

Toxicity Evaluation and Anthelmintic Property of Cebu Cinnamon (*Cinnamomum cebuense* Kosterm.) Leaf Extracts

Genette Chile D. Bilbao, Michael Ryan G. Alimpoos, Jholvenjey N. Baguio, Stephen V. Sabal, Shiela Mae A. Ursal, and Jay P. Picardal
Cebu Normal University
picardalj@cnu.edu.ph

Abstract

Cinnamomum cebuense (Kosterm.) is widely known as stomachache remedy, but adverse reaction to varying amount of herbal extract has also been reported, thus, requiring toxicity-profiling studies. Here, the toxicity of *C. cebuense* leaf extract (CCLE) was evaluated using brine shrimp lethality assay (BSLA) and anthelmintic assay under a complete randomized design with equal replications. Results in BSLA revealed an LC_{50} value of 7.05 $\mu\text{g/ml}$ ($p \leq 0.05$) which is considerably medium-toxic based on standard toxicity criterion. Influence on earthworm activity showed significant difference ($p \leq 0.01$) among the different concentrations of the extract (100 mg/ml, 75 mg/ml, 50 mg/ml, and 25 mg/ml) following a concentration-dependent response trend. Results suggest strong toxicity against earthworms at 75–100 mg/ml while having a moderate to weak toxicity at 25–50 mg/ml. Both assays were able to elucidate toxicity of CCLE which may be due to the presence of cinnamaldehyde, the main phytochemical found in cinnamon leaves. CCLE is toxic at elevated dosages and thus warrants parallel studies using higher-vertebrate models.

Keywords: *Cinnamomum cebuense* (Kosterm.), toxicity study, anthelmintic assay, brine shrimp lethality assay, Philippine cinnamon

1.0 Introduction

In many parts of the world and across civilizations, terrestrial plants remain as the main reservoir of natural products with medicinal benefits (Fennel et al., 2004). Until the present time, the use and practice of herbal medicine among 75–80% of the global population are linked to medicinal plants' efficacy, accessibility, and affordability (Pathare and Wagh, 2012). In the Philippines, among the sources of herbal medicines are the cinnamon species, which are not merely utilized as food spices and seasoning

but also as primary remedy for various medical conditions, particularly in upland and remote villages where commercially available drugs are expensive and unavailable (Research Information Series on Ecosystem, 1992).

Cinnamomum cebuense Kosterm., an endemic cinnamon tree in the Philippines, has long been known for bark-stripping used for treating stomach ache, as well as its leaves being used for curing various illnesses (Del Fierro et al., 2012; Espineli et al., 2014). Recent studies have documented evidence of its pharmacological

activities, such as hepatoprotective, antioxidant (Del Fierro et al., 2012), and antimicrobial potential (Espineli et al., 2014). Anecdotal reports from the montane communities of Cebu also support the use of *C. cebuense* in treating various forms of gastrointestinal disorders and is often given as tea to relieve headache, toothache, and body pains (Lucesio Son, personal communication, 2017).

The use of herbal medicine in the Philippines, however, is confronted with issues on quality control and safety (World Health Organization, 1998). There is a current interest on the regulation and accreditation of traditional healers to ensure that the use, dosage, and administration, as well as production of herbal medicines, will be regulated (Philippine Institute of Traditional and Alternative Health Care, 2018). Since some bioactive constituents of native plants are toxic at elevated dosages (Mounanga et al., 2015), there is a need to address particular safety concerns for phytochemicals, especially for their potential to induce serious harm referred to as toxicity. However, not all pharmacological studies on Philippine plants addressed the issue on toxicity at both cellular and organismic levels. In the US, preclinical studies performed before clinical trials must supply comprehensive information on dosing and toxicity levels of the potential phytomedicine (US Food and Drug Administration, 2018). Although Espineli et al. (2014) already tested the cytotoxicity of five compounds derived from the *C. cebuense's* bark against colon carcinoma (HCT 116), no studies to our knowledge have evaluated the toxicity of the ethanolic extract from the leaves of *C. cebuense*.

Toxicity evaluation using invertebrate bioassays *in vitro* and *in vivo* is needed and is an ideal alternative for vertebrate models which are highly constrained by ethical considerations of

animal welfare (Patwardhan and Ghaskadbi, 2013). This study focuses on evaluating the toxicity of *C. cebuense* leaf extract (CCLE) through a dual assay approach—brine shrimp lethality test (BSLA) and earthworm assay—to address safety and efficacy regulations of potential phytomedicines, which could be developed into commercially-available drugs in the future.

Objectives of the Study

This study aims to determine the toxicity levels of *Cinnamomum cebuense* leaf extracts (CCLE) in terms of its anthelmintic effect on earthworms (*Eudrilus euginiae* Kinberg) and LC50 of brine shrimps (*Artemia salina* L.).

2.0 Materials and Methods

2.1 Plant Material

Mature leaves (~10–18 cm long and dark green) (Barceloux, 2009; Kerala Agricultural University, 2013) of *Cinnamomum cebuense* Kosterm. were collected from the native tree nursery of the Ramon Aboitiz Foundation, Inc. (RAFI), located at Brgy. Busay, Cebu City. In cooperation with Ms. Nena T. Okay, forester of RAFI Nursery, the researchers were given an approval letter to collect leaves of *C. cebuense* on the site. Cinnamon taxonomy experts performed leaf-sample authentication and species verification.

Freshly-collected leaves were repeatedly cleaned with tap water to remove soil debris and were finally rinsed with distilled water before air dried (three days). Leaf samples were cut from its petiole along the midrib, leaving only the lamina needed for the extraction. Leaves were then stored in a room temperature enclosed in a zipper bag (Ziploc™) with silica gel inside until use. Finally, the dried leaves were cut into smaller pieces and

subsequently powdered and homogenized using an electric blender.

