Research Article

Cytotoxicity, Antioxidant Activity, and Total Phenolic Content of the Ethanolic Leaf Extract of *Hornstedtia conoidea* Ridl

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ABSTRACT

This study uncovers the potential benefits of the leaves of the less explored Philippine endemic *Hornstedtia conoidea*. This study aimed to determine the total phenolic content (TPC), total flavonoid content (TFC), total antioxidant activity (TAA), and cytotoxicity of the ethanolic leaf extract of the *H. conoidea*. TPC, TFC, and TAA were determined using the Folin-Ciocalteu method, aluminum chloride method, and phosphomolybdenum method, respectively. Cytotoxicity was determined via brine shrimp lethality assay (BSLA). The TPC, TFC, and TAA of the extract were 13.29 ± 0.32, 6.87 ± 0.26, and 261.60 ± 6.06 mg/g dry sample, respectively. The extract exhibited toxicity with LC_{50} of 24.61 mg/L based on BSLA. Ethanolic leaf extract of *H. conoidea* possesses antioxidative activity and cytotoxic properties.

Keywords: Antioxidant Activity, Cytotoxicity, *Hornstedtia conoidea*, Total Phenolic Content, Brine Shrimp Lethality Test.

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INTRODUCTION

Plants are attractive organisms possessing a variety of specialized features.[1] Plants have an astonishing capacity to synthesize more than 200,000 chemicals with a diverse array of structures, functional groups, solubilities, and reactivities.[2] So far, only 50,000 compounds have been identified from a large number of species.[3] This indicates the existence of thousands of undiscovered compounds with different physical and chemical properties.[4] This calls for further investigation of more indigenous plants.

Hornstedtia conoidea (tagbak) is a Philippine endemic plant that belongs to the Zingiberaceae family. It is the most abundant Philippine *Hornstedtia* species in the Province of Bukidnon and other areas of Mindanao, Philippines. It was reported that ripe fruit seeds of *H. conoidea* are edible. They are eaten by the local people in Bukidnon and are claimed to cure stomach disorders.[5] *H. conoidea* was reported to possess 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.[6] The metabolic study revealed that *H. conoidea* leaves could be a potential productive source of the useful chlorogenic acid and shikimic acid.[7]

There is a continued search for more potential natural sources of compounds with significant bioactivities. The intake of natural antioxidants primarily flavonoids, phenolics, and polyphenols has been effective and reported to have health-protective properties,[8] especially in the prevention of degenerative diseases.[9-10] Polyphenols, which are present in a variety of plants, are utilized as important components of human diets.[11] Various studies have also shown that these antioxidant compounds possess antimutagenic,[12] antitumor,[13] anticarcinogenic,[14] and antiviral activities.[15]

Thus, it is imperative to further investigate the less studied *H. conoidea* which may significantly help in the establishment of its pharmacological importance. This study aimed to determine the total phenolic content (TPC), total flavonoid content (TFC), total antioxidant activity (TAA), and cytotoxicity of the ethanolic leaf extract of *H. conoidea*.

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MATERIALS AND METHODS

Plant Materials

Leaves of *H. conoidea* were collected in Mindanao, Philippines specifically in Purok 17, Musuan, Maramag, Bukidnon (7° 51' 13.9134"N 125° 3' 5.1654"E) in February 2018. Sample collection was done randomly from a sampling site which is located near residential houses.

The collected leaf samples were taxonomically identified by Dr. Fulgent Coritico and Dr. Florfe Acma of the Central Mindanao University Herbarium (CMUH). The washed leaf samples were air-dried for three to four weeks under shade at ambient temperature. The dried leaf samples were powdered using a heavy-duty blender and stored inside an airtight glass container prior to extraction.

Chemicals

The chemicals used in this study were analytical reagent grade of sodium carbonate, Folin-Ciocalteu reagent, gallic acid, aluminum chloride, ethanol, sodium acetate, quercetin, sodium phosphate, ammonium molybdate, sulfuric acid, ascorbic acid, potassium dichromate, and dimethylsulfoxide (DMSO)

Extraction

The powdered dried leaf samples were soaked separately in absolute ethanol (Scharlau) for 48 hr and filtered using Whatman No.1 filter paper. The filtrate (ethanol extract) was rotary-evaporated under a vacuum at 40°C to remove ethanol. The dried crude ethanol extract was kept at -20°C for further analyses.

