Journal on New Biological Reports
JNBR 10 (2) 64 – 71 (2021)

ISSN 2319 – 1104 (Online)

Published by
www.researchtrend.net

Molluscicidal activity of Makabuhay (*Tinospora rumphii* Boerl) stem ethanolic extract against *Radix* (*Lymnaea*) spp. snails

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| **Received**: 18 June 2021 | **Accepted**: 23 August 2021 | **Published Online**: 27 August 2021 |

How to cite Esquilla MG, Sanchez CB, Tenorio JCB. 2021. Molluscicidal activity of Makabuhay (*Tinospora rumphii* Boerl) stem ethanolic extract against *Radix* (*Lymnaea*) spp. snails. J New Biol Rep 10 (2): 64 – 71.

ABSTRACT

Lymnaeid snail infestation and snail-borne parasitic diseases, such as fasciolosis, remain a problem in the livestock industry in the Philippines. Effective molluscicides are thus needed. However, the routine application of commercially available molluscicides may cause unwanted environmental contamination. Natural alternatives to commercially available molluscicides are called for, thus, this study investigated the molluscicidal activity and efficacy of makabuhay (*T. rumphii*) ethanolic stem extract against lymnaeid snails. A total of 150 mature snails from Barangay Guiamalia, Esperanza, Sultan Kudarat were subjected to immersion bioassay with *T. rumphii* ethanolic extracts. There were five treatment groups: Treatment A or negative control (100% tap water), Treatment B (5 000 ppm), Treatment C (7 500 ppm), Treatment D (10 000 ppm) and Treatment E or positive control (10 000 ppm Surekill® 70WP). Snail death was measured 24-, 48- and 72- hours post-exposure. The results revealed a 100% treatment-specific mortality in all *T. rumphii* extract concentrations, and in the positive control at 24-hours post-treatment. Treatments B, C, D and E had an excellent efficacy of 96.67%. These results show that *T. rumphii* ethanolic extracts at 5 000 ppm, 7 500 ppm and 10 000 ppm concentrations are effective natural molluscicides against lymnaeid snails. The promising results suggest that *T. rumphii* stem ethanolic extract may be an excellent natural, inexpensive alternative to commercially available molluscicides.

Key words: Makabuhay, Panyawan, *Tinospora rumphii*, Natural Molluscicide, *Radix* spp., Molluscicide efficacy.

INTRODUCTION

Lymnaeid snail (*Radix* spp.) infestation is a burden to farmers that are engaged with livestock production. The snail serves as an intermediate host of *Fasciola hepatica* and *F. gigantica* in the Philippines (Tenorio & Molina 2021). Fasciolosis is parasitic disease that may cause grave economic losses to cattle, water buffalo, sheep, and goat farmers in the country (Molina et al. 2005; Portugaliza et al. 2019). With the presence of such diseases, animal condition will be affected and production will decline (Molina et al. 2005; Mazeri et al. 2017). In the veterinary practice, taking out the lymnaeid snail is an effective form of disease prevention which lead to the economic benefit of more productive animals. There are several methods to eliminate snail population and one method of choice is application of commercially available molluscicides. However, according to Coelho & Caldeira (2016) these products have multiple lethal effects on other organisms and, aside from being expensive, they require frequent applications to reduce snail population. In the Philippines, control of lymnaeid snails using indigenous plants is an option that farmers can adapt. Thus, these native plants must be studied for their potential molluscicidal activity against snail vectors of economically important veterinary diseases.