2.2 Plant Extraction and Preparation of Test Solutions

For BSLA, finely-powdered leaves (20 grams) were mixed with 95% ethanol (250 mL) in a beaker. For every thirty minutes within six hours, the beaker containing the solution was shaken manually (approximately one minute); and left for forty-eight hours in room temperature afterward. The jar was shaken again (about one minute) and immediately filtered using a Whatmann No. 1 filter paper. Using a rotary evaporator to process the filtrate, the solvent was removed, and the crude extract was obtained under reduced pressure and temperature, <55°C (Olowa and Nuneza, 2013).

DMSO (dimethyl sulfoxide) with v/v % of 1.25% (1.25 mL of DMSO per 100 mL of triple distilled water) was used as the vehicle for the crude extract (Geethaa, Thavamany, Chiew, & Thong, 2013). Twenty milligrams (20 mg) of the crude extract was dissolved in 2 mL of solvent (1.25% DMSO) and served as the stock solution. In preparing different dilutions, the researchers adopted McLaughlin, Rogers, and Anderson's (1998) procedure, yielding four concentrations of the test extract: 1000, 100, 10, and 1 ppm.

For the anthelmintic assay, decoction was utilized in the leaves' phytochemical extraction. Twenty-five grams (25 g) of dried and pulverized plant sample was boiled in 250 mL of triple distilled water, with constant stirring, for forty-five minutes. The solution was allowed to cool down and subsequently filtered by using Whatmann No. 1 filter paper. The same procedure was done consecutively using 18.75 g, 12.5 g, and 6.25 g of leaf powder in 250 mL of triple distilled water to

yield the concentrations 75 mg/mL, 50 mg/mL and 25 mg/mL, respectively.

2.3 Test Organisms and Experimental Design

Brine shrimp eggs (approximately one hundred grams for the entire experiment) ready for hatching were purchased from a registered pet shop in Colon Street, Cebu City. Eggs were stored in the refrigerator (around 10 °C) before incubation (Brine Shrimp Direct, Inc., 2018). The experiment was designed following complete randomized design (CRD), where test organisms were randomly assigned to 1000 ppm, 100 ppm, 10 ppm, and 1 ppm treatments of the test solution, one negative control (artificial seawater), positive control (0.01g/100 mL K₂Cr₂O₇), and a vehicle control (dimethyl sulfoxide, DMSO) in triplicates. Ten live nauplii larvae (larvae of *A. salina*) were placed in each of the three replicate test tubes.

For the anthelmintic assay, African night crawlers (*Eudrilus eugeniae*) of the same batch were procured from the City Agricultural Office, Maguikay, Mandaue City. A total of 252 earthworms (3–5 cm in length; 0.1–0.2 cm in width) were used for the entire experiment. Like in BSLA, a CRD was employed with four treatments of test extracts (T1: 100 mg/mL, T2: 75 mg/mL, T3: 50 mg/mL, and T4: 25 mg/mL) and three control groups (positive control, vehicle control, and solvent control). Albendazole, a standard anthelmintic drug (Jagota, 1986), represented positive control, triple distilled water for the solvent control, and 2% DMSO for the vehicle of albendazole. In each treatment and control group, there were six earthworms allocated in triplicates.

2.4 Experimental Procedures

Each bioassay utilized a combined, modified

experimental protocol after Meyer et al. (1982), McLaughlin et al. (1998), Hamidi et al. (2014), and Sarah, Anny, and Misbahuddin (2017) for BSLA, while for the anthelmintic assay, Ajaiyeoba, Onocha, and Olarenwaju (2001) and Panda, Das, and Tripathy (2011) were utilized.

BSLA procedure comprises of the hatching, incubation, and toxicity testing of the *A. salina* nauplii larvae. In hatching the nauplii larvae, artificial seawater (ASW) in room temperature was prepared by mixing sixty grams of sea salt with two liters of mineral water (Nature's Spring™), pH 9.0 alkaline water, inside a specialized aquarium. An aerator was placed on maintaining proper aeration and ensuring enough oxygen supply. Brine shrimp eggs were then scattered at the top of the water and stirred shortly after. A light bulb (60–100 W) was placed few inches away from the aquarium and was switched on. *A. salina* eggs hatched 20–24 hours after. Several nauplii larvae from the specialized aquarium were collected using a Pasteur pipette and were immediately transferred to a prepared container with artificial seawater and aerator. After 12 hours, 6 mg of dry yeast was added to the tank where nauplii larvae were newly transferred. The one-and-a-half-day-old nauplii larvae were then incubated for another 12 hours to reach second to third larval stage (two-day-old nauplii larvae), which will be used in toxicity test. In toxicity testing, nauplii larvae were exposed to different treatments of plant extracts as well as to control groups. Each test tube, with its respective treatment (0.50 mL), was filled with about 4 mL of artificial seawater, and 10 nauplii larvae were transferred to each test tube using Pasteur pipette. Afterwards, the volume of the seawater was adjusted to 5 mL per test tube. Test tubes were then left uncovered for the whole duration of

toxicity testing (24 hours). Finally, the number of surviving nauplii larvae was counted and recorded using a magnifying glass and flashlight, and the percentage of death after 24 hours was calculated. A dead nauplius was recognized by the absence of controlled forward motion at least within 30 seconds of observation even if light stimulus is present.