Total Phenolic Content

A modified 96-Well microplate Folin-Ciocalteu method[16-17] was employed in the determination of TPC. The ethanol leaf extract (200 µL, 2.5 mg/mL) was mixed with 200 µL 10% Folin-Ciocalteu reagent and 800 mM sodium carbonate. The reaction mixture was incubated for two (2) hr. Two hundred microliters of the reaction mixture were loaded into the microplate well and the absorbance was measured at 750 nm. The standard calibration curve (2.5-15.0 mg/ L) was plotted using gallic acid as standard. TPC results were expressed as gallic acid equivalents per gram sample (mg GAE/g dry sample).

Total Flavonoid Content

The TFC was measured using the aluminum chloride method[18-19] with slight modification. Fifty microliters (0.4 mg/mL) of the ethanol extract was loaded into 96-well microplate and added with 10 µL 10% aluminum chloride, 130 µL 95% ethanol, and 10 µL 500 mM sodium acetate. The mixture was mixed and incubated in the dark for 40 min. Absorbance readings were taken at 415 nm using a 96-well microplate reader. The standard calibration curve (5.0 - 20.0 mg/L) was plotted using quercetin as standard. TFC results were expressed as quercetin equivalents per gram dry sample (mg quercetin/g dry weight sample).

Total Antioxidant Activity

The TAA was determined using the Phosphomolybdenum method[20-21] with slight modification. The ethanol leaf extract (100 µL, 2.5 mg/mL) was combined with 0.9 mL phosphomolybdenum reagent (28 mM sodium phosphate, 4 mM ammonium molybdate, 0.6 M sulfuric acid). The mixture was incubated for 90 min at 95°C dry oven. It was then allowed to cool at room temperature. Two hundred microliters of the mixture were loaded into a 96-well microplate and the absorbance was measured at 695 nm. The standard calibration curve (10.0-400 mg/L) was plotted using ascorbic acid as standard. TAA results were expressed as ascorbic acid equivalents per gram dry sample (mg AAE/g dry sample).

Brine Shrimp Lethality Assay

The cytotoxic effect of *H. conoidea* was determined using brine shrimp lethality assay with slight modifications.[22-23] Brine shrimp (*Artemia salina*) eggs were obtained from Mindanao State University – Naawan Campus, Naawan, Misamis Oriental. The seawater was filtered and then sterilized using an autoclave.

The small tank filled with sterilized seawater was divided into two equal compartments by placing a plastic divider, punched with several two (2) mm holes. The brine shrimp eggs were sprinkled into one of the compartments and were covered with aluminum foil. The eggs were incubated for 24 hr under illumination at room temperature. The resulting nauplii (larvae) were attracted to the other compartment of the tank with a light source and were collected with a Pasteur pipette.

One hundred milligrams of the crude extract was dissolved in 10 mL sterilized seawater to yield a 10,000 ppm sample stock solution. From the stock solution, appropriate volumes (500, 250, 50, 5, and 2.5 µL to prepare 1000, 500, 100, 10, and 5 ppm test solutions, respectively) were placed into separate test tubes. The solvent was evaporated under the fume hood and a minimal amount of DMSO (5% of the volume of the stock solution placed in the test tubes) was added to the dried sample. Ten brine shrimps were transferred to each of the test tubes and sterilized seawater was added

up to five (5) mL mark. Negative and positive controls were prepared using sterilized seawater and aqueous solutions of potassium dichromate, respectively. The test tubes were placed under illumination for 24 hr and survivors were counted. The percentage mortality of nauplii was calculated for each concentration of the sample using the following formula.

$$\% \text{ mortality} = \frac{\text{number of dead nauplii}}{\text{initial number of live nauplii}} \times 100$$

The assessment of risk exposure of *A. salina* to plant extracts can be performed by measuring the concentration of a substance administered to a particular organism and calculating the median lethal concentration (LC_{50}). Probit analysis was used to determine the LC_{50}. LC_{50} is a statistical index that indicates the concentration of a chemical agent capable of causing death in 50% of the organism's population.[24] The lethal concentration for 50% mortality after 24 h of exposure (the chronic LC_{50}) is determined as the measure of the toxicity of the extract or compound. Plant extracts with LC_{50} less than 200 ppm are considered significantly active and have the potential for further investigation.[25]

Statistical Analysis

Analysis of variance (ANOVA) and Pearson correlation analyses were performed using the SPSS version 24.

