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Bioactivity and Synergism of *Tagpo* (*Ardisia tomentosa*) Molluscicidal Extracts

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Population control of golden apple snails (GAS, *Pomacea canaliculata*) is beneficial in rice farming; however, the associated control measures can be expensive and time-consuming. Currently, farmers use synthetic molluscicides such as niclosamide to control GAS infestation, but this has some drawbacks. For example, niclosamide has broad toxicity to non-target organisms and negatively affects the growth of rice seedlings. Plant extracts with potent bioactivity and low toxicity to non-target organisms are attractive alternatives to synthetic molluscicides. In this study, the bioactivity of the crude fruit extract of *tagpo* (*Ardisia tomentosa*) was assessed against GAS and tilapia (*Oreochromis niloticus*) fries. The potential molluscicidal synergism between *tagpo* extract and niclosamide was also assessed by dose-response modeling. To generate data, snails and fish fries were exposed to six or eight concentrations of test substances in one or three trials for 24 h (48 h for fish assay) followed by a 24-h recovery. Snail and fish mortality was assessed by motility criterion to model dose-response curves and estimate lethal concentrations. Lethal ratio tests were performed in R and interpreted at 95% confidence level. The results showed that *tagpo* extract exhibited high molluscicidal activity against GAS (LC_{50} = 27.83 ± 0.53 ppm, value ± standard error, simulated field conditions); however, it showed toxicity to tilapia fries (LC_{50} = 5.36 ± 0.21 ppm). Both *tagpo* extract and niclosamide also exhibited synergistic bioactivity on snails, thus supporting *tagpo* extract as a novel source of potent molluscicides worthy of further development.

Keywords: *Ardisia tomentosa*, golden apple snails, molluscicides, niclosamide, Probit analysis, synergism, *tagpo* extract

INTRODUCTION

Golden apple snails (GAS, *Pomacea canaliculata*) are major rice pests in the Philippines that significantly impact rice production (Jiang et al. 2022). In 1990, the estimated cumulative economic loss from GAS for the Philippines ranged from US$425 M to US$1.2 B (Naylor 1996). In 2022, these estimates were valued at US$994 M to US$2.81 B (US Bureau of Labor Statistics 2022). These economic losses are associated with snail population control measures such as expenses on synthetic molluscicides and low yield (Joshi et al. 2017).

Using synthetic molluscicides in snail population control is still the most popular approach among farmers. As disclosed in a survey among Philippine and Vietnamese rice farmers, more than 70% of the respondents use instant-kill agents to control GAS (Schneiker et al. 2016). Other snail control strategies identified in the survey include employing ducks and fish that prey on snails and manual snail-picking and crushing; however, these strategies are labor-intensive and not easily scalable.

Niclosamide and metaldehyde are two of the most widely used molluscicides. These are synthetic compounds that target vital snail biochemical processes. Niclosamide is a potent molluscicide; however, niclosamide toxicity to a broad range of non-target organisms and its detrimental effect on rice seedlings are major application drawbacks (Joshi et al. 2008). The broad toxicity of niclosamide has been attributed to its capacity to decouple the mitochondrial membrane

potential, which decreases adenosine triphosphate (ATP) levels in cells, and to induce apoptosis and autophagic cell death (Park et al. 2011). Fish and crustaceans are the most vulnerable organisms to niclosamide toxicity due to surface run-offs. As for metaldehyde, its indiscriminate use is feared to result in contamination of lakes and wetlands, which may impact services from these complex ecosystems. In the United Kingdom, it was found that the concentration of metaldehyde in surface waters sampled from 2010 to 2011 frequently exceeded the European Union (EU) pesticide regulatory standard (Kay and Grayson 2014). Green alternatives to niclosamide and metaldehyde are therefore necessary.