For the anthelmintic assay, earthworms were collected, washed, and measured. All the test solutions that were used in the experiment were freshly prepared. The treatment and control solutions were poured in its individual Petri dishes (20 mL). Six earthworms were added to each of the three replicate dishes. Changes in earthworms' behavior and morphology, such as reduced movement, rapid involuntary movement, localized tissue damage and lesions, and fading of body color were recorded. Mean times (in minutes) of the worms' paralysis for each treatment (i.e., when there is no movement within the observation period unless the dish is shaken vigorously) were taken cautiously. As soon as the worms were ascertained to be dead, the mean time (in minutes) was recorded. The loss of their motility characterized death of the earthworm even after being immersed in warm water (approximately 50 °C) and the eventual fading of the body color.

2.5 Data Analysis

For BSLA, the mean number of dead nauplii larvae for the three replicates was used in determining the percent of lethality for every experimental and control group, applying the formula:

$$\% \text{mortality} = \left[\frac{\text{(no. of dead nauplii larvae)}}{\text{(total no. of tested nauplii larvae)}} \right] \times 100$$

Linear regression analysis using Microsoft Excel 2010 was employed to evaluate the relationship between the independent variable (CCLE concentration) and the dependent variable (mortality rate) (Biswas et al., 2011). In calculating median lethality concentration (LC_{50}) value, Probit regression analysis was applied, wherein percent mortality was transformed into probits or probability units based on Finney's table (Finney, 1952). Probit was graphed against Log_{10} concentrations, and thus, yielded a slope-intercept equation. From this equation ($y = ax + b$), the slope (x) was then calculated. Subsequently, LC_{50} was inferred as the antilog of x . The toxicity level of CCLE expressed in lethal concentration 50 or LC_{50} was validated using the toxicity index from Clarkson et al. (2004) which was modified by Hamidi et al. (2014). CCLE, with an $LC_{50} > 1000 \mu\text{g}/\text{mL}$, were considered as non-toxic, while the CCLE with an LC_{50} of 500–1000 $\mu\text{g}/\text{mL}$, 100–500 $\mu\text{g}/\text{mL}$, and 0–100 $\mu\text{g}/\text{mL}$ were considered as low toxic, medium toxic, and highly toxic, respectively.

For the anthelmintic assay, all data were expressed as mean value \pm standard error of the mean ($X \pm \text{SEM}$). F-test in one-way analysis of variance (ANOVA) was used in comparing the mean values of treatment and control groups ($p < 0.01$). Significant ANOVA results were followed with Tukey post hoc test to determine which among the groups' means differ significantly. All data were analyzed using IBM SPSS version 20 software.

2.6 Ethical Consideration

Written permit was provided by RAFI's native tree nursery to allow the use of cinnamon leaves for the purpose of experimentation. Ethical clearance from the CNU research ethics

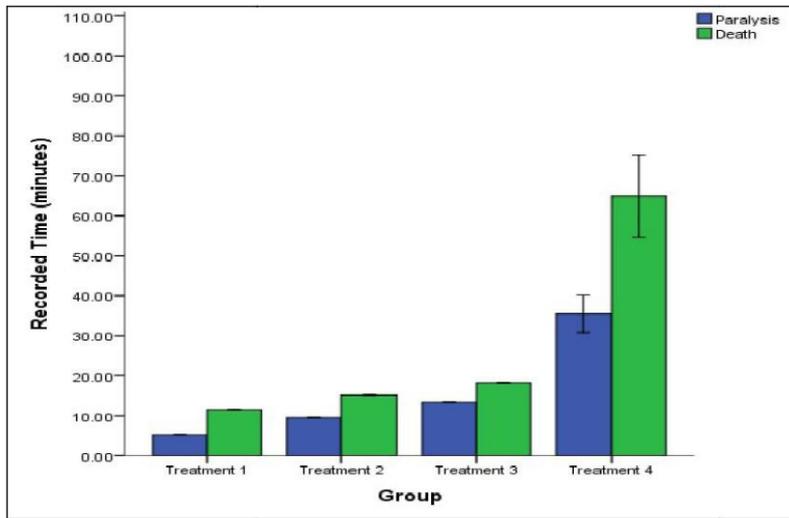
committee indicated proper disposal and handling of invertebrate animal models, such as *A. salina* and widely-distributed *E. eugeniae*, as well as appropriate administration of plant extracts based on recommended concentrations from published references. This toxicity profiling study has not also undergone Food and Drugs Administration (FDA) Philippines' laboratory testing nor received approval of its use as herbal medicine concoction. Though its indigenous use for the treatment of stomachache is known, it could still be toxic at elevated dosages, which may vary from one individual to another.

3.0 Results and Discussion

3.1 Anthelmintic Assay

In this bioassay, the toxic effects of the plant extract were evaluated in terms of the mean time paralysis and death of the earthworms, as shown in Figure 1. An observable, inversely proportional pattern is apparent in the time versus paralysis/death relationship, where the time taken until complete paralysis and death observed are increasing as the CCLE concentration decreases. This result may indicate that CCLE given at a very high level is detrimental to the health and life of the test organism *in vitro* (earthworm).

Soil-transmitted helminthiasis (STH) is among the leading public health concerns causing relentless infections to mankind and is more prominent in developing countries (WHO, 2017). Although synthetic anthelmintics are already commercially available, the reported incidence of severe side-effects and parasite resistance (Drug Office, 2013) associated with these anthelmintic drugs pose alarming threat to the continuous utilization of these compounds.



Legend: T1 (100mg/mL), T2 (75mg/mL), T3 (50mg/mL), and T4 (25mg/mL)

Figure 1. Toxicity of CCLE against earthworms in terms of its paralysis and death time (in minutes). Results presented as mean±SEM. Time taken until paralysis or death are inversely proportional to the level of concentration of CCLE in the test solution.

