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In vitro evaluation of the molluscicidal activity of *Euphorbia tirucalli* latex extract against the mollusk rice pest *Pomacea canaliculata* (Caenogastropoda: Ampullariidae)

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Abstract

The latex of *Euphorbia tirucalli* ("Pobreng Kahoy") is widely known for its medicinal, antimicrobial, and insecticidal properties. Observations on the plant's molluscicidal potency were reported by local farmers. In this study, the molluscicidal activity of aqueous latex extract (ALE) of *E. tirucalli* was evaluated against adult Golden apple snail ("Kuhol", *Pomacea canaliculata*), a rice seedling pest. This research employed a Completely Randomized Design in triplicates (3 trials, N=450) under 3 experimental treatments (T_1= 8.75 ppm, T_2= 7.5 ppm ALE, T_3= 6.25 ppm ALE) and two control groups (Positive Control=Niclosamide [Snail Shatter™] and Negative Control= distilled H_2O [Absolute™]). After 96-hours experimental period, mortality data across treatment groups were analyzed using Probit analysis, One Way ANOVA ($p<0.05$) followed by Tukey's HSD test. Results revealed that there is a positive correlation between ALE concentration and snail mortality and T_1 (8.75 ppm) was reported to have a comparable lethal effect to that of the commercially-available molluscicide (Niclosamide). Lethal dosages of the aqueous extract of the *E. tirucalli* latex was LD_{50}=6.33 ppm ALE and LD_{90}=9.36 ppm ALE. For future research, it is recommended to have phytochemical screening of similar ALE concentrations and toxicity level assessment to economically important non-target organisms (Tilapia *Oreochromis niloticus* and Water fleas *Daphnia* spp.) under field trials.

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Introduction

Euphorbia tirucalli Linnaeus (1753) is renowned because of its vast and numerous medicinal, antimicrobial, and insecticidal benefits (Silva *et al.,* 2007; Mwine *et al.,* 2010). However, there has been a scanty literature elucidating the molluscicidal potency of *Euphorbia tirucalli* Linn. (Mwine and Van Damme, 2011). Initial reports of the plant being a molluscicide were revealed in the records of Vanderplank (1944) in Tanganyika, Africa wherein the natives utilize its fresh branches to protect juvenile plant seedlings against snails and slugs. In 1997, Clark *et al.* included *E. tirucalli* as a part of the list of those plants that have molluscicidal properties but results of *in vivo* assay were scant in scientific publications. According to Jurberg *et al.* (1985), the latex of the plant could also be used as molluscicide against schistosome-carrying snail *Biomphalaria glabrata* with comparable effect to that of chemical molluscicides like niclosamide and copper sulfate, however it has high toxicity to fishes. When Tiwari *et al.* (2003) tested the plant's extract against fresh water snails *Lymnaea acuminata* and *Indoplanorbis exustus,* it resulted to mortality possibly by blocking enzymatic activity in their nervous systems. Meanwhile, the extract resulted to low mortality when tested against non-target organisms.

Euphorbia tirucalli is a member of the plant family Euphorbiaceae whose molluscicidal property is known globally (WHO, 1983; Wal *et al.,* 2013). The rice pest *Pomacea canaliculata* is a gastropod that continuously cause damage to juvenile rice seedlings, leading to poor standing of crop production, decline of natural and endemic fauna, water quality deterioration caused by application of synthetic molluscicides in the rice fields, and parasitism in humans (Dancel and Joshi, 2000; Cagauan and Joshi, 2003; Joshi, 2005). This research evaluates the molluscicidal activity of the aqueous extract of the latex (ALE) of *Euphorbia tirucalli* against the rice pest *Pomacea canaliculata*. Specifically, it aims to (i) determine the concentration of the aqueous extract of the latex that can yield to 50% snail mortality (LC_{50}); (ii) determine the concentration of the aqueous extract of the latex that can yield to 90% snail mortality (LC_{90}); and (iii) determine the relationship of concentration of ALE and snail mortality.

Materials and method

Verification of identity of Golden Apple Snail

Before the actual collection process, a handful of snail specimens from the rice field in Linao, Minglanilla, Cebu were brought to the Department of Agriculture–7 [DA] at Maguikay, Mandaue, Cebu for verification process. The specimen validation was done by an expert on agricultural pests from the Regional Crop Protection Center of DA – 7.