Makabuhay or panyawan (*Tinospora rumphii* Boerl*)* is a creeping plant known for its medicinal uses. It is a climber plant belonging to the family Menispermaceae (Salvaña et al. 2020, Dapar 2020). *Tinosphora rumphii* is a native plant and is distributed throughout the Philippines. The plant contains many bitter principles such as columbine, picroretine, berberine, and diterpenes (Hanthanong et al. 2021). It has glucoside and traces of alkaloids (Galia & Galia 2016, Stuart 2019). The plant has been evaluated for its antiparasitic action against gastrointestinal helminths (Fernandez 1997; Ramada et al. 2018). It has also been studied for its potential to control head lice (Torre et al. 2017). Its molluscicidal activity has also been studied in various species of snails in the Philippines. It is among the twenty-three plants that exerted a 100% mortality rate to golden apple snails (*Pomacea canaliculata*) at a concentration below 10, 000 ppm (Rejesus & Punzalan 1995). Hence, Dela Cruz et al. (2000), recommend the use of *T. rumphii* in land preparation as one of the management options for controlling *P. canaliculata*. Makabuhay is abundant in various metabolites and phytochemicals (Chi et al. 2016). Among these are alkaloids (i.e., tannins, saponins, terpenoids and flavonoids) that exhibit potential for molluscicidal effect on lymnaeid snails of the *Radix* (*Lymnaea*) spp. (Chi et al. 2016).

This study introduces the molluscicidal activity of makabuhay ethanolic stem extract against lymnaeid snails. It aimed to investigate the molluscicidal activity and efficacy of *T. rumphii* stem ethanolic extract against *Radix* (*Lymnaea*) spp. snails. Specifically, the study intended to determine the following: the treatment-specific mortality (TSM) among lymnaeid snails treated with various concentrations of *T. rumphii* stem extract 24h post-exposure, the residual activity of the makabuhay extracts by determining the TSM at 72 hours post-exposure, and the molluscicidal efficacy of various concentrations of makabuhay stem extract against lymnaeid snails after 24 hrs exposure.

MATERIALS AND METHODS

Snail Collection and Storage

A total of 150 adult lymnaeid snails were collected from ponds in Barangay Guiamalia, Esperanza, Sultan Kudarat (6.6989° N, 124.5449° E) using a clean bucket with natural water from the snails' habitat (Fig. 1). Collection of all test snails were done within one day, four days prior to the immersion bioassay Snail species were identified based on geographic distribution, shell length, shell color, shell aperture and whorl pattern. Morphological descriptions provided by Monzon et al. (1993) and Martin & Cabrera (2018) were used to identify snails to the genus level – *Radix* (*Lymnea*) spp.

Test snails were stored in a basin with aged tap water and stones (Fig. 1B). The basin was placed in a shaded open quarter without fluorescent lights to attain a room temperature with 12-hour light and 12-hour dark photoperiod. Test snails were fed with partially boiled dried lettuce and three days were given for the snails to acclimatize during storage (Mandefro et al. 2017).

Collection of plant material and preparation of treatments

Fresh *T. rumphii* stems were collected from Purok Sinamar 1, Barangay Poblacion, Kabacan, Cotabato and the USM Agricultural Research Center, University of Southern Mindanao, Kabacan, Cotabato (7.1072° N, 124.8403° E). Ethanolic extraction and preparation of various treatment concentrations were conducted at the College of Science and Mathematics, and the College of Veterinary Medicine, respectively, of the University of Southern Mindanao in Kabacan, Cotabato (7.1072° N, 124.8403° E).

Collection of plant material was conducted early in the morning. Clean cutting instruments and chemical free containers were used and washed. Collected *T. rhumphii* were washed with distilled water. These were then chopped to about half an inch in length and subjected to air drying to reduce its moisture content. The air-dried material was subjected to mechanical grinding in order to attain a fine powder with a mesh size of about 200 µm (Ndamukong et al. 2006). The ground material was placed inside a clean glass container and stored at a room temperature before extraction. A pure concentration was obtained via ethanolic extraction by soaking the material in 100% ethanol for 48 hours, followed filtration using Buchner funnel, and subjecting the extract to rotary evaporation. A 10 000ppm stock solution was prepared by adding 100 grams of pure ethanolic extract to 10 liters of sterile distilled water. From the prepared solution, three different concentrations of *T. rumphii* stems were made and a total of five treatment solutions were prepared to wit:

Treatment A or Negative control (100% tap water) or 0 ppm.
Treatment B (50% water and 50% stock solution) or 5 000 ppm.
Treatment C (25% water and 75 % stock solution) or 7 500 ppm.
Treatment D (100 % stock solution) or 10 000 ppm.
Treatment E or Positive control (Commercial molluscicide: Surekill® 70WP dissolved at 10 000 ppm).

