Asian Science and Technology Innovation Journal

ASTIJ

Research Article

Phytochemical analysis and insecticidal potential of *Tinospora crispa* (L.) Hook. f. & Thomson stem extract using Madagascar hissing cockroach

Gelster C. Paquibo[1*] and Lorelie Gloria A. Samaniego[1]

[1]Animal Biology Division, Institute of Biological Sciences, College of Arts and Sciences, Central Mindanao University, Maramag, Bukidnon, Philippines.
*Correspondence: gelspaquibo@gmail.com

Citation: Paquibo G C, Samaniego L G A. *Phytochemical analysis and insecticidal potential of Tinospora crispa (L.) Hook. f. & Thomson stem extract using Madagascar hissing cockroach.* ASTIJ. 2025; **1**(1): 24-35

Received: June 11, 2025
Revised: June 24, 2025
Accepted: June 24, 2025
Published: June 27, 2025

Abstract: The undesirable effects of repeated use of synthetic insecticides had led to the conceptualization of this study which aimed to evaluate the potential of *Tinospora crispa* (L.) Hook f. & Thomson (Panyawan) stem extract as insecticidal agent using Madagascar hissing cockroach as model. Thirty-six (36) female and thirty-six (36) male cockroaches were used in the study. Ethanol was used as extracting solvent, and the extract was concentrated following standard procedure. The crude extract was subjected to qualitative and quantitative phytochemical analysis. From the fresh extract, four concentrations (5%, 25%, 45%, and 65%) were prepared for the insecticidal assay. Results showed that *T. crispa* extract contains flavonoids, alkaloids, saponins, phenolics and tannins. In addition, the concentrations of *T. crispa* extract used had caused 100% mortality after 21 hr and 24 min of observation for females and 17 hr and 36 min for males. The plant extract showed significant insecticidal potential at 0.05 level of significance. All the concentrations used showed insecticidal activity; however, the effect of the plant extract is concentration dependent. The knockdown time of the cockroaches significantly decreased as the concentration of the extract is increased. From the results, it can be inferred that *T. crispa* stem extract has an insecticidal effect against Madagascar hissing cockroach.

Keywords: *Tinospora crispa*; Phytochemical analysis; insecticide; Madagascar hissing cockroach

1. Introduction

The repeated use of synthetic insecticides for insect pests and vectors control has disrupted natural biological control systems. It has also resulted in the development of resistance, undesirable effects on non-target organisms and fostered environmental and human health concern. Hence, a search for alternative control measures has been initiated [1 & 2]. Natural insecticides such as pyrethrum, nicotine and rotenone, have been extensively used until recently for insect control [3]. It has been reported that essential oils of leaf and bark of some plants demonstrated high larvicidal and insecticidal activity against insect pests [4].

Insects are very important as primary or secondary decomposers. Without insects to help break down and dispose of wastes, dead animals and plants would accumulate in our environment. However, not all insects are beneficial to human; they can be harmful [5]. Cockroaches spread filth and destroy fabrics, food, and book-bindings. They release portions of their partially digested food at intervals and drop excretions. They also expel a nauseous secretion from both their mouth and skin through gland openings which gives an enduring, aggressive smell to food or in any area they visited [6].

Gromphadorhina portentosa, commonly known as Madagascar hissing cockroach belongs to the family Blaberidae. Unlike many cockroach species, Madagascar hissing cockroaches do not possess wings which makes them flightless roach [7]. Its population is widely spread throughout the Philippines which is mostly found in dry lowland forests. Some are kept and cultured by exotic animal collectors for feeders. These are also used as alternative non-mammalian test organisms in research.

