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Phytochemical Screening of *Nepenthes Bellii* Kondo Phytochemical Analysis And Evaluation of The Cytotoxic And Antioxidant Activities of Leaf Ethanolic Extract Of The Carnivorous Plant *Nepenthes Bellii* Kondo (Nepenthaceae)

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ABSTRACT
Nepenthes spp. is an important tropical carnivorous pitcher plant in the Philippines, which has been utilized within the conventional pharmaceutical framework to treat and control distinctive afflictions. However it lacks information on its pharmacological properties, hence in this study, we investigated the phytochemicals, antioxidants, and cytotoxicity activity of the leaf ethanolic extract of Nepenthes bellii. Leaf ethanolic extracts were prepared to identify the phytochemicals present, DPPH assay was performed to determine the free radical scavenging activity and extracts were prepared at 10-, 100- and 1000- ppm for the cytotoxicity screening through Brine Shrimp Lethality assay. Results revealed that alkaloids, anthraquinones, flavonoids, saponins, steroids, and tannins are present in the N. belii leaf extracts. Strong free radical scavenging activity was observed with an LC_{50} value of 2.55 µg/mL and the brine-shrimp lethality assay revealed an LC_{50} value of 5.41 ± 0.73 which indicates highly toxic to the Artemia salina nauplii. Thus, the results of this study highlight the properties of N. Bellii extract supporting its traditional medicinal uses reported by the local people.
Keywords: *Nepenthaceae, Nepenthes bellii, Phytochemicals, Pitcher plants.*

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INTRODUCTION

Traditional medicine plays an important role in several communities in meeting primary health care across the world most especially in developing countries.[1] In cultural traditions, different medicinal plants usually undergo extraction or decoction of the different parts of the plant as their means of preparation for a treatment.[2] At present, interest in using medicinal plants is increasing worldwide due to their safety, efficacy, cultural acceptability and lesser side effects as compared to synthetic drugs.[3] However, herbal medicine needs to be tested for efficacy using conventional trial methodology while several specific herbal extracts has been demonstrated to be efficacious for specific conditions.[4]

In the Philippines, many plants have been reported as herbal medicine but only few had undergone conventional trials for pharmacological research. Pitcher plants for instance, have long been used as traditional medicine by many aboriginal communities and have attracted renewed pharmaceutical interest due to recent investigations revealing their cytoprotective activities in cell models.[5] A study in North America supports these findings where they revealed that preparations from the leaves and the plant's long slender pitchers are beneficial in treating symptoms of diabetes, and in particular slow healing infections.[6] *Nepenthes bellii* Kondo, locally known as -Hara-Hara‖, is an endemic and vulnerable species of carnivorous pitcher plant in the Philippines. *Nepenthes* spp. can be found in the provinces of Surigao del Sur and Surigao del Norte and an ultramaficolous micro-endemic from Dinagat Islands Province, northern Mindanao, Philippines.[7] *Nepenthes bellii* together with other known *Nepenthes* species (e.g. *N. khasiana, N. ampullaria and N. gracilis, N. ampularia, N. mirabilis*) has been

one of the plants that are used as an alternative medicine to treat constipation, urinary tract problems, digestion problems, fluid retention, and other conditions.[8] Although several species of *Nepenthes* have carried out pharmacological screening, there is a lack of information regarding the pharmacological assessment of *Nepenthes belii*. Thus, this study prompted us to evaluate *Nepenthes bellii*'s phytochemical components of certain bioactive compounds that will help us researchers understand the nature on how it can cure and heals;[9] antioxidant property to determine if the substances present possess free radical chain reaction breaking properties;[10] and the brine shrimp lethality assay to investigate the cytotoxic activity.[11]

MATERIAL AND METHODS

Collection of Plant Material

Permits to the local government of Carrascal were acquired prior to collection of samples. Study proposal were also presented prior to the conduct of this study. Fresh leaves of *Nepenthes bellii* were collected in April 2020 in Barangay Adlay, Carrascal, Surigao del Sur. This plant material was authenticated by a botanist and a herbarium specimen was prepared and was deposited in Surigao del Sur State University, Cantilan Campus, Surigao del Sur, Philippines.

Preparation of Plant Extract

The plant leaf samples were processed following the protocol[12] described as, leaf samples were air-dried under the shade for 3 weeks at room temperature until crispy, powdered using a blender, and before extraction, kept in an airtight plastic container. In preparing the ethanolic extract, 100 grams of the powdered leaf samples were soaked in 200 ml of pure ethanol for 7 days. Mixtures were placed in a container covered with black cloth and a foil and stored in a locker with room temperature. Residue were filtered and re-extracted with ethanol. The ethanol (solvent) was separated using Whatman No. 1 filter paper. After filtration, the solvent was removed *in vacuo* by using a rotary evaporator and the concentrated leaf ethanolic crude extract of *N. bellii* were obtained. The crude extracts were stored in a tight glass container and refrigerated at 7°C until used.