As alternatives to synthetic molluscicides, plant extracts have been widely studied with some encouraging results. For example, saponins derived from quinoa husks by alkaline hydrolysis have been assessed for their snail-killing effect on apple snails in the Philippines (Joshi et al. 2008). Quinoa saponins were shown to be molluscicidal at 13 ppm against juvenile snails under simulated field conditions and were shown to be ovicidal to 1–5-d old snail eggs. These plant-based molluscicides have been shown to have a minor effect on developing rice seedlings, unlike niclosamide which significantly reduces seedling survival. It has been reported that quinoa saponins were non-toxic to goldfish and tilapia fish at their maximal molluscicidal concentration: 33–54 ppm (San Martín et al. 2008). Additionally, saponins from tea (*Camellia sinensis*) seeds were assessed for their molluscicidal efficacy against GAS and toxicity to red swamp crayfish (*Procambarus clarkii*) and redear sunfish (*Lepomis microphus*) in the United States (Olivier et al. 2016). The plant-derived molluscicide has been shown to induce 100% snail mortality at 15 and 30 ppm. The active compound in tea seeds has been shown to be non-toxic to crayfish and toxic to redear sunfish. Tea seed saponins have also been shown to be efficacious against different snail species that are intermediate hosts of the human schistosomiasis parasites (Jia et al. 2019).

An understudied plant native to the Philippines is *tagpo* (*Ardisia tomentosa*) which is widely distributed in the primary forests of the country. The conservation status of *A. tomentosa* is non-threatened (POWO 2024). As shown previously, its crude water extract has potential molluscicidal activity against *Oncomelania hupensis* quadrasi snails, the obligate intermediate host of *Schistosoma japonicum* in the Philippines (Gomez and Anacta 2020). Many novel natural products from *Ardisia* species have been discovered (Kobayashi and de Mejía 2005; Liu et al. 2022). For example, Raga et al. (2011) have shown that *Ardisia squamulosa* hexane leaf extract influences the spermatogenesis in rats, revealing that the extract affects sperm count but not sperm morphology and viability. To date, at least seven Philippine *Ardisia* species have been studied for their pharmacological activities, including cytotoxicity to different cancer cell lines, anti-angiogenic property, and apoptotic activity (Tang et al. 2009; Zhang et al. 2010; Herrera and Amor 2011; Molina-Magtoto and Buot 2020). The majority of Philippine *Ardisia* species are endemic. With numerous reports on the pharmaceutical potential of natural products from this genus, it is therefore important to study these plants.

While there is plenty of published work on plant molluscicides, studying the activity of novel plant sources is still relevant because new investigations may unveil novel structures of active compounds and mechanisms of action (Joshi et al. 2017; Chuong Nguyen et al. 2022; Kamari et al. 2023; Zaib et al. 2023). Therefore, this study aimed to evaluate the bioactivity of *tagpo* extract against GAS and tilapia fries. Moreover, the study sought to assess the molluscicidal synergism between *tagpo* extract and niclosamide against GAS.

MATERIALS AND METHODS

Identification of *Ardisia tomentosa*

Plant samples were sent to the Jose Vera Memorial Herbarium, University of the Philippines, Diliman, Quezon City, Philippines for identification. The plant was identified to the genus level (*Ardisia*). For species level identification, photographs of the plant were sent to Prof. Liezel M. Magtoto of the University of the Philippines, Baguio City, an expert in Philippine *Ardisia* plants. The plant was then identified as *Ardisia tomentosa*. The photographs of *A. tomentosa* samples used in this study are presented in Fig. 1. Fruit samples were collected from a domesticated tree which was propagated by stem cutting.