Table 1. Anthelmintic activity of CCLE-treated groups based on time until paralysis and time recorded until death (mean±SEM)

GROUP	CONCENTRATION (mg/mL)	EARTHWORM (<i>Eudrilus eugeniae</i>)	
		Time taken until paralysis (P) in mins (mean ± SEM)	Time taken until death (D) in mins (mean ± SEM)
CCLE	100	5.20±0.08 ^a	11.41±0.10 ^a
	75	9.56±0.10 ^a	15.15±0.10 ^a
	50	13.29±0.01 ^a	18.15±0.02 ^a
	25	35.46±4.66 ^{b,c}	64.86±10.23 ^{b,c}
Positive Control	20	272.65±8.57 ^{b,d}	336.22±3.89 ^{b,d}
Vehicle Control	-
Solvent Control	-
ANOVA (p≤0.01)	-	.000*	.000*

Different letter superscripts (a,b,c,d) within groups indicate significant difference at p≤0.01.

CCLE = *C. cebuense* leaf extract; Positive Control = Albendazole™; Vehicle Control for PosCon = 2% DMSO; Solvent Control = Triple-Distilled Water

In Table 1, all concentrations of CCLE exhibited significant anthelmintic effects to the earthworms, which generated the mean time paralysis ranging

from 5.20 to 35.46 min and subsequent mean time for death ranging from 11.41 to 64.86 min. The anthelmintic activity of the leaf extract also

shows a concentration-dependent response trend, with faster paralytic effect and shorter death time observed at higher concentrations (75–100mg/mL), while the longer time for paralysis and death noticed at lower concentrations (25–50mg/mL).

These results may show strong toxicity against earthworms at higher concentrations (75–100 mg/mL), while moderate to weak toxicity at lower concentrations (25–50mg/mL). Statistical analysis of the data further revealed that anthelmintic activities of the plant extract at levels of 100, 75, 50, and 25 mg/mL were statistically different from the standard anthelmintic drug, Albendazole™ ($p \leq 0.01$). *C. cebuense* at 50, 75, and 100 mg/mL concentrations were found to be extremely more potent than Albendazole™, whereas 25 mg/mL showed most substantial activity comparable to the standard Albendazole™. Better anthelmintic activity against earthworms and tapeworms *in vitro* was also observed in *Cinnamomum tamala*'s essential oils compared to the standard Piperazine citrate (Akhtar, Iqbal, Khan, & Lateef, 2001). The discrepancy between the effectiveness of these synthetic drugs and cinnamon species may have been due to different target mechanisms. Albendazole™ inhibits tubulin polymerization, reducing energy metabolism and cellular transport in worms, hence, starving and killing it eventually (Vercruysse & Claerebout, 2016); whereas CCLE-treated worms manifest muscle spasms and faster paralysis, suggesting CCLE's target mechanism towards the worm's muscles and nerves.

Meanwhile, both 2% DMSO (used as the vehicle for Albendazole™) and triple distilled water (used as the solvent of plant extract) did not induce paralysis and death towards the earthworms within the observation period. Therefore, it can be inferred that Albendazole™ certainly caused the anthelmintic effects observed in the positive control group, while the toxic compounds present in the leaves of *C. cebuense* caused paralysis and death in the CCLE-treated group.

Cinnamaldehyde, the main component of the essential oil found in the bark and leaves of the cinnamon tree (Wong, Ahmad-Mudzaqqir, & Wan-Nurdiyana, 2014), may have been one of the toxic compounds that elucidated remarkable anthelmintic activity in *C. cebuense*. Previous studies utilizing the leaf extracts of *C. camphora* (Rabiu, Subasish, & Parag, 2011; Singh & Jawaid, 2012), *C. tamala* (Ahmed et al., 2013), and *C. zeylanicum* (Rakhshandehroo, Asadpur, Jafari, & Malekpour, 2016) attributed their significant anthelmintic properties to cinnamaldehyde. Along with cinnamaldehyde, cinnamic acid is also one of the characteristic secondary metabolites in the cinnamic acid pathway that effectively works against goldfish ectoparasite (*Dactylogyrus intermedius*). The presence of other bioactive compounds may have indeed, at least partially, contributed to the toxicity of CCLE towards the earthworms.

According to Ragasa et al., (2013), *C. cebuense* leaves yielded squalene, β -caryophyllene, and a mixture of bauerenol, α -amyirin, and β -amyirin which are sesquiterpenes and triterpenes. In an anthelmintic study using holy basil (*Ocimum sanctum*), the effectiveness of the extract was attributable to the presence of eugenol and β -caryophyllene (Asha, Prashanth, Murali, Padmaja, & Amit, 2001). Bauerenol, α -amyirin, and β -amyirin isolated from *Rotula aquatica* Lour. also exhibit significant anthelmintic activity against earthworms (*Pherethima posthuma*).

In addition to paralysis and death, some morphological changes in the earthworms were observed after several hours of exposure to various concentrations of *C. cebuense* leaf extract, as presented in Figure 2. It was noted further that the severity of these morphological effects magnified at an even more extended time exposure of the earthworms to the different *C. cebuense* concentrations.