Phytochemical screening

Phytochemical screening of the ethanolic leaf extracts of *N. bellii* were processed following the protocol[13] with modifications. Ethanolic leaf extracts of *N. bellii* were subjected to qualitative phytochemical screening for the determination of the presence of various classes of active chemical constituents such as alkaloids, anthraquinones, cyanogenic glycosides, flavonoids, saponins, steroids, tannins and other phenolic compounds.

DPPH radical scavenging activity

The chemicals used are of high grade and monitoring was observed for proper chemical preparation. The antioxidant capacity of *N. bellii* leaf extracts were determined by 2,2- diphenyl-1-picrylhydrazyl (DPPH) on the ability of free radicals to decolorize in the presence of antioxidants following the protocol designed by[14-15] with slight modifications. The stock solution was prepared by dissolving 24 mg DPPH with 100 mL ethanol and then refrigerated to -10C until used. Reaction mixture was prepared using 2.5 ml of 6.5x10-5 M DPPH solution and 0.5 ml of sample extracts dissolved in ethanol, ethanol being the control. Leaf extracted with concentrations, 1 μg/mL, 2 μg/mL, 3 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, 30 μg/mL, 50 μg/mL) were tested in DPPH solution for 12 h in the dark place of room temperature. Absorbance was measured at 515 nm using UV-Vis spectrophotometer (SHIMADZU UV mini 1240). The percentage of DPPH radical scavenging activity was determined using the equation mentioned below. The concentration of sample required for 50% inhibition was determined and represented as IC50 value for each test solution.

% DPPH scavenging activity = (A control – A sample / A control) x 100

Where, Acontrol and Asample are the absorbance values of the test and of the blank sample, respectively. The DPPH radical has odd electrons that act on absorbing in a 517 nm for purple coloration to be visible. The decrease in absorption was taken as a measure on the extent of radical scavenging. All determinations were carried out at three trials.

Brine shrimp lethality assay

The New Aqua Laboratory in Naawan, Misamis Oriental provided brine shrimp eggs. *Artemia salina* were hatched in artificial sea water prepared from commercial sea salt 40 g/1 and supplemented with 6 mg/1 dried yeast in regulation of the salinity and pH. A plastic chamber was used for hatching with a regulation of an electric errator. The eggs were sprinkled into the plastic chamber. The set-up was placed in a room temperature. After 48 hr, nauplii larvae were pipetted and transferred to the vials for treatments of different concentrations for the bark and leaf extracts. The procedure for BSLT was modified from the assay described by.[16-17] Five milligrams of the extracts were made up to 2 mg/ml in artificial sea water. Extracts were serial diluted in four different concentrations in three replicates (10 ppm, 100 ppm and 1000 ppm) and placed in the transparent vials for observation. Ten (10) nauplii were added to each vial. The vials were covered by a foil and placed in a

room temperature for a 6 hr and 24 hr observation. After which, numbers of dead (non-motile) nauplii in each vial were counted.

Statistical Analysis

Analysis was carried out in a protocol by.[18-20] The percentage mortality (% mortality) was calculated using the formula mentioned below. This is to ensure that the death (mortality) of *A. salina* nauplii is attributed to the bioactive compounds present in the leaf extracts of *N. bellii.*

$$\% \text{ mortality} = (\text{no. of dead nauplii} / \text{initial no. of live nauplli}) \times 100$$

Microsoft excel 2016 and Probit Analysis by [21] was used to determine the lethality concentration (LC_{50}) of *A. salina* at 95% confidence intervals. Plant extracts with an LC50 value of less than 100 ppm was considered as potent or active. LC_{50} value of less than 1000 µg/mL is toxic while LC50 value of greater than 1000 µg/mL is non-toxic.[22]

RESULTS

Phytochemical Screening

Nepenthes spp. is an essential medicinal plant with broad pharmacological spectrum. Different species such as *N. bicalcaratas, N. khasiana, N. mirabilis*, showed the presence of various phytochemical constituents responsible for various pharmacological and ethnomedicinal properties.[8] Screening of the phytochemical constituents of *Nepenthes bellii* ethanolic leaf extract revealed the presence of bioactive compounds such as alkaloids, anthraquinones, flavonoids, saponins, steroids and tannins. As shown in Table 1, N. *bellii* ethanolic leaf extract is rich in anthraquinones, flavonoids, steroids and tannins. Our result is in congruence with different species of *Nepenthes* subjected to phytochemical analysis which revealed that *N. khasiana* have presence of flavonoids, tannins, alkaloids and saponins from the pitcher and leaf extracts.[23] And another species, the *Nepenthes bicalcarata* also revealed high presence of anthraquinones, flavonoids, steroids and tannins,[24] the same results with the present study.