Tinospora crispa (L.) Hook f. & Thomson, commonly known as Panyawan, is a claimed medicinal plant under the family of Menispermaceae or the moonseed family. It is used in traditional medicine to treat numerous health conditions such as jaundice, rheumatism, urinary disorders, fever, malaria, diabetes, internal inflammation, fracture, scabies, and hypertension. It was proven that *Tinospora crispa*

stem extract has an insecticidal effect against *Macrotermes gilvus* [8] and it also exhibits insecticidal and pesticidal potential against rice weevil [9]. *T. crispa* contains high flavonoids which is responsible in suppressing cell and promotes apoptosis which is essential for a plant to be an effective insecticide [10 & 11].

There is a need to find an alternative, organic, biodegradable and affordable insecticides derived from plant products that are easy to utilize, effective, and safe to the environment as well as to human health. Hence, this study explored the potential of *T. crispa* as a natural insecticide using *G. portentosa* as a model. The null hypothesis that guided this study was "*Tinospora crispa* stem extract has no insecticidal effect on Madagascar hissing cockroach".

2. Objectives

Generally, the study aimed to evaluate the potential of *Tinospora crispa* (L.) Hook f. & Thomson (Panyawan) stem extract as an insecticidal agent using Madagascar hissing cockroach (*Gromphardorhina portentosa*) by determining the secondary metabolites present in *Tinospora crispa* (Panyawan) stem extract, the knock-down time of Madagascar Hissing Cockroach exposed to different concentration of *T. crispa* stem extract, and identifying the concentration of *T. crispa* stem extract that has an insecticidal effect on Madagascar Hissing Cockroach.

3. Methodology

3.1 Place of the Study

The insecticidal assay was conducted at the researcher's residence in Agusan Pequeño, Butuan City, Agusan del Norte. The qualitative and quantitative phytochemical analysis experiment was conducted by a research assistant at the Natural Product Research and Development Center (NPRDC) in Central Mindanao University, University Town, Musuan, Maramag, Bukidnon.

3.2 Collection and Identification of Plant Sample

Ten (10) kilograms of *Tinospora crispa* (L.) stem (Fig. 1) were collected from the researcher's garden located in P-Maabi-abihon, Agusan Pequeno, Butuan City. The collected samples were washed with tap water to remove dirt, then with distilled water. Excess water was removed by wiping the samples with clean tissue paper. Representative vegetative and reproductive organs of the plant were brought to the Central Mindanao University Museum for confirmation of the identity of the plant.

Figure 1. Collected stem plant samples.

3.3 Preparation of Plant Samples

Five (5) kilograms from the harvested samples were chopped/sliced thinly (Fig.2A) for air-drying. The sliced samples were evenly spread in winnowing trays (Fig. 2B). During the day, the winnowing trays

were hung in a well-ventilated area where there is no direct sunlight exposure. At night, winnowing trays were kept and covered with clean cloth. Drying was done for two (2) weeks. When samples were brittle and moisture-free, these were packed in a sealed zip lock to be used for phytochemical analysis.

Figure 2. Preparation of plant samples. A-Freshly chopped stem of *T. crispa*; B-Air-drying of chopped stem samples.

3.4 Plant Extraction for Phytochemical Analysis

Plant extraction was conducted at NPRDC by a researcher assistant following the procedures of Zhang et al. (2018) [12] and Abubakar (2020) [13]. The air-dried stem samples were pulverized using an electric grinder. The powdered samples were placed in a clean glass jar with lid and soaked in 95% ethanol for twenty-four (24) hours, and then filtered using a filter paper. Filtrate was placed in a beaker with magnetic stirrer and then placed in a magnetic plate. The magnetic plate was set with a stirring speed of 250 to 1000 rpm, temperature below 78.5°C (below boiling point of ethanol) and with an extraction time of thirty (30) minutes to one (1) hour until crude extract was produced. Crude extracts were placed in a sealed container and refrigerated until used.

3.5 Qualitative Phytochemical Analysis

The phytochemical screening was conducted according to the standard procedure for the qualitative determination of major phytochemical constituents, including flavonoids, alkaloids, saponins, phenolics, tannins, and glycosides.