RESULTS

Total phenolics and total flavonoid content

Mean TPC and TFC values of the ethanol leaf extract are summarized in Table 1. As shown in Table 1, the TPC and TFC value of the ethanolic leaf extracts of *H. conoidea* is 13.29 ± 0.32 mg GAE/g sample and 6.87 ± 0.26 mg quercetin /g sample, respectively. Figure 1 graphically compares TPC and TFC. It is evident that TPC is higher than the TFC value.

Total Antioxidant Activity

As shown in Table 1, the ethanolic leaf extract of *H. conoidea* has a TAA of 261.6 ± 6.06 mg AAE/g dry sample.

Table 1: Mean TPC,TFC, and TAA of ethanol leaf extract of *H. conoidea*.

Plant	Mean TPC ± SD (mg/g dry sample)	Mean TFC ± SD (mg/g dry sample)	Mean TAA±SD (mg/g dry sample)
H. conoidea	13.29 ± 0.32	6.87 ± 0.26	261.60 ± 6.06

TPC and TFC values, expressed as mg GAE/g dry weight sample and mg quercetin / sample, respectively. TAA value is expressed as mg AAE/g dry weight sample. Values are expressed in mean ± standard deviation (SD), n=4.

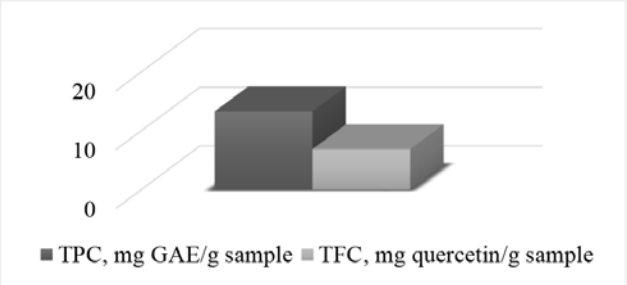

Figure 1: Graphical comparison between TPC and TFC values of the ethanolic leaf extract.

Cytotoxicity

The LC_{50} of the ethanolic leaf extract of *H. conoidea* was determined via BSLA. The obtained percent mortality at different concentrations of the plant extracts was subjected to Probit analysis to calculate the LC_{50} values. The obtained data are summarized in Table 2. The results of the BSLA indicated that the ethanolic leaf extracts and the positive control ($K_2Cr_2O_7$) produced a dose-dependent cytotoxicity effect on brine shrimp nauplii as revealed in Figure 2. The LC_{50} of the ethanolic leaf extract of *H. conoidea* was 24.61 mg/L. The positive control, on the other hand, gave the lower LC_{50} value of 9.68 mg/L.

Table 2: Mean LC_{50} value of the ethanolic leaf extract of *H. conoidea*.

Sample	LC_{50}, mg/L	Inference*
H. coinodea	24.61 ± 7.21[a]	Cytotoxic
$K_2Cr_2O_4$	9.68 ± 1.74[b]	Cytotoxic

*LC_{50} < 200 ppm (mg/L) is cytotoxic[25]
Median lethal concentration (LC_{50}) values in mg/L, are expressed in mean ± standard deviation (SD), n=3. For the second column, values followed by same letter (a - b) are not statistically different at $p < 0.05$, as measured by the Tukey HSD Test.

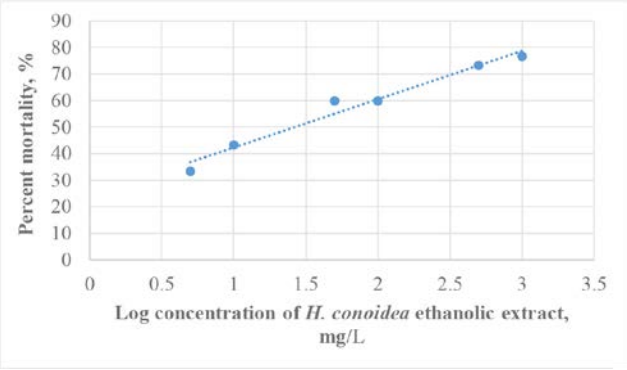

Figure 2: Dose-dependent cytotoxicity effect of the *H. conoidea* ethanolic leaf extracts on brine shrimp nauplii.