DPPH radical scavenging activity

For the radical scavenging activity in this study, result shows that rapid assessment for antioxidant property using DPPH assay demonstrated that the ethanolic extracts of N. *bellii* possess strong free radical scavenging activity with LC_{50} value of 2.55 µg/mL. As shown in the Table 2 and Figure 1, the free radical scavenging activity had stabilized at concentrations 10 µg/mL to 50 µg/mL, thus at this concentrations, optimum activity of the *Nepenthes bellii* ethanolic extract was reached.

Brine shrimp lethality assay

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.[45] The LC_{50} value of the brine shrimp obtained for *N. bellii* is 5.41 ± 0.73 which indicates highly toxic to the *Artemia salina* nauplii upon exposure at different concentrations. Maximum mortality was observed in 100 ppm and 1000 ppm of the ethanolic extracts while the lowest mortality was observed in 10 ppm (Table 3). The result showed that the toxicity of the leaf was directly proportional to the different concentrations used in the assay.

DISCUSSION

Phytochemical Screening

Anthraquinones are an important group of bioactive components that are found not only in *N. bellii* but also in many other species of medicinal herbs, such as rhubarb, aloe, senna, and purslane.[25] It has an anti-fungal, anti-bacterial effects which also shows potential protective properties for gastrointestinal and renal systems and even a potential treatment for cancer.[26- 29] In addition, anthraquinones have known properties of anti-termites as it is known to be a major factor in determining the resistance of wood to some specified termites' species.[30] Flavonoids, a group of natural substances with variable phenolic structures, are found in leaf, fruit, vegetable, grain, bark, root, stem and flower parts of a plant. They are now considered as an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications.[31] Thus, natural products and medicinal plants with flavonoids have great beneficial effects on health for it possess medical properties such as antioxidant,[32] anti- allergic and reduce risk for heart disease[33] and exhibits a number of *in vitro* and *in vivo* anti- inflammatory and anticancer actions.[34] Another bioactive compound, the steroids had immense pharmacological activity like any other compound, however, steroids have several restrictions.[35] Santos *et al*.[36] demonstrates that there is antinociceptive actions or the analgesic effect of the steroid compounds isolated from the leaves, stems, and roots of a certain medicinal plant *in vivo* using mice. Another rich bioactive compound in *Nepenthes bellii* is tannins. Tannins are produced by plants in adverse environmental conditions, being responsible for their protection against herbivores and pathogenic diseases and are essential for the growth and reproduction of the plants.[37] Medicinally, tannins are employed in antidiarrheal, haemostatic, and

antihemorrhoidal compounds and significant properties as anti-viral,[38] anti-bacterial[39] and anti-parasitic effects.[40] Henceforth, the presence of these bioactive compounds that had been identified using phytochemical screening presumes that *Nepenthes bellii* is a potential plant with pharmacological properties in searching for cures and treatment of several target diseases respectively.

DPPH radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method is an approach to evaluate antioxidant potential of a compound, an extract or other biological sources. A process wherein the prospective compound or extract is mixed with DPPH solution and absorbance is recorded after a defined period.[41] The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine[42] or simply to evaluate antioxidants by spectrophotometry.[25] In confirmation to the study in 2014,[43] samples only exhibiting LC50<10 µg/mL are considered very active antioxidants as they have the potential comparable to the antioxidant standards of quercertin, β-carotene, ascorbic acid, gallic acid and the plant extracts exhibiting the greatest antioxidant potential were those with the highest levels of total polyphenols. Screening of phytochemicals in *N. bellii* is a basis for antioxidant activity of the leaf ethanolic extract. Polyphenolic compounds such as flavonoids and tannins that are abundant in *N. bellii* which are contributors of the high free radical scavenging activity since chemical activities of polyphenols in terms of their reducing properties as hydrogenor electron-donating agents predicts their potential for action as free-radical scavengers.[44]

Brine shrimp lethality assay

Crude plant extract is toxic if it has an LC50 value of less than 1000 µg/mL while non-toxic if the value is greater than 1000 µg/mL.[22] As mentioned on the results, 100 ppm and 1000 ppm concentrations of the ethanolic extract of *N. bellii* are highly toxic having LC50 of >1000 which is confirmed on a mortality rate of 100% and probits value of 5.18 for both respectively. In this study, distilled water was used as a control for nauplii treatments and in 24 hr the count of live nauplii accounts to approximately 100%, confirming that there is no presence of any bioactive compounds on the water. In brine shrimp assay, cytotoxicity of bioactive compounds from the plant extracts is being evaluated.[46] And as mentioned, *N. bellii* have secondary metabolites that contribute to the cytotoxic activity such as anthraquinones, flavonoids, tannins and steroids. This concludes that the ethanolic leaf extract of the *N. bellii* have potential value in pharmacological uses in known dosages. On the other hand, findings in this study show that in 10 ppm, the cytotoxic activity was less but still had presence of potent cytotoxic components. Hence, this cytotoxic activity in 10 ppm that shows low mortality indicated that upon administration, the risk is low compared to the highly toxic concentrations. This is in congruence to the study in 2006,[47] in the evaluation for general toxicity using brine shrimp, maximum mortalities took place at a concentration of 1000 µg/ml whereas; least mortalities were at 10 µg/ml concentration. Although, BSLA is inadequate in determining the mechanism of action of the bioactive substances in the plant, it is very useful by providing a preliminary screen that can be supported by a more specific bioassay, once the active compound has been isolated.[19]