3.5.1 Test for Flavonoids: Alkaline Reagent Test

A two (2) ml of 2.0% NaOH mixture was mixed with the aqueous plant crude extract. When added with two (2) drops of diluted acid to mixture, formation of pale yellow to colorless solution showed the presence of flavonoids [14].

3.5.2 Test for Alkaloids: Dragendorff's Test

Two (2) ml of stem extract was added with one (1) ml of Dragendorff's reagent along the side of the test tube. Formation of orange or reddish-orange brown precipitate indicated the presence of alkaloids [15].

3.5.3 Test for Saponins: Froth/Foam Test

Five (5) mL of the extract was placed in a test tube and was shaken well for five minutes. Formation of stable foam or persistent froth for ten (10) minutes indicated the presence of saponins [15].

3.5.4 Test for Phenolics and Tannins: Ferric Chloride Test

This detection was based on black-green, brownish green, blue-green, violet, purple, or red-brown solution or precipitate formation by adding few drops of 5% ferric chloride solution to 2 mL of the extract solution [14, 16, & 17].

3.5.5 Test for Glycosides: Keller-Kiliani Test

A solution of glacial acetic acid (4.0 ml) with 1 drop of 2.0% FeCl3 mixture was mixed with the 10 ml aqueous plant extract and 1 ml H2SO4 concentrated. A brown ring formed between the layers which showed the entity of cardiac steroidal glycosides [14 & 16].

3.6 Quantitative Phytochemical Analysis

3.6.1 Total Phenolic content

The amount of phenol in the aqueous extract was determined by Folin-Ciocalteu reagent method. Two and a half (2.5) ml of 10% Folin-Ciocalteu reagent and two (2) ml of 2% solution of Na2CO3 was added to 1ml of plant extract. The resulting mixture was incubated for 15 minutes at room temperature. The absorbance of the sample was measured at 765nm. Gallic acid was used as standard (1mg/ml). All the tests were performed in triplicates. The results were determined from the standard curve and were expressed as gallic acid equivalent (mg/g of extracted compound) [18].

3.6.2 Total Flavonoid content

Aluminum chloride colorimetric method was used to determine flavonoid content. One (1) ml of sample plant extract (made from one (1) gram of crude extract dissolved in a one (1) ml distilled water) was mixed with three (3) ml of methanol, 0.2ml of 10% aluminum chloride, 0.2ml of 1M potassium acetate and 5.6ml of distilled water and remains set at room temperature for thirty (30) minutes. The absorbance was measured at 420nm. Quercetin was used as standard (1mg/ml). All the tests were performed in triplicates. Flavonoid contents were determined from the standard curve and were expressed as quercetin equivalent (mg/g of extracted compound) [19].

3.7 Preparation of Plant Extract for Insecticidal Assay

Five (5) kilograms of fresh samples were sliced at least one 1cm or less (Fig.3-A). The juice was extracted using an electric juicer (Fig. 3-B). Extracted juice was filtered using a filter paper to absolutely separate solid substance from the filtrate.

Figure 3. Preparation of plant extract for insecticidal assay. A – Sliced *T. crispa* stem; B – Juice extraction using electric juicer.

Using the freshly extracted samples, four (4) different concentrations were prepared: 5%, 25%, 45%, and 65% (Fig. 7) [20]. Fifty (50) ml solution of each treatment were prepared following the proportion below:

T1 – 5% concentration (2.5 ml fresh extract + 47.5 ml distilled water)
T2 – 25% concentration (12.5 ml fresh extract + 37.5 ml distilled water)
T3 – 45% concentration (22.5 ml fresh extract + 27.5 ml distilled water)
T4 – 65% concentration (32.5 ml fresh extract + 17.5 ml distilled water)