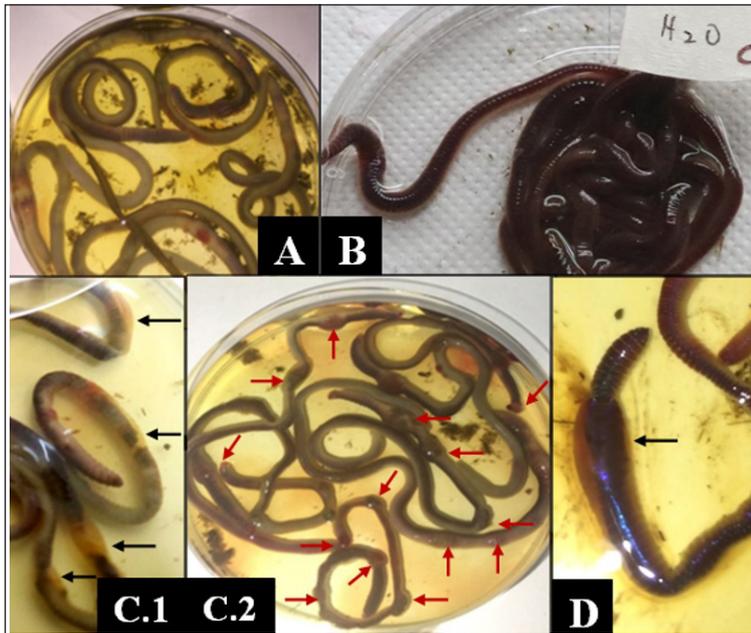


Figure 2. A–D. Morphological changes in the earthworm after exposure to CCLE. (A) The pale body color of earthworms from 100-mg/mL-treated group; (B) Control group (H₂O); (C.1) Localized tissue damage (black arrows) and (C.2) lesions (red arrows) in the body of earthworms from 100-mg/mL-treated group; (D) Slight erosion of the epicuticle from 50-mg/mL-treated group.

In an anthelmintic study conducted by Ling, Jiang, Liu, Li, and Wang (2015), cinnamaldehyde remarkably caused tegumental damage to *D. intermedius*. Accordingly, slight erosion of the epicuticle of the earthworm induced by the CCLE may be related to the strong anthelmintic effect of the cinnamaldehyde. Similar instances were also prominent in other studies, wherein researchers (Martin, Robertson, & Bjorn, 1997; Kundu, Roy, & Lyndem, 2012) suggested that tegument surface was a vital target organ for natural anthelmintic products. Also, *Ascaris suum* exposed to trans-cinnamaldehyde derived from *C. verum* manifested ultrastructural damages, such as localized tissue damage and lesions in the muscular layer, as well as striking damages in the internal digestive tissues

(Williams et al., 2015). These compelling evidence further imply that the morphological damages induced by CCLE to the earthworms in this experiment may have been at least partly due to the anthelmintic efficacy of the cinnamaldehyde.

Nevertheless, it is a limitation of the study as to whether inapparent damages to the morphology of the earthworms have something to do with their death. Therefore, this study warrants further verification and isolation of the toxic compounds responsible for the significant anthelmintic activity of *C. cebuense*. Moreover, the anthelmintic assay was able to disclose the toxicity profile of CCLE against the African night crawlers and observed a significant difference among the different concentrations.

Table 2. Number of brine shrimp nauplii larvae (*Artemia salina*) that survived after treatment with *Cinnamomum cebuense* leaf extract in different concentrations and percent mortality with the median lethal concentration (LC_{50}) value.

Plant Extract	Concentrations (ppm or µg/mL)	Log10 Concentration	Experimental Group			Total no. of live nauplii larvae	Total no. of dead nauplii larvae	Total no. of nauplii larvae samples	% Mortality	LC_{50}
			No. of surviving nauplii larvae after 24 hours							
			R1	R2	R3					
			<i>n</i> =10	<i>n</i> =10	<i>n</i> =10					
Cinnamomum cebuense Leaf Extract (CCLE)	T1	1000	3	0	0	0	30	30	100%	7.050 µg/mL
	T2	100	2	1	0	0	1	29	97%	
	T3	10	1	3	4	3	10	20	67%	
	T4	1	0	10	9	9	28	2	30	

T = Treatment; n = number of samples; no. = number; R = replicate

3.2 Brine Shrimp Lethality Assay

The toxicity of the leaves of *C. cebuense* was investigated in vivo against the brine shrimp (*A. salina*) (Table 2).

Results showed that treatment 1, with the highest concentration (1000 ppm), exhibited the highest mortality rate (100%), whereas treatment 4, with the lowest level (1 ppm), yielded the least mortality rate (7%), revealing further that the toxicity of *C. cebuense* leaf extract (CCLE) follows a linear dose-response trend. Thus, as the concentration/dose of CCLE increases, the percent mortality also increases.

The LC_{50} value, which reads 7.050 µg/mL in Table 2, represents the lethal concentration of *C. cebuense* leaf extract that could kill 50% of the test population within the incubation period. The LC_{50} value was estimated using a probit regression analysis, and this concentration value is considered to possess medium-toxic effect based on Clarkson et al. toxicity criterion (Hamidi et al., 2014).

The median lethal concentration value of *C.*

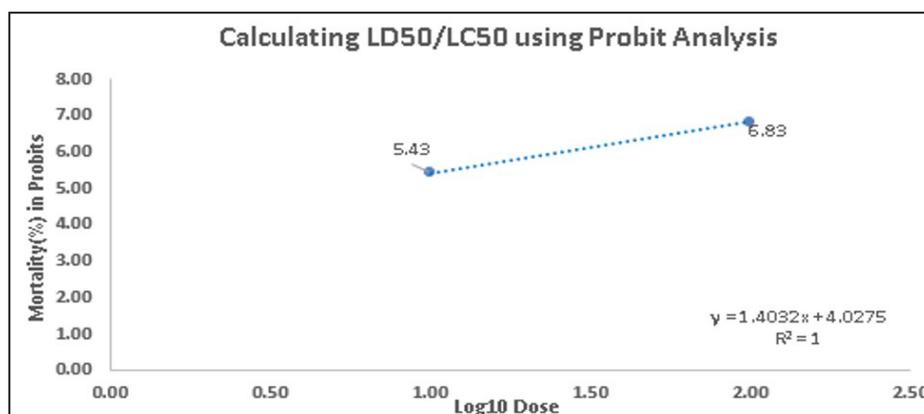
cebuense corroborates with the study of Maridass (2008), which investigated the toxicity of the leaf extracts of eight cinnamon species using brine shrimp lethality assay, namely, *C. travancoricum* (226.5 µg/mL), *C. walaiwarens* (156.0 µg/mL), *C. wightii* (256.4 µg/mL), *C. sulphuratum* (132.4 µg/mL), *C. riparium* (112.3 µg/mL), *C. perrottetii* (189.0 µg/mL), *C. verum* (196 µg/mL), and *C. glaucescens* (136.5 µg/mL). The corresponding numerical values represent their LC_{50} , which are all medium toxic (Maridass, 2008), similar to the toxicity level in *C. cebuense* leaf extract of the present experiment.