The study utilized a commercial molluscicide (Surekill® 70WP) with an active ingredient niclosamide that belongs to amide group of pesticides (DIY Pest Control Expert, 2020). Ten grams of the molluscicide powder was dissolved to one liter of sterile distilled water to obtain 10, 000 ppm molluscicide concentrations.

Experimental Design

The molluscicidal activity of *T. rumphii* against lymnaeid snails was assessed by measuring the mortality across different treatments. Efficacy was determined by evaluating mortalities in different treatment concentrations and was compared to the established value: 80% efficacy (WHO 2019). Procedures used in the study were based on the guidelines for laboratory and field testing of molluscicides for control of schistosomiasis set by the World Health Organization (2019). The study followed a completely randomized design and five treatments with three replications were used. Ten snails were randomly allocated per treatment replication.

Application of extracts and treatments followed the test conditions for immersion bioassay set by the World Health Organization (2019): water temperature was maintained to 24-26ºC, acidity was kept to a pH range of 6.5-7.7 and a 12-hour light and 12-hour dark photoperiod was provided and monitored until the end of the observation period. 30 mL clear glass bowls used and filled with treatment solutions and were aptly labeled based on their concentrations and replications.

Each snail study group was immersed to assigned treatment concentrations (Fig. 1C). Thirty (30) mL of each treatment was used. A period of five minutes was allocated for every immersion. The recovery medium was placed 20 inches away from the treatment medium and a net was put as a mechanical barrier to actively moving snails (Fig. 1D). After exposure to the treatments, the snails were removed, rinsed and transferred to new set of containers with natural habitat water for recovery. Assessment of snail deaths within treatment groups was conducted by the first author. Death of snails were assessed using the following criteria:

a. Shell Color (snail should change color from dark red or black to yellowish red or pale red)
b. Movement (snail should not move or manifest membranous action for a minute of plate observation)
c. Response to needle pocking (snail should not show response to repeated needle pocking)

The study was conducted at the College of Veterinary Medicine, University of Southern Mindanao, Kabacan, Cotabato from November to December 2020.

Data gathered and statistical analysis

The mortality induced by all treatments on the experimental snails were noted and analyzed. Specific data such as the number of snails that died under different treatments, the number of snails that died in different post-exposure times, and the molluscicidal efficacy of each treatment concentration were also noted.

Statistical tools that were used included frequency counts, means, and percentages. One-way ANOVA was not used to analyze significant differences in the mortality since all snails in the treated groups had died. Instead, only the treatment-specific mortality (TSM) and molluscicidal efficacy were determined.

To test the efficacy (E) of molluscicidal treatments, the following formula was used:

$$E = \left(\frac{\text{n in Control Group} - \text{n in Treatment Group}}{\text{N in Control Group}} \right) \text{X } 100$$

Where: n = Number of survivors
N = Population

RESULTS AND DISCUSSION

This study was conducted to determine the molluscicidal activity of the locally found *T. rumphii* stem extracts against *Radix* (*Lymnaea*) spp. snails. A total of 150 mature snails from ponds in Barangay Guiamalia, Esperanza, Sultan Kudarat were used in the study. The snails were subjected to a three-day acclimatization process with environmental condition successfully maintained before treatment application.

Assessment of Snail Death: Changes in Shell Color, Immobility, Response to needle pocking

The change in shell color was significantly observed with the increasing concentrations of *T. rumphii* stem extract (Fig. 2A). Similarly, the positive control had also displayed a significant change in snail shell color which was observed within 24h after treatment exposure. Most of the snails in Treatment E (Surekill® 70WP) had changed their shell color, while all snails in Treatment A (tap water) did not change their shell color within 24h. It was after 48h and 72h of the treatment exposure that the snails in Treatment A were observed to slowly change their shell color. A 100% immobility was observed in the treated groups (Treatments B, C, D and E) after 24h of treatment exposure (Fig. 2B). Response to needle pocking is the final test to assess snail death. This test distinguished dead immobile snails from alive non-responsive ones. Alive snails responded to painful stimulus by contracting their membranes (Figs. 2C1 & C2). It was observed that 100% of the test snails in the treated groups (Treatments B, C, D and E) were non-responsive to needle pocking after 24h post treatment exposure. These snails that did not respond to needle pocking and were thus determined to be dead. Only one snail in Treatment A died during the observation period.