Preparation of Plant Extracts

Mature *tagpo* fruits (Fig. 2; 500–1000 pieces per sampling) were collected in Sta. Fe, Leyte (11°12′24″ N, 124°56′09″ E, 15 m altitude) in April and May 2021, and were dried overnight at 60ºC in a drying oven (Binder, Germany). The endosperm was separated, crushed with a mortar and pestle, sieved, and stored in an ordinary freezer until use. To prepare the extract, 1 g of powder was mixed with 100 mL of distilled water and

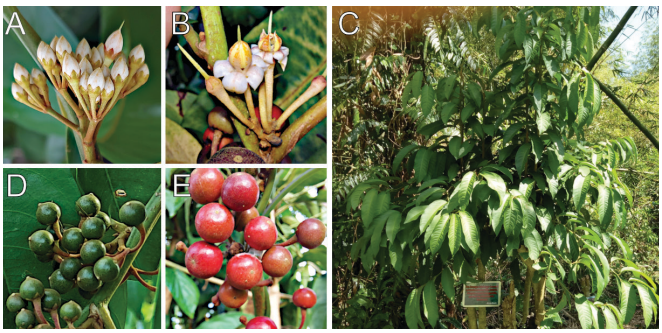

Fig. 1. Photographs of *Ardisia tomentosa* used in the study. A and B: inflorescence; C: *tagpo* tree; D: mature and unripe fruits; and E: ripe fruits. The tree is in a garden owned by one of the authors of this study.

stirred for 1 h. The mixture was allowed to sit for another hour and the liquid layer was decanted and spun at 4000 rotations-per-minute for 4 min using a benchtop centrifuge (Labnet, USA). The centrifugate was used as the stock solution of *tagpo* extract and used in all downstream experiments. To determine the concentration of *tagpo* extract, the solvent from the centrifugate was evaporated and the mass of residue was measured gravimetrically, i.e., monitoring the change in the mass of a pre-weighed evaporating dish (Rice et al. 2012). Ten mL (extract volume) of *tagpo* extract were transferred into a pre-weighed (initial mass) evaporating dish (ED) and then heated in a drying oven (Binder, Germany) at 105ºC for 1 h. The evaporating dish was then cooled in a desiccator for another hour and weighed after. The heating-cooling-weighing cycle was repeated until the difference in two consecutive masses was less than 0.5 mg. The final mass of ED was used in the calculation. Total solids value was calculated using the following equation:

$$Total\ solids\ (ppm) = \frac{ED\ initial\ mass\ (g) - ED final\ mass\ (g)}{extract\ volume\ (mL)} \times 10^6 \qquad (Eq.1)$$

Fig. 2. *Tagpo* (*Ardisia tomentosa*) fruit. A: mature fruit; B: cross-section; and C: endosperm.

Based on four measurements, the total solids content in *tagpo* extract was calculated as 2222 ppm. This value reflects that only 22.22% of *tagpo* powder is soluble in water or only 0.2222 g of 1.000 g of *tagpo* powder dissolves in 100 mL of water.

Preparation of Stock Solution of Niclosamide

The niclosamide stock solution (100 mg/L) was prepared by dissolving 0.1400 g of a commercial 70% wettable powder formulation (NiclosM®, Leads AGRI) with distilled water in a volumetric flask to make a 1-L solution.

Spectrophotometric Determination of Saponins

For the quantitative estimation of saponins in *tagpo* extract, the method of Madhu et al. (2016) was used with minor modifications. *Tagpo* extract (0.25 mL) was mixed with 0.25 mL vanillin solution and 2.50 mL aqueous sulfuric acid (72% by volume acid). To develop color, the reaction mixture was heated in a 60°C water bath for 10 min. The absorbance of samples at 560 nm was measured using a 10-mm quartz cuvette with a UV-Vis spectrophotometer (Millipore, Germany) against a reagent blank. A calibration curve was prepared using aescin standard solutions with the following concentrations: 100, 250, 500, 750, 1000, and 1500 ppm. Saponin content was expressed as mg of aescin equivalent per g of *tagpo* powder.