Table 3 shows the different control groups used in the experiment. A maximum rate of mortality (100%) was evident in the positive control, i.e., potassium dichromate ($K_2Cr_2O_7$), as this was expected to be a toxic chemical. On the other hand, negative control and nonmanipulated control exhibits 0% mortality rate, which enabled the elimination of other factors that may have contributed to the total number of dead brine shrimp nauplii larvae.

Table 3. The number of brine shrimp nauplii larvae (*A. salina*) that survived in different control groups.

Control Groups	No. of surviving nauplii larvae after 24 hours			Total no. of alive nauplii larvae	Total no. of dead nauplii larvae	Total no. of nauplii larvae samples	% Mortality
	R1	R2	R3				
	n=10	n=10	n=10				
Positive Control	0	0	0	0	30	30	100%
Negative Control	10	10	10	30	0	30	0%
Non Manipulated Control	10	10	10	30	0	30	0%
Vehicle Control	10	10	9	29	1	30	3.33%

R = replicate; no. = number; n = number of sample; positive control = potassium dichromate ($K_2Cr_2O_7$); negative control = distilled water (dH_2O); nonmanipulated control = artificial seawater; and vehicle control = 1.25% dimethyl sulfoxide (DMSO)

**Figure 3.** Percent mortality of brine shrimp nauplii larvae (*Artemia salina*) at 24 hours after being exposed to different concentrations of *Cinnamomum cebuense* leaf extract.

However, the 3.33% mortality observed in the vehicle control (1.25% DMSO) is relatively low and will be disregarded because, in replicates 1 and 2, no dead nauplius was observed. In using Abbott's formula, WHO (2016) also stated that no correction of the test results is needed in cases where the vehicle control's mortality is less than 5%.

In Figure 3, the percent mortality of the brine shrimps was plotted against the log₁₀ of the sample

concentration. The x-axis shows the independent variable (log concentration), while the y-axis shows the dependent variable (mortality rate). The graph depicts a clear linear dose-dependent toxicity trend indicating that the two variables are directly proportional to one another. The R^2 value of 1 or 100% measures the closeness of the data to the regression line (solid black line). The higher the R^2 value, the better the linear model fits with the data.

The slope in the graph's equation, $y = 1.4032x + 4.0275$, represents the steepness of the line and defines a linear relationship between the variables (dose and response). The slope estimates the average rate of change in y (the mortality rate) as x (log concentration) changes. Y-intercept value of 3.591 represents the average mortality rate of brine shrimp, while the slope value (1.661) predicts that, as the concentration increases, the percent mortality increases on the average by 1.661. Nevertheless, the maximum mortality rate of brine shrimp lethality assay is only 100%, hence, at an even higher concentration, 100% of brine shrimp mortality would still be observed.

The observed lethality of the different concentration of CCLE may suggest the presence of potentially toxic phytochemicals. Ragasa et al. (2013) and del Fierro et al. (2012) reported the presence of different phytochemicals such as bauerenol, squalene, humulene, β -caryophyllene, α -amyrin, and β -amyrinin in the leaves of *C. cebuense*, and some of which are toxic at elevated dosages. Ragasa et al. (2013) noted that the higher concentration of bauerenol in a plant extract there is, the greater is the toxicity. This phytochemical has the potential to prevent the metabolism of carbohydrates that can cause a condition where there will be an abnormal deposition of harmful chemicals, such as glycogen, in the different organs of an organism. According to Chudzik, Korzonek-Szlacheta, and Krol (2015), cytotoxic activity of squalene has been observed in many cancer cell lines by inducing apoptosis. Another study by Espineli et al. (2014) suggests that humulene, a sesquiterpene, exhibits cytotoxic activity. These different studies support that the leaves of *C. cebuense* indeed contains toxic phytochemicals responsible for the death of the brine shrimps

nauplii larvae.

Although brine shrimp lethality assay is just a preliminary tool in testing the toxicity of natural plant products, without a doubt, it reveals a significant toxicity profile of CCLE. However, the researchers cannot positively identify which potent toxic phytochemicals are present in the crude extract. Thus, this study urges further investigation of the *C. cebuense* leaves, especially for the toxicity-profiling of each of its bioactive compounds.

4.0 Conclusion

This study successfully elucidated the toxic effects of *Cinnamomum cebuense* leaf extract (CCLE) using the dual invertebrates bioassays approach: brine shrimp lethality assay (BSLA) and anthelmintic assay. Both assays manifested a linear concentration-response trend, suggesting that toxicity and mortality increased with concentration of test solutions. CCLE registered as medium toxic to *Artemia salina* as evidenced by LC_{50} value of $7.050\mu\text{g/mL}$, while its highest concentration manifested higher potency than standard drug, as evidenced by shorter time taken until paralysis and death.