Collection of Golden Apple Snail

The snails were collected through handpicking from a rice field in Linao, Minglanilla, Cebu. Only the snails with a shell height ranging 15-20 mm with mass ≥ 4 grams were collected (Joshi *perscomm,* 2017). The shell height is known by measuring with a caliper as the distance between the apex of the shell and the lower margin of aperture (Joshi *et al.,* 2005).

The snails were allowed to adapt to the laboratory conditions through acclimatization for 48 hours. During the acclimatization process, the snails were placed and reared in clean containers containing dechlorinated water and were given green papaya leaves, *ad libitum,* as food. The acclimatization process was adopted from Joshi *et al.* (2005) and Dai *et al.* (2011) with modifications, as suggested by local farmers. Only those active and healthy golden apple snails were subjected to experimentation. There were about 450 snails that were subjected for 3 laboratory trials in the study.

Verification of identity of Pencil-tree (Euphorbia tirucalli)

Before the latex collection process, a plant specimen from Danao City was brought to the Department of Agriculture at Maguikay, Mandaue, Cebu for verification of species identity. The specimen validation was done by the taxonomy division of the department.

Collection of plant latex

The latex was collected from the *Euphorbia tirucalli* found in Taytay, Danao City, Cebu. The procedure employed in the collection of plant latex was adopted from Jurberg *et al.* (1985). Briefly, incisions were made in the branches and stem of *E. tirucalli* in order to collect the exudate. The collected latex (1 ml) from the plant was placed in a test tube containing 3mL of distilled water, was covered with cork and then transported to the laboratory. It was mixed in the laboratory with 96 mL of distilled water to make the latex solution (100 mL of 10 ppm aqueous latex extract solution).

Research Protocol

Preliminary screening and range finding test

From the latex solution, a 50 ml aqueous latex extract (ALE) solution was aliquoted and served as the stock solution. From this stock solution, all serial dilutions were made and the following concentrations were prepared: 10 ppm ALE, 5 ppm ALE, 2.5 ppm ALE, and 1.25 ppm ALE. These concentrations were used during the preliminary screening. It was found that the two highest snail mortality rates were observed in the solutions containing 10 ppm ALE and 5 ppm ALE.

Preparation of controls

The negative control that was utilized in this experiment was distilled water and the positive control was a powdered form of niclosamide (Snail Shatter™) which was prepared at the recommended rate of 35 grams per 16 liters of water (2.19 g/L).

Bioassay procedure for molluscicidal activity

The definitive test procedures suggested by WHO (1983) and Joshi *et al.* (2005) were adopted, with slight modifications. There were 3 replicates per treatment, and under each replicate, there were 10 snails placed in a container containing 400 mL of distilled water and 50 mL of the ALE solution. The concentrations of the aqueous latex extract used during the actual bioassay are the following: 6.25 ppm ALE, 7.5 ppm ALE, and 8.75 ppm ALE. The three treatment solutions were determined through the results of preliminary screening.

The golden apple snails were exposed to three different concentration of aqueous latex extract inside a transparent plastic container covered with plastic screen (to allow entry of oxygen and prevent exit of the snails). Mortality of snail was simultaneously observed for a total of 4 days with 24 hours interval. For the first day of laboratory trial, there were 3 observations that were performed and one observation for the remaining days. Every after 24 hours, all snails were subjected to recovery process where all snails were rinsed and submerged in distilled water for 30 minutes to allow them to recover. Mortality is observed and confirmation test of mortality was performed to all snails. A snail is considered dead if there is no muscular contraction/movement elicited after probing with a needle (Massaguni and Latip, 2015). All dead snails were removed from the container to avoid contaminating the water and affect other snails. The dead snails were also crushed as suggested by WHO (1983). The water was changed with distilled water with the solution of latex (same volume) after mortality confirmation. The LD_{50} and LD_{90} were determined through Probit analysis (WHO, 1983).

Statistical analysis

Mortality data were used to calculate for LC_{50} and LC_{90} values, along with its 95% fiducial upper and lower confidence limits, Chi-square (χ^2), coefficient of correlation (R^2) and also slope values through Probit analysis using MS Excel Stat v2015.1 (Massaguni and Latip, 2015). To further determine if there is statistical significance between and among treatment and the control values, data were subjected to One-way Analysis of Variance (ANOVA). A *post hoc* analysis was also done (Tukey's Honestly Significant test at 0.05 level of significance) to further verify statistical significance between treatment and control values.