TSM of lymnaeid snails after 24h of treatment exposure

Treatment-specific mortality (TSM) is essential in providing the actual percentage of snails that died under different treatments. Table 1 shows that the TSM in Treatment A (Negative) after 24h was 3.33%, while the rest of the treatment groups obtained a TSM of

Table 1. The treatment-specific mortality among *Radix* (*Lymnaea*) spp. snails in triplicate treatments of various makabuhay (*T. rumphii*) stem extract concentrations 24-hour after exposure.

TREATMENTS	N	R_1	R_2	R_3	TOTAL	TREATMENT- SPECIFIC MORTALITY AFTER 24H
A: Negative control (100% tap water)	30	0	1	0	1	3.33%
B: 5 000 ppm of makabuhay stem extract	30	10	10	10	30	100%
C: 7 500 ppm of makabuhay stem extract	30	10	10	10	30	100%
D: 10 000 ppm of makabuhay stem extract	30	10	10	10	30	100%
E: Positive control (10 000 ppm Surekill 70WP)	30	10	10	10	30	100%

Table 2. Snail death counts after 24-, 48- and 72-h post-exposure to various treatments and the treatment-specific mortality after 72 hours

TREATMENTS	N	24H	48H	72H	TOTAL	TREATMENT-SPECIFIC MORTALITY AFTER 72H
A: Negative control (100% tap water)	30	1	0	0	1	3.33%
B: 5 000 ppm of makabuhay stem extract	30	30	0	0	30	100%
C: 7 500 ppm of makabuhay stem extract	30	30	0	0	30	100%
D: 10 000 ppm of makabuhay stem extract	30	30	0	0	30	100%
E: Positive control (10 000 ppm Surekill 70WP)	30	30	0	0	30	100%

Table 3. The molluscicidal efficacy of *T. rumphii* stem extracts in triplicate treatments of varying concentrations after 24-hour exposure

TREATMENTS	N	NUMBER OF SURVIVED SNAILS			TOTAL	EFFICACY
		R_1	R_2	R_3		
A: Negative control (100% tap waterwater)	30	10	9	10	29	0%
B: 5 000 ppm of makabuhay stem extract	30	0	0	0	0	96.67%
C: 7 500 ppm of makabuhay stem extract	30	0	0	0	0	96.67%
D: 10 000 ppm of makabuhay stem extract	30	0	0	0	0	96.67%
E: Positive control (10 000 ppm Surekill 70WP)	30	0	0	0	30	96.67%

100%. The 3.33% TSM in Treatment A was attributed to a single mortality in the second replicate.

Residual activity of *T. rumphii* stem extract after 72 Hours

The study intended to record and analyze snail deaths in parallel observations across the different post-exposure times of 24h, 48h and 72h. The result showed a 100% mortality in all the treated groups 24h after exposure to *T. rumphii* stem ethanolic extract. The negative control group has only one snail that died during the entire observation period. This did not significantly affect nor invalidate the results because the number did not exceed the 10% (WHO 2019).

