nauplii for 24 hrs. However, the ethyl acetate extract when further subjected to a two-round gravity column chromatography, the cytotoxicity activities in terms of percent mortality out of 30 exposed for 24 hrs nauplii of most sub-fractions were observed to be lower than 50%. Although not conclusive, this may be due to the spreading of activity among several components or loss of synergistic effect. The data are summarized in Table 1.

Table 1: Brine shrimp larvae mortality after 24 hr exposure to1000 ppm of the leaf extracts of *N. fruticans*

Extract	Percent mortality
Ethanol (NFEt)	94.00
Ethyl acetate (NFE)	92.00
Hexane (NFH)	90.00
Dichloromethane (NFD)	78.00
NF Fractions	
NFE 1	50.00
NFE 2	73.30

Cont…

Extract	Percent mortality
NFE3	23.30
NFE 4	13.30
NFE 5	10.00
NFE 6	6.67
NFE 2.2	40.00
NFE 2.3	16.67
NFE 2.4	6.67

The BSLT assay has proven to be a convenient system for screening bioactive natural products. It has the complete and effective range to test the toxicity as well as for various assays[6]. 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[11].

The antibacterial screening of the crude extracts from *Nypafruticans* showed no inhibition against *B. subtilis* and *E. coli.* Moreover, the ethyl acetate was chosen for antioxidant screening on the basis of its cytotoxicity data and the result showed it has an antioxidant activity expressed by its ascorbic acid (AAE) values (Table 2). Total antioxidant capacity by Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo(V)-complex at acidic pH.

Free radicals are constantly generated resulting in extensive damage to tissues and biomolecules leading to various disease conditions. So the medicinal plants are employed as an alternative source of medicine to mitigate the diseases associated with oxidative stress[12].

Table 2: Antioxidant activity (AAE, ug/mL) of the ethyl acetate fractions of *nypafruticans* using phosphomolybdenum method

Extract	Concentration (ppm)			
	25	**50**	**100**	**500**
NFE	< 5	5.3	14.1	243.4
NFE 2.2	123.9	473.8	482.5	482.5

CONCLUSION

The leaf extracts of *N. fruticans* demonstrated the presence of secondary metabolites with potential biological activities. No antibacterial activity was observed. All plant extracts obtained from the plant sample exhibited bioactivity against the brine shrimp *A. salina* and the chosen ethyl acetate extract is a promising source of antioxidant compounds. With further fractionation and more advanced chromatographic techniques, there are certainly many biologically active compounds with interesting activities yet to be discovered from *N. fruticans* and these of course should be subjected to as wide a range of bioassays as possible in order to confirm this conclusion.

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