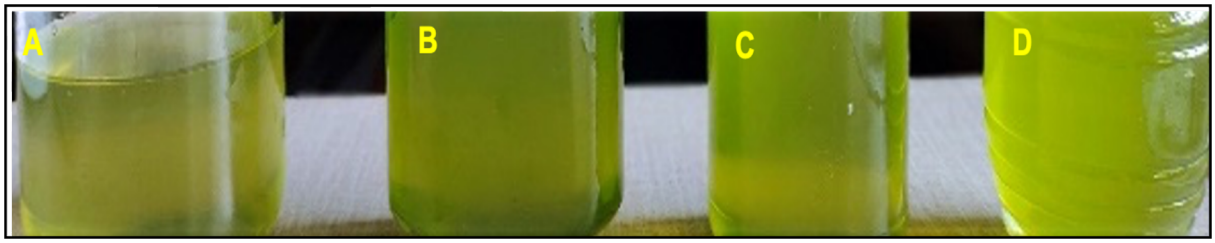


Figure 4. Treatment concentrations. A-5%; B-25%; C-45%; D-65%.

3.8 Preparation of Madsagascar Hissing Cockroach for Insecticidal Assay

G. portentosa were obtained from an authorized exotic animal seller from Iligan City. Thirty-six (36) matured male and thirty-six (36) matured female hissing cockroaches (Fig. 5) were purchased and brought to Butuan City.

Figure 5. Mature male and female Madagascar Hissing Cockroach used in the study.

3.9 Acclimatization

Acclimatization was done for seven (7) days. The purchased cockroaches were placed into a clean well-ventilated round plastic container with cover. During the acclimatization period, five (5) grams of rodent feeds softened with five (5) distilled water were fed daily every 9:00 o'clock in the evening (Fig. 6).

Figure 6. Acclimatization

3.10 Sex Identification of Madagascar Hissing Cockroach

The sex of the cockroach was identified by observing the head. Male cockroach has two (2) large tubercles or bumps on the dorsal surface of the prothorax (Fig. 7A) while female cockroach lacks the bump (Fig. 7B)

3.11 Experimental Procedure

After acclimatization, on the 8th day, male and female cockroaches were randomly placed into separate plastic containers. Each container was properly labelled with the treatment number and replicate number. Assignment of treatment numbers and replicate numbers to the containers was done randomly by drawing lots. Six (6) treatments, with six (6) replicates each, were prepared. The treatments were as follows:

To+ - positive control (Greenleaf Powder Cockroach Killing Bait solution)
To- - negative control (distilled water)
T1 - 5% fresh extract
T2 - 25% fresh extract
T3 - 45% fresh extract
T4 - 65% fresh extract

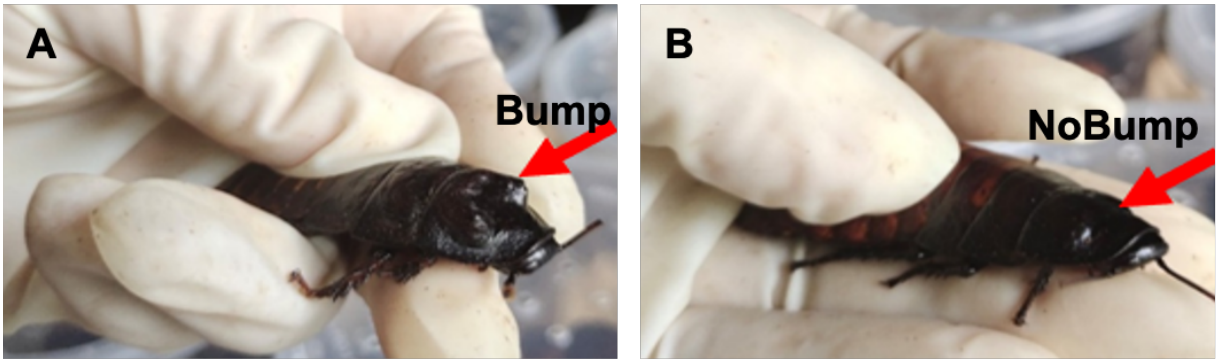


Figure 7. Madagascar Hissing Cockroach sex identification. A – Male with two (2) bumps; B – Female with no bump.