DISCUSSION

Total phenolic content

Phenolic compounds are commonly found in both edible and inedible plants and they have been reported to have biological effects, including antioxidant activity[26-27] and antimicrobial properties.[28] These physiological properties are responsible for the preventive activity of phenolic compounds against infectious and degenerative diseases, inflammation, and allergies via antioxidant, antimicrobial, and protein or enzyme neutralization or modulation mechanisms.[28] Flavonoids and tannins are among the classes of phenolic compounds. Accordingly, flavonoids have many favorable medicinal and physiological properties and are proven to exhibit antioxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties. They are also known to be modulators of key cellular enzyme functions and potent inhibitors of xanthine oxidase, cyclo-oxygenase, lipoxygenase, and phosphoinositide 3-kinase.[29-31]

Apart from flavonoids, saponins, and tannins, the water and ethanolic extracts of *H. conoidea* leaves have been found to contain alkaloids.[6] In this present study, the TFC value in the ethanolic leaf extract of *H. conoidea* is lower in concentration than the phenolic compounds as shown in Figure 1. This can be explained considering that flavonoid is a subclass phenolic compound.[32]

Total Antioxidant activity

Antioxidants are known to delay or prevent free radical formations which thereby suppresses life-threatening diseases for instance cancer. It had been shown that the presence of antioxidants prevents cancer progression by maintaining normal cell cycle regulation.[33] Numerous studies about plants and their active compounds are reported to possess antioxidant activity.[34] Thus, the total antioxidant activity has been investigated for various types of quantification techniques.

The phosphomolybdenum method is the most common and established technique used in the determination of the total antioxidant activity in plants such as the methanolic (101.82± 1.75 mg AAE/g) and acetone (85.71± 1.35 mg AAE/g) leaf extracts of *Marrubium vulgarae* (Horehound),[35] *Halimeda opuntia* (Linnaeus) Lamouroux (57.36± 0.004 mg AAE/g),[36] ethanol extracts of *S. wightii* (47 mg AAE/g), *H. gracilis* (35.9 mg AAE/g),[37] methanolic extracts of *Hypnea pannosa* (7.31 mg AAE/g), and *Sargassum coriifolium* (28.08 mg AAE/g).[38] The same method was employed in this study to quantify the total antioxidant activity of ethanolic leaf extracts of *H. conoidea* which obtained a high amount of 261.60 ± 6.06 mg AAE/g dry sample. Hence, it is evident that *H. conoidea* ethanolic extract has higher antioxidant activity than the abovementioned previously investigated plants. Moreover, this result is supported by the previously reported high DPPH radical scavenging activities, another form of antioxidant quantifying technique, in the water extracts of *H. conoidea* leaves.[6] Chlorogenic acid was also found higher in *H. conoidea* leaves than that in *Zingiber officinale* leaves.[7] These three chlorogenic acid isomers, namely; 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, and 5-4-O-caffeoylquinic acid were also reported to contribute to the antioxidative activities of prune (*Prunus domestica* L.).[39]

Correlation of Total Phenolic and Total Flavonoid in Total Antioxidant activity

The antioxidant activities of plant extracts are correlated to the number of phenolic compounds and flavonoids. An imperative study revealed that the antioxidant activity of plants is primarily due to the amount and type of polyphenolic and flavonoid compounds present in them.[40] For example, marginal and positive correlation between TPC and antioxidant activity in terms of DPPH, hydroxyl, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging and phosphomolybdate assays were observed in the whole plant extracts of *Torilis leptophylla* L.[41] Weak and positive correlation was also observed between TFC and antioxidant activity in terms of superoxide anion and hydroxy radicals.[41] TPC also showed a strong association (R^2 = 0.90) with antioxidative activity as determined using the Ferric thiocyanate method and thiobarbituric acid methods.[42] Antioxidant activity and phenolic content in selected medicinal and culinary herbs were also found to have a linear relationship.[43] Antioxidant activity was found strongly correlated with TPC in fresh strawberries (*Fragaria x ananassa* Duch), raspberries (*Rubus idaeus* Michx), and highbush blueberries (*Vaccinium corymbosum* L.), and lowbush blueberries (*Vaccinium angustofolium* Aiton).[44] There was a strong positive correlation between TPC and antioxidant activity as determined using ferric reducing potential, DPPH, and ABTS assay.[45]

In Zingiberaceae plants, a significant positive correlation between TAA and TPC was observed in the alcoholic extract of the *Hedychium coronarium*,[46] and *Etlingera philippinensis*.[47] However, in this study, the phenolics and flavonoids did not display a significant degree of association with antioxidant activity as shown in Table 3 (p<0.05). A contradiction to the other Zingiberaceae plants. The same trend was also reported that phenolic compounds were not the major contributor to the

Table 3: Association of TPC and TFC in TAA of *H. conoidea*.