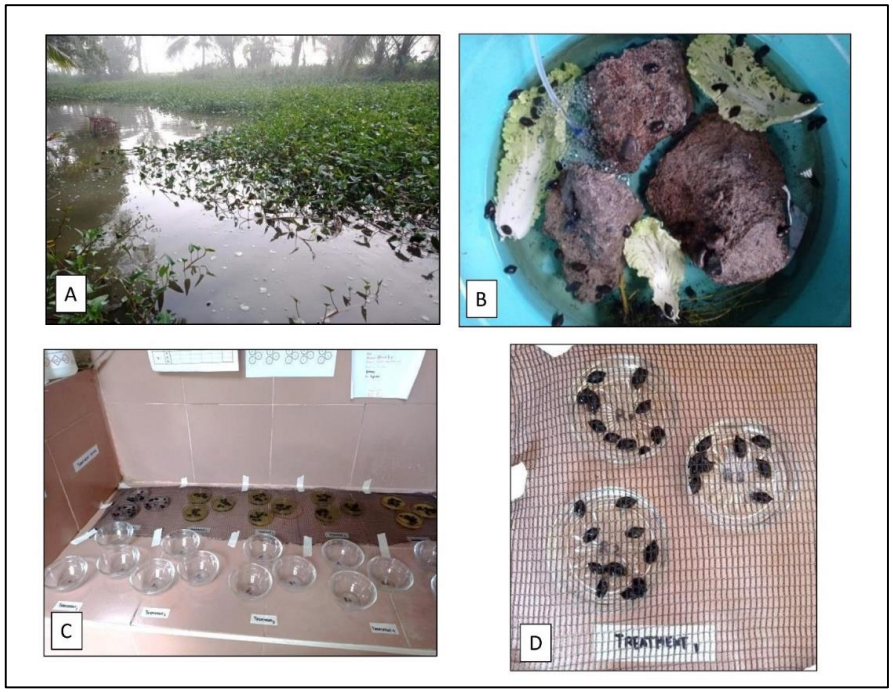

Fig 1. Snail collection and molluscicide bioassay. A: *Radix* (*Lymnaea*) spp. infested pond in Brgy. Guiamalia, Esperanza, Sultan Kudarat where the experimental snails were collected. B: The basin where lymnaeid snails were stored for the three-day acclimatization period. C: Application of treatment extracts via snail immersion bioassay. D: The net was placed above the treatment container during immersion procedure.

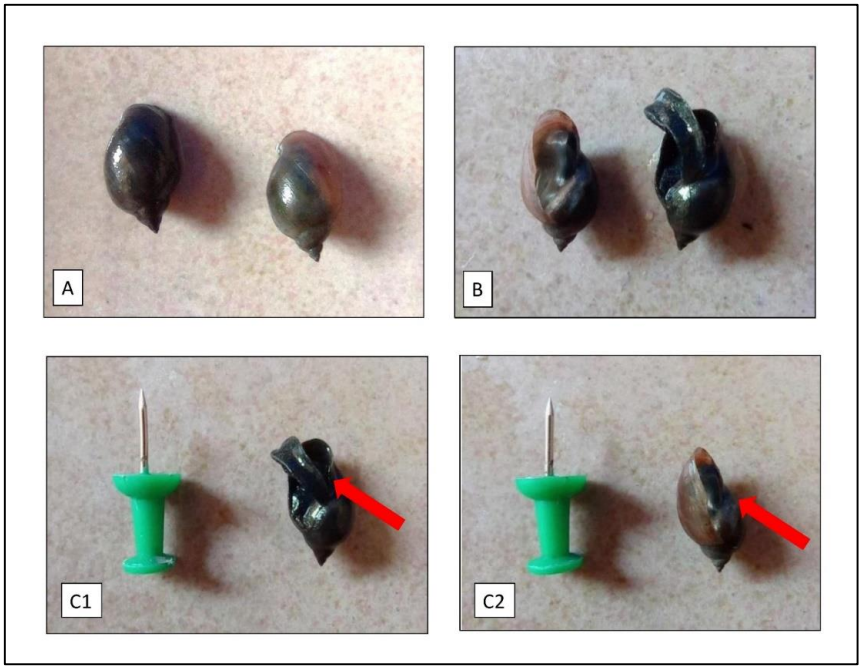

Fig 2. Assessment of Snail Death. A: Changes in *Lymnaea* spp. shell color-alive snails were dark brown (Left) and dead snails were yellowish-brown (Right) in color after 24h post-treatment exposure; B: Dead, non-moving snail (Left) and alive, actively- moving snail (right); C1: Alive snails (red arrow) responded to needle pocking by moving its foot; C2: Dead snails (red arrow) did not respond to needle pocking.

Molluscicidal efficacy of *T. rumphii* stem extract

Compared to the TSM, molluscicidal efficacy gives a percentage result in relation to the controlled groups. When the number of survivors in the controlled group decreases, supposing that many of the snails in the treatments died, the molluscicidal efficacy in the treated groups will also decrease since the result will be corrected by the mortalities in the controlled groups. The World Health Organization (2019) has set an efficacy cut-off of 80% in any malacological bioassay studies in order to assess molluscicidal actions of novel plants extracts.