Each roach was given a daily ration of five (5) grams of rodent feed wet with five (5) ml of the assigned treatment concentration to soften the feed. The feeds were given at night since roaches are nocturnal. To determine the daily amount of feed consumed by the cockroaches, the left-over feed was weighed and subtracted from the initial feed given.

3.12 Knock-down Time Determination

From the start of feeding (Fig. 8), the cockroaches were observed every ten (10) minutes until 50% and 100% mortality were achieved [21]. The time of mortality of the cockroaches was recorded. A cockroach was concluded dead if the cockroach does not regain consciousness within 1-2 minutes of probing with a clean forceps [6].

3.13 Statistical Analysis

The study used One Way Analysis of Variance (ANOVA) to determine whether there is a significant difference in the mortality rate of cockroaches when exposed to different *Tinospora crispa* stem extract concentrations, negative control and positive control. To confirm the differences that occur between groups, Homogeneous Subsets derived from Tukey's Honest Significant Difference were used. This was referred to and done by a statistician, Mr. Neal V. Quizon of the Mathematics Department, College of Arts and Sciences, Central Mindanao University.

Figure 8. Feeding of cockroaches.

3.14 Documentation

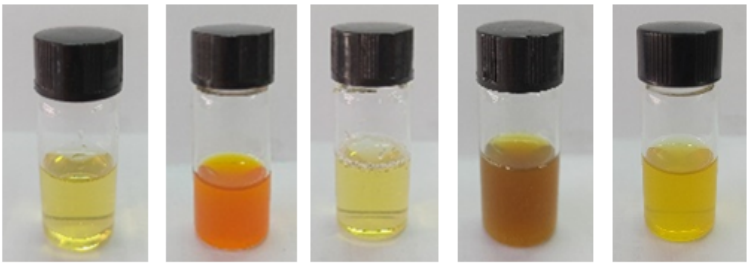
Photographs were used to document the activities done during the study. Data gathered was recorded in a record book for future reference and analysis.

4. Results and discussion

4.1 Phytochemical Analysis

Tinospora crispa contains chemical components as shown after the phytochemical screening was done (Appendix B). The qualitative analysis results are summarized in Table 1, whereas the quantitative analysis for total phenolic and flavonoid contents is presented in Table 2.

Table 1. Phytochemicals present in *Tinospora crispa* stem extract.

Plant Sample	Flavonoids	Alkaloids	Saponins	Phenolics & Tannins	Glycosides
***Tinospora crispa* stem**	+	+	+	+	-
					

Note: (+) Not detected (-) Present

Among the various tests done, *Tinospora crispa* stem extract showed positive result including flavonoids, alkaloids, saponins, phenolics and tannins. Glycosides were not detected in the sample extract (Table 1). Quantitative determination showed that the total phenolic and flavonoid content of the stem extract is relatively low (1.70 + 0.0222 mg GAE/g DW sample and 0.40 + 0.0031 mg QE/g sample, respectively) (Table 2). According to Gutierrez (2016), phytochemicals found in plants provide various biological effects such as being medicinal, insecticidal, antimicrobial, repellent, larvicide and many others. In the study of Palma (2017) [22] together with his colleagues, they stated that flavonoids are one of the major components in development of synthetic pesticides. *T. crispa* extract contains flavonoids, which maybe one of the factors for the insecticidal potential of this plant. As reported, flavonoids inhibit enzymatic activity, which prevents the growth of larvae of different insect species and some flavonoids interfere with the process of moulting. Flavonoids also inhibit the formation of juvenile hormone (ecdysone) and inhibit transcription of ecdysone receptor-dependent genes which is essential for initiating the larval to adult transition. The study of Khanna and Kannabiran (2007) [23] also revealed that alkaloids and tannins (also detected in this study) demonstrated pesticidal activity and are potential compounds to kill mosquito larvae. Lee (2000) [24] also stated in his study that tannins can be used as insecticide against *Culex quinquefasciatus* larvae.