Results

Molluscicidal action of aqueous extract of Euphorbia tirucalli latex

Table 1 shows the average adult *P. canaliculata* mortality when continually subjected to different concentrations of ALE of *E. tirucalli* (in ppm) for 96 hours.

The positive control (niclosamide, Snail Shatter™) recorded the highest percentage snail mortality (100%). Snails that have died due to exposure to niclosamide solution were often found with their opercula permanently close and their feet usually shrunk. Aqueous extracts of *E. tirucalli* latex (in ppm) recorded mean percentage snail mortality of 40%, 60% and 70% on adult *P. canaliculata* for 6.25 ppm ALE, 7.50 ppm ALE, and 8.75 ppm ALE, respectively. Lowest percentage snail mortality was observed in the negative control (distilled water) where other snails exposed with distilled water were often found freely moving.

Table 1. The average mortality rates of adult Golden Apple Snail (GAS) *Pomacea canaliculata* after 96 hours of continued application of the varying concentrations of aqueous latex extract of *Euphorbia tirucalli* across all trials (n=150).

Treatment/Controls	Ave. no. of living GAS (before treatment) n=150	Ave. no of living GAS (after treatment)	Ave. no. of dead GAS after 96 hrs of observation	% Mortality
		Control Groups		
NC Distilled water	30	24	6	20%
+C Niclosamide (2.19g/L)	30	0	30	100%
		Experimental Groups		
T_1 – 6.25 ppm ALE	30	18	12	40%
T_2 – 7.50 ppm ALE	30	12	18	60%
T_3 – 8.75 ppm ALE	30	9	21	70%

For each trial, 150 snails were used. For the three trials (conducted on separate days), the grand total of the snails used is 450 snails. Each snail represents 1 sample

Mortality refers to the number of dead adult GAS *Pomacea canaliculata*

ALE = aqueous latex extract.

Death from the negative control was possibly attributed to stress and inability of some snails to fully acclimatized to the lab condition. Few minutes after exposure of snails in the treatments, behavioral changes were also observed. Snails whose bodies were exposed in the solution manifested a sudden twisting motion and crawling towards other snails. Formation of white and sticky substance (most probably mucus) in the bodies of the snails (or around the operculum if the shell is close) was also evident. Snails' feet that have lost their turgidity were also observed in the experimental treatments (i.e. observation similar to those snails in the positive control).

Table 2. One-way Analysis of Variance (ANOVA) of the snail mortality of the varying concentrations of Aqueous Latex of *Euphorbia tirucalli.*

source	Sum of squares	df	Mean square MS	F statistic	p-value
Between groups	1064.4	4	266.1	221.750	0.000*
Within groups	12.000	10	1.200		
total	1076.4	14			

*significant at $p \leq 0.05$.

Based on the statistical treatment of data (ANOVA) (Table 2), the computed p-value is 0.000 at 0.05 level of significance. This means that the null hypothesis (i.e. there is no difference in the snail mortality rates between and among the treatment and control values) is rejected. This result implies that the snail mortality rates exposed to the negative control (distilled water), positive control and treatments (aqueous extract of *E. tirucalli* latex of varying concentration in ppm) significantly differ from each other in terms of the % mortality. Post hoc test results (Table 3) also revealed that the snail mortality across groups was statistically

significant to each other, suggesting that each treatment was not comparable in terms of inducing mortality to the snails. As reflected in the same table (Table 3), there is an increasing trend of snail mortality as the concentration of ALE increases. Treatment 3 (8.75ppm ALE) recorded the highest mortality among experimental groups but was not even comparable to the positive control, as the latter posted 100% mortality at 96h. The pattern of increasing mortalities is indicative of a direct proportionality of dose to % mortality pattern.

Table 3. The average mortality of adult Golden Apple Snail (GAS) *Pomacea canaliculata* after 96 hours of continued application of the varying concentrations of aqueous latex extract of *Euphorbia tirucalli* per trial (n=150)

Treatment / Controls	Ave. no. of dead GAS after 96 hrs of observation per trial			Snail Mortality across all Trials (mean±S.D.)
	Trial 1	Trial 2	Trial 3	
Control Group				
NC Distilled water	7	5	6	6.00 ± 1.00^{a}
+C Niclosamide (2.19g/L)	30	30	30	30.0 ± 0.00^{b}
Experimental Group				
T_1 – 6.25 ppm ALE	14	11	11	12.0 ± 1.73^{c}
T_2 – 7.50 ppm ALE	19	18	17	18.0 ± 1.00^{d}
T_3 – 8.75 ppm ALE	22	20	21	21.0 ± 1.00^{e}

Values followed with the same superscript letter are insignificant at 0.05 level of significance according to *post hoc* analysis (Tukey's test).