Table 3 depicts that the molluscicidal efficacy of Treatment A or negative control is 0%, since this group served as the basis in solving the efficacy in the treated groups. The molluscicidal efficacies of Treatment B (5 000 ppm makabuhay stem extract), Treatment C, (7 500 ppm makabuhay stem extract), Treatment D (10 000 ppm makabuhay stem extract) and Treatment E as the positive control (10 000 ppm Surekill® 70 WP) were all 96.67%.

As observed in the results, the molluscicidal efficacies of 5 000 ppm, 7 500 ppm and 10 000 ppm concentrations of makabuhay stem extract suggest that it is a suitable and effective alternative to commercially available molluscicides against *Radix* (*Lymnaea*) spp. The resulting efficacy of 96.67% meets and surpasses the required 80% WHO standard for molluscicidal efficacy. Also, the molluscicidal efficacies of makabuhay extract treatments tested was comparable to the efficacy of 10 000 ppm commercial molluscicide Surekill® 70WP. These results show the great promise of *T. rumphii* stem extract as a natural molluscicide against *Radix* (*Lymnaea*) spp.

DISCUSSION

Based on the results, all the treatment concentrations (5 000 ppm, 7 500 ppm and 10 000 ppm) of *T. rumphii* stem extracts eliminated all the treated snails 24h post treatment exposure. The result is the same as those reported by Rejesus & Punzalan (1995). They found that *T. rumphii* leaves extract at concentrations of 4 000- 8 000 ppm had resulted in a promising 100% mortality in Golden apple snails (*Pomacea cannaliculata*) within 24h after treatment. Similarly, Mula et al. (2019) reported that *T. rumphii* water extracts had a 96. 7 % mortality rate among *P. canniculata*. Consequently, Stuart (2019) had emphasized that the active molluscicidal phytochemicals were contained in *T. rumphii* stems, with the exemption of some of its bitter components such as picroretine alkaloid which can only be found in leaves. Hence, makabuhay stem extracts were used in this study. Extracts from *T. rumphii* stems may have stronger molluscicidal activities that its leaves, which could explain the excellent result in this study. Moreover, Singh et al. (1997) noted that the molluscicidal action of makabuhay (*T. rumphii*) is attributed to the combined activities of its phytochemical components such as tannins, saponins, alkaloids, flavonoids and terpenoids, which are found to be an effective molluscicide at the dose of only <1-100ppm. Some of these are alkaloids that can readily pass through the biologic membranes within its neutral and protonated forms (Verpoorte 2005). The promising result of 100% mortality 24h after treatment exposure indicates that the molluscicidal phytochemicals in the *T. rumphii* extracts are probably fast-acting compounds.

The test snails were discolored, immobilized, and did not respond to needle pocking. A change in the shell color is an indication that the snail is dead. Dead snails undergo a rapid process of decomposition because of their delicate soft tissues. After then, ammonia will be released in the water and is most likely accompanied by an extreme rotting odor (Page 2021). However, a color change may occur in response to environmental threat, stress, and chemicals. With regards to the makabuhay treatments, the change in shell color can be attributed to the presence of tannins in *T. rumphii* stem extracts. Tannins are classified as an astringent that have the capability of binding to various organic compounds and even minerals in snail tissues (USDA 2020). Because of their unique properties, tannins are capable of contracting the snail tissues and bind with tissue proteins and minerals, thus leaving a yellowish hue to the snails' shell after the treatment exposure.

A 100% immobility within 24h post-treatment was observed indicating snail death due to the combined actions of phytochemicals such as tannins, saponins and alkaloids. Tannins, with its astringent effect, can disrupt protein functions relative to movement (Martin et al. 2009, Encyclopedia Britannica 2020, USDA 2020). Moreover, the presence of tannins in water can also the affect feeding sensory pathways and response to available food, which may had rendered the snail immobile while conserving much of its energy (Vehovszky et al. 2019). These tannins present in the *T. rumphii* can readily bind precipitate proteins, cellulose and starches which are found in the snail leading to digestive problems which may affect the feeding behavior and eventually leading the snails to death (USDA 2020). Similarly, direct tissue destruction was observed by Al-sayed et al. (2014), which had resulted in decrease in tissue protein and hemolymph. Because of these, the snails could be rendered immobile and eventually die.