Table 2. Quantitative phytochemicals analysis *Tinospora crispa* stem extract.

Plant Sample	Parameters	Results
Tinospora crispa stem	Total Phenolic Content	1.70 ± 0.022 mg GAE/g DW sample
	Total Flavonoild Content	0.40 ± 0.031 mg GAE/g DW sample

Note: Values were performed in triplicates and represented as mean ± SD

4.2 Knock-down time of Madagascar Hissing Cockroach

Tables 3 and 4 and Figures 9 and 10 display the average knockdown time for the female and male Madagascar hissing cockroach.

Table 3. Average Knock-down time (50% and 100% mortality) of female Madagascar hissing cockroach given with different concentration of *Tinospora crispa* stem extract.

Treatment (n = 6)	Average Knock-down time (50% mortality)	Average Knock-down time (100% mortality)
To^- (Negative control)	0	0 [a]
To^+ (Positive control)	7 hrs and 18 mins	10 hrs and 42 mins [d]
T_1 (5% stem extract)	20 hrs and 36 mins	21 hrs and 24 mins [b]
T_2 (25% stem extract)	19 hrs and 36 mins	20 hrs and 18 mins [b]
T_3 (45% stem extract)	17 hrs and 48 mins	19 hrs and 30 mins [b]
T_4 (65% stem extract)	10 hrs and 48 mins	12 hrs and 15 mins [c]

Note: Means with different letters are significantly different at $P < 0.05$

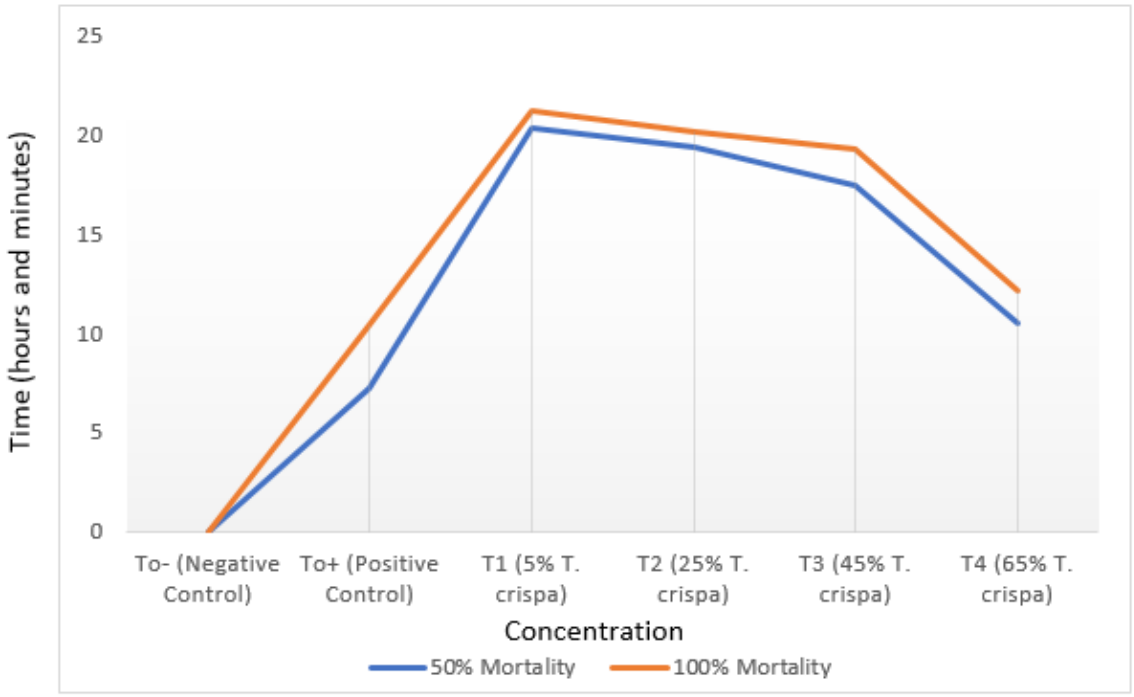


Figure 9. Average Knock-down time (50% and 100% mortality) of female Madagascar hissing cockroach given with different concentration of *Tinospora crispa* stem extract.