Toxicity of aqueous extract of Euphorbia tirucalli latex

The lethal dosages (along with its limits) (Table 4) were calculated and estimated using Probit analysis. Under laboratory conditions, the dosage that can cause 50% snail mortality at a given *P. canaliculata* sample is 6.33 ppm of aqueous extract of *E. tirucalli* latex while 90% snail mortality can be manifested if the solution used is 9.36 ppm ALE. An extract from a plant that has LD_{50} ranging from 0-500 ppm is known to possess high level of toxicity. By the standards of World Health Organization (1965) the lethal dosages fit to the norm where an aqueous extract must exhibit lethality at a dose lower than 20 ppm.

It was also revealed in the Probit analysis that the slope value is positive (Fig. 1).

The coefficient of correlation of the treatments and mortality rates is 0.995 which can be interpreted as "very high correlation" This result may suggest that there is a direct relationship between the concentration of treatment and snail mortality where the concentration of the aqueous extract of *E. tirucalli* latex increase together with the increase of mortality of *P. canaliculata*.

Discussion

In the present study, the aqueous extract of the *E. tirucalli* latex presented molluscicidal activity wherein the increasing concentration of aqueous latex extract of *E. tirucalli* is positively correlated with snail mortality rate.

The resulting trend of this study is similar and parallel to that of the works of Jurberg *et al.* (1985) and Tiwari *et al.* (2003) although Jurberg *et al.* (1985) used different concentration of aqueous extract (target organism is *Biomphalaria glabrata*) while Tiwari *et al.* (2003) used lyophilized latex (target organisms are *Indoplanorbis exustus* and *Lymnaea acuminata*). Behavioral changes were also

observed during the course of exposure of adult *P. canaliculata* to the treatments. Those behavioral changes noticed include twisting motion, hyperactive crawling, and formation/release of a white, sticky coagulates. The twisting motion and hyperactivity were also observed in the research of Tiwari *et al.* (2003) on the effect of lyophilized latex to target organisms *Indoplanorbis exustus* and *Lymnaea acuminata*.

Table 4. Calculated lethal dosages (in ppm) after the 96th hour of continued exposure of adult *Pomacea canaliculata* to the varying concentrations of aqueous extract of *Euphorbia tirucalli* latex.

Lethal Doses of aqueous extract of *E. tirucalli* latex (ppm)		R^2	Chi-square value	Slope value	Fitting
LD50	LD90				
6.33 ppm	9.36 ppm				
*95%LCL=5.46 ppm	*95%LCL=8.07 ppm	0.9	0.95	7.54	Good fit
*95% UCL =7.35 ppm	*95% UCL =10.86 ppm	95			

*95% Fiducial LCL (Lower Confidence Limit) and UCL (Upper Confidence Limit), values significant at $p<0.05$.

The formation/release of white, sticky coagulates was probably induced by the reaction of the polar compounds (e.g. phorbol and ingenol esters) of *E. tirucalli* to the mucus membrane of the snail (Alves, 2013; Aljabarin *et al.,* 2014). The latex of *E. tirucalli* is known to be highly vesicant in nature and induces extreme irritation to skin and mucosal membranes (Furstenberger and Hecker, 1986).

The reactions of adult *P. canaliculata* upon exposure to the aqueous latex extract and its subsequent mortality can be attributed to the chemical composition of the latex in *E. tirucalli*. Mwine and Van Damme (2011) reported that the latex of *E. tirucalli* is toxic in nature and it is manifested in the low pressure of herbivory of the plant. Among the bioactive compounds identified to possess molluscicidal properties are saponins, tannins, phorbol, ingenol, steroids, flavonoids, and terpene esters (Nielsen *et al.,* 1979; WHO, 1983; Khan and Ahmed, 1988; Khan, 2010; Khan and Malik, 1990; Morallo-Rejesus, 2000; Upadhyay *et al.*, 2010; Niño *et al.,* 2012; Alves, 2013; Wal *et al.,* 2013; Aljabarin *et al.,* 2014). Saponins, for instance, are known to affect cholesterol level in the gills of *P. canaliculata*, suppress feeding activities in other slugs, and are membranolytic to epithelial lining in nature (San Martin *et al.,* 2008; Cruz and San Martin, 2013).