	TAA	TPC	TFC
TAA	1.000	0.772	0.805
TPC	0.772	1.000	0.866
TFC	0.805	0.866	1.000

Pearson Correlation coefficients (p<0.05) 2-tailed.

antioxidant capacities of the microalgae as evidenced by the low correlation coefficients between antioxidant capacity and phenolic content. It was stated that the microalgae could have contained different antioxidant compounds.[48] The antioxidant activity of plant extracts is not limited to phenolics.[49] Antioxidant activity may also be due to the presence of other antioxidant secondary metabolites, such as volatile oils, vitamins, carotenoids, and others.[49]

Cytotoxicity

BSLA is a simple and high throughput cytotoxicity test of bioactive chemicals. BSLA is an important preliminary tool to screen plant extracts for cytotoxic properties. It is based on the killing ability of test compounds on a simple zoological organism like brine shrimp (*Artemia salina*).[50] BSLA is used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds.[51]

LC_{50} value of *H. conoidea* is statistically different from the control $K_2Cr_2O_7$ (Table 2). The standard $K_2Cr_2O_7$ exhibited more potent cytotoxicity against the *A. salina* nauplii as compared to the *H. conoidea* extract. Apparently, a lesser concentration of $K_2Cr_2O_7$ is needed to cause 50% mortality among the test organisms. The cytotoxicity and LC_{50} value have an inverse relationship, meaning plant extract having LC_{50} values < 200 ppm are considered highly active.[25] Thus, the plant leaf extract studied in this study is potentially cytotoxic which may exhibit a potential anti-tumor or anticancer activity, as previous studies have reported a positive correlation between brine shrimp toxicity and 9KB (human epidermoid carcinoma of the nasopharynx) cytotoxicity (p=0.036 and Kappa=0.56). Moreover, the usefulness of brine shrimp as a prescreen for antitumor activity was confirmed in a blind comparison with *in vitro* cytotoxicity and 3PS (*in vivo* P388 murine leukemia) activity.[25,52]

Phytochemical analysis revealed the presence of alkaloids, flavonoids, saponins, tannins, and steroids in both water and ethanol extracts of *H. conoidea* leaves. Ethanol extracts of *H. conoidea* rhizomes were also found to contain these phytochemicals.[6] The presence of these phytochemicals in the ethanolic leaf extracts of *H. conoidea* may provide additional scientific evidence for their cytotoxic property.

It is noteworthy, however, that to date only very few literature on the bioactivity of *H. conoidea,* an endemic ginger species in the Philippines, is available. A study on the determination of the antioxidant property of *H. conoidea* showed high DPPH radical scavenging activity of the water leaf extracts of *H. conoidea* with a percentage DPPH inhibition of 92.97 ± 0.23 at 500 μg/mL.[6] Related studies on other *Hornstedtia* species were also conducted. The n-hexane, ethyl acetate, and methanol extracts of the rhizome, leaf, and stem of *Hornstedtia leonurus* Retz. from Malaysia were screened for antimicrobial and antifungal activities. It was found that the ethyl acetate and methanol extract of the rhizome showed potent activity against *Candida albicans* (MIC 225 μg/mL) and *Aspergillus niger* (MIC 450 μg/mL) and *Staphylococcus aureus* (MIC value of 450 μg/mL), respectively.[53] *Hornstedtia pinga* from West Java, Indonesia exhibited strong antimicrobial activity against *Bacilus subtilis* (MIC at 500 *μg*/mL) and *E. coli*.[54] No reports on the cytotoxicity of *H. conoidea* were found in the literatures. Thus, the promising result of this study of the cytotoxicity of *H. conoidea* and the limited information on its other medicinal properties would warrant further investigation.

CONCLUSION

Ethanolic leaf extract of *H. conoidea* displayed cytotoxic property and antioxidant activity. It is worth emphasizing that the Philippine endemic *H. conoidea* is a potential source of natural antioxidants and cytotoxic compounds.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DPPH: 1,1-Diphenyl-2-picrylhydrazyl; **ABTS:** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid **DMSO:** dimethylsulfoxide; **GAE:** gallic acid equivalents; **AAE:** Ascorbic acid equivalent; **BSLA:** Brine shrimp lethality assay; **LC_{50}:** concentration of a chemical agent capable of causing death in 50% of organism's population.

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