Table 4. Average Knock-down time (50% and 100% mortality) of male Madagascar hissing cockroach given with different concentration of *Tinospora crispa* stem extract.

Treatment (n = 6)	Average Knock-down time (50% mortality)	Average Knock-down time (100% mortality)
To^- (Negative control)	0	0 [a]
To^+ (Positive control)	6 hrs and 24 mins	7 hrs and 36 mins [d]
T_1 (5% stem extract)	16 hrs and 42 mins	17 hrs and 36 mins [b]
T_2 (25% stem extract)	15 hrs and 6 mins	16 hrs and 24 mins [c]
T_3 (45% stem extract)	12 hrs and 42 mins	13 hrs and 48 mins [c]
T_4 (65% stem extract)	8 hrs and 42 mins	10 hrs and 30 mins [d]

Note: Means with different letters are significantly different at $P < 0.05$

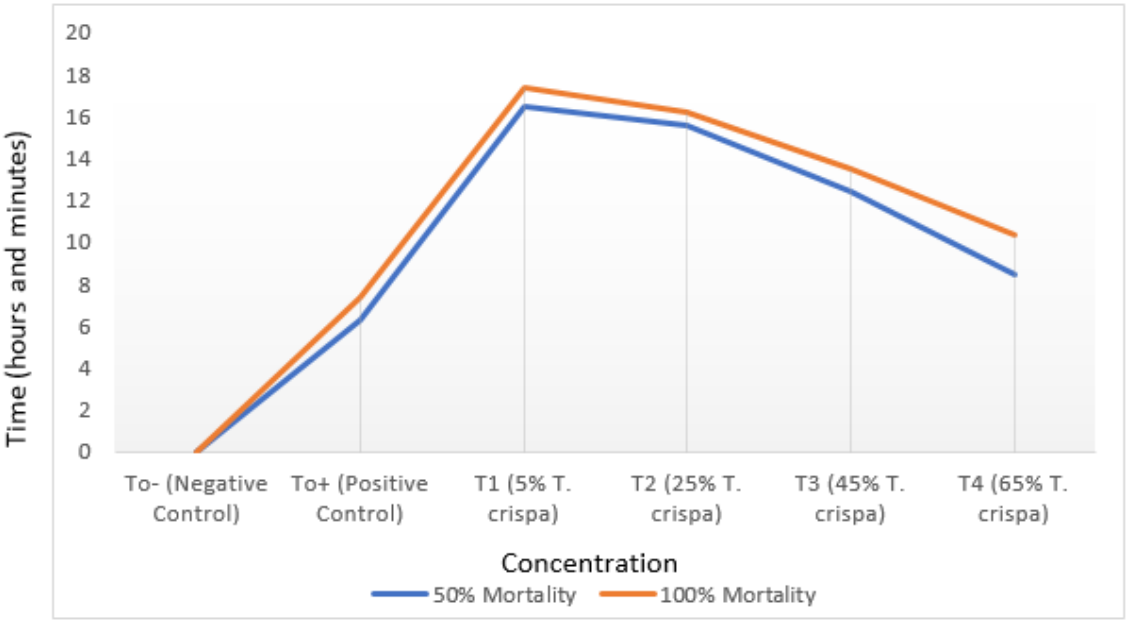


Figure 10. Average Knock-down time (50% and 100% mortality) of male Madagascar hissing cockroach given with different concentration of *Tinospora crispa* stem extract.