Nepenthes spp. as carnivorous plants is characterized by the synthesis of secondary metabolites in the insect-trap tissues, which are used for self-defense.[48] This study presented specific secondary metabolites in *Nepenthes bellii* that are essential for attacks of parasites and medicinal property. Although many medicinal plants had undergone assays, *Nepenthes* spp. that is endemic in Mindanao, especially *Nepenthes bellii* that are first found in Surigao Provinces has not been elucidated. And for the record, this is the first evaluation of the phytochemical screening, antioxidant capacity and cytotoxicity examination of *N.bellii*.

Study in Israel in 2010,[49] revealed that *Nepenthes* spp. have antifungal property by bioactive compound naphthoquinone and a case had been reported also that another species of *Nepenthes* is confirmed to have anti-malarial property with presence of bioactive compound naphthoquinone. Naphthoquinones, is a bioactive compound that is considered to be potential antifungal drugs and are produced by many plants that belong to the *Nepenthaceae*[50-51] is absent in *Nepenthes bellii*. Thus, the differences in location and endemicity of Nepenthes spp., gives different bioactive compounds present in the plant. However, a thing in common for the *Nepenthes* spp. that exists worldwide together with the six endemic species that are found in the Philippines esp. *N. bellii* in Surigao is that, they have exemplary medicinal properties.

Alkaloids	Anthraquinones	Cyanogenic glycosides	Flavonoids	Saponins	Steroids	Tannins
+	+++	–	+++	++	+++	+++

Table 1: Phytochemical constituents of *N. bellii* ethanolic leaf extract.

Table 2: DPPH radical scavenging assay of the *Nepenthes bellii* ethanolic extract.

Percent (%) inhibition at different concentration	
control	0 ± 0.3
1 µg/mL	26.11 ±0.2
2 µg/mL	38.3 ± 0.2
3 µg/mL	59.98 ± 0.1
5 µg/mL	93.84 ± 0. 02
10 µg/mL	96.50 ± 0.01
20 µg/mL	96.50 ± 0.01
30 µg/mL	96.50 ± 0.01
50 µg/mL	96.50 ± 0.01
IC_{50} µg/mL	2.55

Mean ± SD.

Table 3: Effects of *N. bellii* ethanolic leaf extract on exposure to *A. salina* in Lethality Assay.

Concentrations of the Extract (ppm)	Number of Dead Nauplii After24 hr			Brine Shrimp Mortality (%)	LC_{50} (µg/ml)	Probit
	T1	T2	T3			
10	5	4	8	57	24	4.29
100	10	10	10	100	>1000	5.18
1000	10	10	10	100	>1000	5.18

CONCLUSION

Nepenthes bellii ethanolic leaf extract have bioactive compounds such as alkaloids, anthraquinones, flavonoids, saponins, steroids and tannins. These bioactive compounds are contributors to the high activity of antioxidant and cytotoxic property of the plant. Leaf ethanolic extracts of N. belii possess strong free radical scavenging activity with LC_{50} value of 2.55 µg/mL. And based on the brine-shrimp lethality assay, LC_{50} value of the brine shrimp obtained for *Nepenthes bellii* is 5.41 ± 0.73 which indicates highly toxic to the *Artemia salina* nauplii However, toxicity of the leaf was identified to be directly proportional to the different concentrations used in the assay. The bioactive compounds present in the plant indicate medicinal potential that can be utilized by the locals for their folkloric medicine by proper administration. Hence, this study has proffer a remarkable phytochemical properties information regarding *Nepenthes belii* and can be used as baseline data to various aspects of research of this medicinal plant species.

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CONFLICT OF INTEREST

The authors declare no competing interest.

ABBREVIATIONS

2,2-diphenyl-1-picrylhydrazyl **(DPPH)**

AUTHOR CONTRIBUTIONS

JMB: Conceptualization, methodology, writing - (original draft and editing), visualization, funding acquisition. MAKN: Methodology, investigation, formal analysis, visualization, supervision, writing - (review and editing). RCMHO: Conceptualization, methodology, investigation, formal analysis, visualization, writing - (original draft, review and editing).

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