Collection and Acclimatization of Snails

Golden apple snails were collected in Sta. Fe, Leyte (11°11′55″ N, 124°56′08″ E, 11 m altitude) on multiple occasions. Juvenile snails (1.5–2.0-cm shell diameter) were handpicked and washed with Artesian well water (Tamburi and Martin 2009). Snails were acclimatized in plastic basins covered with nets to prevent them from escaping. This was done for 2 d using the same well water, changing the water every 12 h. The snails were fed *Ipomea batatas* leaves ad libitum. Only active snails (motility criterion) were used in the molluscicidal assays.

Acclimatization of Tilapia Fries

Nile tilapia fries were sourced from the hatchery of the Bureau of Fisheries and Aquatic Resources Regional Office VIII, Babatngon, Leyte. Fish fries were acclimatized in aerated 45-L rectangular glass aquariums for 1 wk. Excess feed and feces were siphoned out twice daily while the water was changed daily. The fries were fed with commercial feeds four times a day at 20% of their biomass (Hussain 2004). The fries were kept in holding tanks for 1 wk before being used in assays. Feeding was stopped 24 h before the assay (Oluwatoyin 2011). Physico-chemical parameters of water such as dissolved oxygen, salinity, pH, and temperature were monitored every 12 h until the end of the acclimatization period. Dissolved oxygen was maintained at 7–9 ppm. The protocol used in fish rearing and assays was registered under IACUC Protocol No. 2019-052-023 from the University of San Carlos, Cebu City.

Preparation of Test Solutions for Laboratory Assays

Six *tagpo* extract concentrations were tested in laboratory assays, namely 13.2, 17.6, 22.0, 26.4, 35.0, and 43.6 ppm, wherein 3, 4, 5, 6, 8, and 10 mL of *tagpo* extract were added to 500 mL of Artesian well water (pH 6.30 – 6.50), respectively. Based on a 2222-ppm stock solution, the concentration was computed using Eq. 2:

$$Concentration\ (ppm) = \frac{2222 \times extract\ volume\ (mL)}{500 + extract\ volume\ (mL)} ppm \qquad (Eq.2)$$

Artesian well water was used for negative controls, and 2.00 ppm of niclosamide was used for positive controls which was prepared by mixing 10 mL of 100-ppm stock solution and 500 mL of well water.

Preparation of Test Solutions for Simulated Field Assays

For simulated field assays, six concentrations were used, namely 13.2, 22.0, 30.7, 39.3, 47.8, and 56.3 ppm. These concentrations were prepared by mixing 12, 20, 28, 36, 44, and 52 mL of *tagpo* extract and 2000 mL of Artesian well water, respectively. Artesian well water was used as negative control, while 2.00 ppm of niclosamide was used as positive control which was made by mixing 40 mL of 100-ppm niclosamide stock solution and 2000 mL of Artesian well water. Each test concentration was paired with negative and positive controls.

Preparation of Test Solutions for Fish Toxicity Assays

For toxicity assays, eight concentrations were used: 1, 3, 5, 6, 7, 8, 9, and 11 ppm. These concentrations were prepared by adding 0.23, 0.68, 1.13, 1.35, 1.58, 1.81, 2.03, and 2.49 mL of *tagpo* extract, by means of an automatic pipet, into 500 mL of distilled water, respectively. Each test concentration was paired to negative and positive controls. Distilled water was used as negative control, whereas 1 ppm of niclosamide was used as positive control (5 mL of 100 ppm stock dissolved in 500 mL of distilled water).

Preparation of Test Solutions for Synergy Experiments

For synergism experiments, a different batch of *tagpo* powder was used; hence, new dose-response data was collected for *tagpo* extract and niclosamide and was the basis of LC_{50} used in mixed *tagpo* extract-niclosamide assays. This ensures that the statistical inference is valid. For synergism experiments, the niclosamide concentrations used were 0.40, 0.60, 0.81, 1.01, 1.26, 1.51, 2.02, and 2.52 ppm. To prepare these niclosamide solutions, 2, 3, 4, 5, 6.25, 7.5, 10, and 12.5 mL of 101 ppm stock solution of niclosamide was added to Artesian well water to make a final volume of 500 mL, respectively. A different equation (Eq. 3) was used to calculate the final concentrations of test solutions, since for synergy experiments, the final solution volume was fixed at 500 mL. All solutions were made using 500-mL volumetric flasks.

$$Concentration\ (ppm) = \frac{Y\ conc.\ (in\ ppm) \times volume\ of\ Y\ (mL)}{500} ppm \qquad (Eq.3*)$$

* Y is either concentration of niclosamide stock solution or *tagpo* extract in ppm.