For both sexes, a similar trend was observed. Shortest knock-down time for 50% and 100% mortality is noted in the positive control group (To+). For the treated groups, a decreasing trend can be seen in the knock-down time as the concentration of the extract given to the roaches increases (Fig. 9 and 10). The differences in the knock-down time is significantly different. Similar to the study of Pregoner and his colleagues (2019) [20] who used *Periplaneta americana*, the results showed that the highest concentration of *Tinospora crispa* had the fastest average knock-down time. In addition, Gutierrez (2016) [9] stated that the highest concentration of *Tinospora crispa* proved to be the most effective in protecting grains from rice weevils. In Abdullah's study (2012) [8], the mortality of both *Macrotermes gilvus* soldiers and workers increased as *T. crispa* extract also increases. Probably, the higher the concentration of the treatment used; the more secondary metabolites are present. Hence, better results (i.e. less knock-down time) was achieved.

Table 5 showed that female cockroaches had a longer knock-down time average compared to the male cockroaches. This implies that female cockroaches were more resistant to the given treatments compared to male cockroaches. The difference of knock-down time average between sexes is significantly different.

Table 5. Average Knock-down time (50% and 100% mortality) of female and male Madagascar hissing cockroach treated with *Tinospora crispa* stem extract (n=36).

Gender	Knock-down time average
Female	16 hrs and 49 mins [a]
Male	13 hrs and 12 mins [b]

Note: Means with different letters are significantly different at $P < 0.05$

Cha et al. (1970) [25] reported the same results that female cockroach was more resistant than male when exposed to various insecticides. Hostetler & Brenner (1994) [26] also revealed that female cockroaches were more behaviorally and physiologically resistant to insecticides than male. Accord-

ingly, this result was probably due to the female cockroach's larger body size, which is generally attributed to more body fat than that of the male cockroach. In fat body cells, lipids, carbohydrates and proteins are the substrates and products of many pathways that can be used for energy production and determine the survival of an insect. The fat body is the main tissue responsible for innate and acquired humoral immunity of insects (Skowronek, 2021) [27]. Probably, these are the reasons why the female cockroaches which were relatively larger than the male are more resistant to the treatment concentration than the male cockroaches.

5. Conclusions

Based on the results, the following are inferred from the study.

5.1 *Tinospora crispa* contains secondary metabolites that have potential insecticidal activity.

5.2 The highest concentration of *T. crispa* stem extract had the shortest knock-down time in both female and male Madagascar hissing cockroaches; and

5.3 The insecticidal effect of *T. crispa* stem extract on the Madagascar hissing cockroach is concentration-dependent.

The results of the study reject the null hypothesis which is "*Tinospra crispa* stem extract has no insecticidal effect on Madagascar hissing cockroach".

6. Recommendations

To the future researchers, the following recommendations are suggested:

6.1 isolation of bioactive compounds found in *T. crispa* stem extract should be done.

6.2 conduct an insecticidal experiment using larger population; and

6.3 conduct a phytochemical analysis using different extracting solvents.

7. Patents

Author Contributions: G.C.P. conceptualized and conducted the study, performed data curation, formal analysis, and prepared the original draft. L.G.A.S. supervised the research and provided critical review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Department of Science and Technology – Science Education Institute (DOST-SEI) through the provision of a thesis allowance under the scholarship grant. No additional external funding was received.

Acknowledgments: The author gratefully acknowledges the invaluable guidance of Dr. Lorelie Gloria A. Samaniego and the support of the Thesis Advisory Committee—Dr. Aprille Joy M. Luceño-Tenizo and Ms. Felisa Jyl C. Marfil—as well as the Institute of Biological Sciences and the Natural Product Research and Development Center (NPRDC) of Central Mindanao University. Sincere thanks are also extended to Mr. Neal V. Quizon for his assistance with the statistical analysis, to the Department of Science and Technology (DOST) for the scholarship grant, and to the author's family and friends for their unwavering encouragement and support throughout the study.

Conflicts of Interest: The authors declare that they have no known financial or personal conflicts of interest that could have influenced the work reported in this paper.

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