Alkaloids and saponins in the *T. rumphii* extract may also have exerted some effects in snail mortality within the treatments. Alkaloids affect protein functions through inhibiting protein synthesis (Ke et al. 2017). The phytochemical tends to inhibit various enzyme activities related to protein synthesis. Also, saponins contained in the extracts could have hastened snail immobility and death via membranolytic activity (Cruz & San Martin 2013). Like tannins, saponins can also cause damage to snail tissues, especially the gastric epithelial membrane (Cruz & San Martin 2013). Moreover, Martin et al. (2009) reported that the death of snails could be attributed to the effect of saponins in the snail's breathing mechanism. The phytochemical was observed to successfully prevent snails from breathing through their siphon.

The study was not able to assess the 72-hour post-treatment residual activity of *T. rumphii* extract on the snails due to the experimental animals' death within 24h. Also, the excellent molluscicidal efficacy of *T. rumphii* in this study was maybe due to the high concentration of the test treatments. Hence, a study utilizing lower concentrations may be of value so as to determine the minimum lethal concentration of *T. rumphii* ethanolic extract against *Radix* (*Lymnaea*) spp.

snails. Nevertheless, the death of snails in this study, which was assessed via observation of shell discoloration, immobility and non-responsiveness to needle pocking, is attributed to the molluscicidal activities of the phytochemicals in the *T. rumphii* stem ethanolic extract.

CONCLUSIONS AND RECOMMENDATIONS

A total of 150 mature lymnaeid snails were collected, acclimatized, and subjected to different treatments. An immersion bioassay was done during the treatment applications. Immersion was done for 5min. Efficacy of each treatment was determined. The results revealed that *T. rumphii* stem ethanolic extract at concentrations of 5 000 ppm, 7 500 ppm and 10 000 ppm had rendered an excellent 100% mortality among lymnaeid snails 24h post-exposure. 96.67% molluscicidal efficacy was observed in all the *T. rumphii* treatments.

In conclusion, the *T. rumphii* ethanolic stem extract concentrations studied herein provided an excellent molluscicidal activity against lymnaeid snails. All the concentrations tested had exhibited an outstanding 96.67% molluscicidal efficacy against the lymnaeid snails; the result surpassed the 80% minimum molluscicide efficacy set by WHO (2019). Hence, *T. rumphii* stem ethanolic extracts may be a great natural alternative to commercially available molluscicides.

The researchers recommends that the concentrations to be used in future investigations on the molluscicidal effect of *T. rumphii* be lowered than those used herein to determine the lowest effective concentration. It is also recommended a field study be conducted to determine the efficacy of *T. rumphii* stem ethanolic extract in the snail's normal environment and record its effects on non-target organisms, if any. Lastly, the specific mechanisms by which the *T. rumphii* stem extract render death among lymnaeid snails should also be explored.

ACKNOWLEDGEMENTS

The 1st author would like to thank Dr. Elizabeth C. Molina and Dr. Josephine R. Flores for the insights they shared as part of his thesis examination committee. Similarly, the 1st author would like to acknowledge Prof. Lovielle A. Gonzaga, Ms. Belene Esmejarda, and Dr. Lydia C. Pascual of the USM Department of Chemistry for the time and equipment they offered for the ethanolic extraction of *T. rumphii* stems used in this study. The authors would also like to thank the faculty and staff of the College of Veterinary Medicine for aiding them during the conduct of the study. Lastly, the 1st and 3rd authors dedicate this scientific article to Dr. Carlito B. Sanchez (the 2nd author) who has met his untimely demise during the peer review period of this publication.

AUTHORS' CONTRIBUTIONS

This research was part of a thesis written and conducted by the 1st author. The research was under the supervision of the 2nd author. Critical review and revisions were done by the 2nd and 3rd authors. All the authors had read and approved the manuscript for publication.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

FUNDING

This study did not receive any external funding. Reagents and materials were provided by the USM College of Veterinary Medicine Central Laboratory.

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