This study no longer delves in screening and identifying bioactive compounds mainly responsible for its toxicity and its potential anthelmintic activity. Also, invertebrate bioassays do not necessarily indicate similar bioactivity among vertebrate models. This study generated relevant information and preliminary data that can be correlated with other biological assays, and hence, serves as a convenient platform for future relevant toxicological studies. In the meantime, additional preclinical studies that would isolate specific compounds and further validate the toxicity profile of CCLE using higher animal models

are needed to establish safer treatment and gain approval of its clinical and medical application.

Acknowledgment

The authors acknowledge the technical support of the following institutions: CNU Biology Department for the laboratory facilities, as well as, the CNU Research Institute of Tropical Biology and Pharmacological Biotechnology, and Dr. Glenn G. Oyong of the DLSU Center for Natural Science and Environmental Research for the technical expertise. We wish to extend our gratitude to Forester Nena Okay for the provision of *C. cebuense* leaf samples from the native tree nursery of Ramon Aboitiz Foundation, Inc. (RAFI) and the anonymous reviewers for their comments and suggestions.

References

- Ahmed, J., Sultana N., Dewan S.M.R., Amin, M.N. & Uddin, S.M.N. (2013). Determination of Chemical Groups and Investigation of Anthelmintic, Cytotoxic, and Antibacterial Activities of Leaves of *Cinnamomum tamala* (Family: Lauraceae). *International Journal of Pharmamedix*, 1, 222–232.
- Ajaiyeoba, E.O., Onocha, P.A. & Olarenwaju, O.T. (2001). In vitro anthelmintic properties of *Buchholzia coriacea* and *Gynandropsis gynandra* extracts. *Pharmaceutical Biology*, 39, 217–220.
- Akhtar, M.S., Iqbal, Z., Khan, M.N. & Lateef, M. (2001). Anthelmintic activity of medicinal plants with particular reference to their use in animals in the Indo–Pakistan subcontinent. *Small Ruminant Research*, 38, 99–107.
- Asha, M.K., Prashanth, D., Murali, B., Padmaja, R., & Amit, A. (2001). Anthelmintic activity of essential oil of *Ocimum sanctum* and eugenol. *Fitoterapia*, 72, 669–70.
- Barceloux, D.G. (2009). Cinnamon (*Cinnamomum* species). *Dis Mon: DM*, 55, 327–335.
- Biswas, S.K., Chowdhury, A., Das, J., Karmakar, U.K., Shill, M.C., & Raihan, S.Z. (2011). Assessment of toxicity, antibacterial activity and phytochemical screening of ethanol extract of *Phyllanthus acidus* L. bark. *Journal of Applied Pharmaceutical Science*, 1, 112–114.
- Brine Shrimp Direct Inc. (2018). Hatching Brine Shrimp Cysts. Retrieved from <https://www.brineshrimpdirect.com/about-us/articles/hatching-brine-shrimp-cysts/>.
- Chudzik, M., Korzonek-Szlacheta, I. & Król, W. (2015). Triterpenes as potentially cytotoxic compounds. *Molecules*, 20, 1610–1625.
- Clarkson, C., Maharaj, V.J., Crouch, N.R., Grace, O.M., Pillay, P., Matsabisa, M.G., Bhagwandin, N., Smith, P.J. & Folb, P.I. (2004). *In vitro* antiplasmodial activity of medicinal plants native to or naturalized in South Africa. *Journal of Ethnopharmacology*, 92, 177–191.
- Del Fierro, R.S., Maquilang, Q.M., Sanjorjo, R.A., Tradio, M.D., Shen, C.C. & Ragasa, C.Y. (2012). Secondary metabolites from *Cinnamomum cebuense*. *Journal of Medicinal Plant Research*, 6, 2146–2149.
- Drug Office. (2013). Oral Anthelmintics. Available

- at: https://www.drugoffice.gov.hk/eps/do/en/consumer/news_informations/dm_19.html.
- Espineli, D.L., Agoo, E.M., del Fierro, R.S., Shen, C.C., & Ragasa, C.Y. (2014). Cytotoxic and antimicrobial compounds from *Cinnamomum cebuense* Kosterm. (Lauraceae). *Pharmaceutical Chemistry Journal*, 48, 598–602.
- Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M. & Van Staden, J. (2004). Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethnopharmacology*, 94, 205–217.
- Finney, D. J., Ed. (1952). Probit Analysis. Cambridge, England, Cambridge University Press.
- Geethaa, S., Thavamany PJ, Chiew SP, and Thong OM. 2013. Interference from ordinarily used solvents in the outcomes of *Artemia salina* lethality test. *J Adv Pharm Tech Res*, 4:179–182.
- Hamidi, M, Jovanova, B. & Panovska, T. (2014). Toxicological evaluation of the plant products using Brine Shrimp (*Artemia salina* L.) model. *Macedonian Pharmaceutical Bulletin*, 60, 9–18.
- Jagota, S.C. (1986). Albendazole, a broad-spectrum anthelmintic, in the treatment of intestinal nematode and cestode infection: a multicenter study in 480 patients. *Clinical Therapeutics*, 8, 226–231.
- Kerala Agricultural University (KAU). (2013). Cinnamon. Retrieved from <http://www.celkau.in/crops/spices/Cinnamon/cinnamon.aspx>.
- Kong, J.O., Lee, S.M., Moon, Y.S., Lee, S.G. & Ahn, Y.J. (2007). Nematicidal activity of cassia and cinnamon oil compounds and related compounds toward *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). *Journal of Nematology*, 39, 31–36.
- Kundu, S., Roy, S.& Lyndem, L.M. (2012). Cassia alata L: potential role as anthelmintic agent against *Hymenolepis diminuta*. *Parasitology Research*, 111, 1187–1192.
- Ling, F., Jiang, C., Liu, G., Li, M. & Wang, G. (2015). Anthelmintic efficacy of cinnamaldehyde and cinnamic acid from cortex cinnamon essential oil against *Dactylogyrus intermedius*. *Parasitology*, 142, 1744–1750.
- Maridass, M. (2008). Evaluation of brine shrimp lethality of *Cinnamomum* species. *Ethnobotany Leaflets*, 12, 772–775.
- Martin, R.J., Robertson, A.P.& Bjorn, H. (1997). Target sites of anthelmintics. *Parasitology*, 114, 111–124.
- McLaughlin, J.L., Rogers, L.L. & Anderson, J.E. (1998). The use of biological assays to evaluate botanicals. *Drug Information Journal*, 32, 513–524.
- Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. & McLaughlin, J.L. (1982). Brine shrimp: a convenient general bioassay