E. tirucalli belongs to the family *Euphorbiaceae*. In 1983, World Health Organization enlisted *Euphorbiaceae* as one of those plant families that possess molluscicidal potential against harmful snails (e.g. schistosome-carrying snails). *Euphorbiaceae* is next to the family *Leguminosae* which is known to have the most numerous members of plant having molluscicidal activity.

Ecotoxicological assessments for the *E. tirucalli* have already been done using non-target organisms. In the study of Jurberg *et al.* (1985) it was revealed that the latex is highly toxic to fish. However, unparalleled results have been observed in the ecotoxicity assessment of Tiwari *et al.* (2003) who also used fish as non-target co-inhabitant. The lethal dosage (90ppm) of the latex manifested low mortality on fish. Differences between these two researches can be attributed to the species of fish used in the studies Jurberg *et al.*(1985) utilized *Lebistes reticulatus* while Tiwari *et al.*(2003) used *Channa punctatus*, the nature of the solute (Jurberg *et al.,* 1985 utilized fresh latex while Tiwari *et al.,* 2003 used dried one), and the concentrations of solute in the solution. However according to Jurberg *et al.* (1985), *Euphorbia tirucalli* can still be considered as a potential molluscicide even with its ability to induce irritability on other organisms since it conforms to the standards for

plants that can be used as molluscicide set by Kloos and McCullough (1982). Among the properties making *E. tirucalli* a good candidate molluscicide include widespread distribution of the species, easy method of propagation, molluscicidal compound can be extracted and isolated from regenerating plant part and the method of extracting the compound is easy.

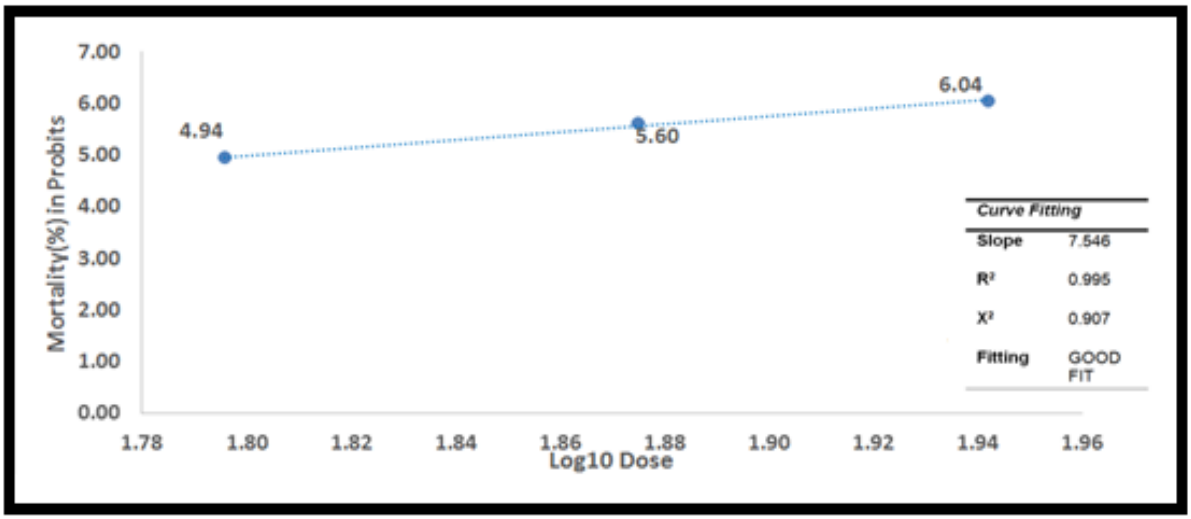

Fig. 1. The relationship between the ALE concentrations of *Euphorbia tirucalli* (Log_{10} Dose) and mortality rate of adult *Pomacea canaliculata* (% mortality in probits).

Based on the findings of the study, this research concludes that the aqueous extract of *Euphorbia tirucalli* latex can be considered as a potential molluscicide for the control of infestation of *Pomacea canaliculata* snail under laboratory conditions with lethal dosages of 6.33 ppm (LD_{50}) and 9.36 ppm (LD_{90}). The solution is toxic at high concentrations, induces behavioral changes and reactions consequently causing the mortality of the snail, and has comparable toxic effect to that of the commercially-available chemical control. Future research directions may consider exploring other indigenous species of *Euphorbia* or its relatives, and exploring a much bigger experimental set-up under field conditions with the non-target organisms is recommended.

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