- for active plant constituents. *Planta medica*, 45:31–34.
- Moshi, M.J., Innocent, E., Magadula, J.J., Otieno, D.F., Weisheit, A., Mbabazi, P.K. & Nondo, R.S. (2010). Brine shrimp toxicity of some plants used as traditional medicines in Kagera region, northwestern Tanzania. *Tanzania Journal of Health Research*, 12, 63–67.
- Mounanga, M.B., Mewono, L. & Angone, S.A. (2015). Toxicity studies of medicinal plants used in sub-Saharan Africa. *Journal of Ethnopharmacology*, 174, 618–627.
- Olowa, L.F. & Nuñeza, O.M. (2013). Brine shrimp lethality assay of the ethanolic extracts of three selected species of medicinal plants from Iligan City, Philippines. *International Research Journal of Biological Sciences*, 2:74–77.
- Panda, S.K., Das, D. & Tripathy, N.K. (2011). Evaluation of Anthelmintic activity of *Chlorophytum borivilianum santapau* & *fernandes*. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 2:676–679.
- Pathare, Y.S. & Wagh, V.D. (2012). Herbal medicines and nutritional supplements used in the treatment of glaucoma: A review. *Research Journal of Pharmaceutical, Biological, and Chemical Sciences*, 3, 331–339.
- Patwardhan, V. & Ghaskadbi, S. (2013). Invertebrate alternatives for toxicity testing: hydra stakes its claim. *ATEX*, 2, 69–76.
- PITAHC. (2018). Department of Health - Philippine Institute of Traditional and Alternative Health Care. Retrieved from <http://pitahc.gov.ph/pitahc-remains-strong-as-a-research-institute-for-tcm/>.
- Rabiu, H., Subasish, M. & Parag, G.H. (2011). Investigation of in vitro anthelmintic activity of *Cinnamomum camphor* leaves. *International Journal of Drug Development Research*, 3:295–300.
- Ragasa, C.Y., Espineli, D.L., Agoo, E., Del Fierro, R.S., Don, M.J. & Shen, C.C. (2013). Chemical constituents of *Cinnamomum cebuense*. *Chinese Journal of Natural Medicine*, 11, 264–268.
- Rakhshandehroo, E., Asadpour, M., Jafari, A. & Malekpour, S.H. (2016). The effectiveness of *Cinnamomum zeylanicum*, *Punica granatum* flower, and *Capsicum annum* extract against *Parascaris equorum* infective larvae. *Journal of the Faculty of Veterinary Medicine - Istanbul University*, 42, 132–137.
- Research Information Series on Ecosystems [RISE] (1992). Distribution and Site Requirements of Some Reforestation Species. Special Issue No. 4. ERDB, College, Laguna 12 pp.
- Sarah, Q.S., Anny, F.C. & Misbahuddin, M. (2017). Brine shrimp lethality assay. *Bangladesh Journal of Pharmacology*, 12, 5-2017.
- Schultz, E. & Joutti, A. (2007). Arsenic Ecotoxicity in Soils, Geological Survey of Finland. Miscellaneous Publications, 53 p.

- Singh, R. & Jawaid, T. (2012). *Cinnamomum camphora* (Kapur). *Pharmacognosy Journal*, 4, 1–5.
- U.S. Food and Drug Administration (USFDA). (2018). The Drug Development Process – Step 2: Preclinical Research. Retrieved from: <https://www.fda.gov/ForPatients/Approvals/Drugs/ucm405658.htm>.
- Vercruyse, J. & Claerebout, E. (2016). Mechanisms of Action of Anthelmintics. Retrieved from: <http://www.merckvetmanual.com/pharmacology/anthelmintics/mechanisms-of-action-of-anthelmintics>.
- Williams, A.R., Ramsay, A., Hansen, T.V., Ropiak, H.M., Mejer, H., Nejsun, P., Mueller-Harvey, I. & Thamsborg, S.M. (2015). Anthelmintic activity of trans-cinnamaldehyde and A-and B-type proanthocyanidins derived from cinnamon (*Cinnamomum verum*). *Scientific Reports*, 5, 14791.
- Wong, Y.C., Ahmad-Mudzaqqir, M.Y. & Wan-Nurdiyana, W.A. (2014). Extraction of essential oil from cinnamon (*Cinnamomum zeylanicum*). *Oriental Journal of Chemistry*, 30, 37–47.
- World Health Organization. (1997). Working Group on Herbal Medicines. Western Pacific Regional Office, Manila, Philippines. 206 pp. Retrieved from <http://www.who.int/iris/handle/10665/207932>.
- World Health Organization. (2016). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes 2nd ed. Geneva, Switzerland: WHO Document Production Services.
- World Health Organization. (2017). Soil-transmitted helminth infections. Retrieved from <http://www.who.int/mediacentre/factsheets/fs366/en/>.