For *tagpo* extract, the following concentrations were used: 13.3, 17.8, 22.2, 26.7, 31.1, 35.6, 40.0, and 44.4 ppm. These concentrations were made by mixing 3, 4, 5, 6, 7, 8, 9, and 10 mL of *tagpo* extract with Artesian well water to make a final volume of 500 mL, respectively. To prepare different niclosamide solutions in 24 ppm of *tagpo* extract, 2, 3, 4, 5, 6.25, 7.5, and 10 mL of a 103.3 ppm niclosamide stock solution was added to distilled water. Each of these concentrations was supplemented with 5.5 mL of *tagpo* extract and the solution was diluted to make a 500-mL final solution volume. To prepare *tagpo* extract solutions supplemented with 1.00 ppm niclosamide, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0 mL of *tagpo* extract was added each with 5.0 mL of 100 ppm niclosamide stock solution and each was diluted with distilled water. The concentration of niclosamide and *tagpo* extract in synergy assays were based on their respective LC_{50} values. In all four assays, each test concentration was paired with a negative control made from only Artesian well water.

Bioassays

In all assays, there were as many negative control setups as the number of test concentrations for each assay type or test substance. The laboratory, simulated field, and toxicity assays were performed in three trials using three, four, or five replicates per test concentration. For both the laboratory and simulate field assays, snails were exposed to test solutions for 24 h and observed for initial reactions in the first 5 min. Afterwards, the snails were washed with well water and allowed to recover in favorable conditions for another 24 h. The snails were assessed for mortality after the recovery stage using motility criterion. With a needle, the snail foot muscle was prodded and the slightest reaction to prodding was used to count live snails, else they were counted as dead. For laboratory assays, tests were performed using 500 mL of test solution placed in 1.5-L polyethylene terephthalate bottles (soda bottles). For simulated field assays, tests were performed using 2 L of test solution and nonsterile clay loam soil collected from a rice field in Sta. Fe, Leyte. Ten-day-old rice seedlings were sown in the soil 5 cm apart. Tests were performed using plastic boxes with the following dimensions: 20 x 18 x 28 cm (L x W x H) and soil volume of 2753 cm^3 which corresponded to a soil height of 7.6 cm. For toxicity assays, tilapia fries were exposed for 48 h. Dead and moribund fish fries were separated every 3 h. Fish fries were considered dead or moribund if they were unable to swim and were unresponsive to moderate prodding. The loss of equilibrium, defined as the inability to move in a coordinated manner and maintain a normal upright posture, was not considered as mortality, although it was noted.

Statistical Treatment

All statistical analyses were performed in R (Ritz et al. 2015; R Core Team 2018; Gomez and Anacta 2020). LC_{50} and LC_{90} estimations were performed via Probit analysis. Lethal

ratio tests were also done. Interpretation of lethal ratio tests was based on the confidence interval of the ratio, where if the value 1.000 is included in the interval, the difference between the lethal concentrations compared was interpreted as not significant. All statistical tests were assessed at a 95% confidence level.

RESULTS AND DISCUSSION

Table 1 summarizes the effect of different concentrations of *tagpo* extract on GAS under laboratory conditions. Results showed that snail mortality was directly proportional to *tagpo* extract concentration. These results were reproduced all in three trials. A maximal molluscicidal effect was observed at 43.6 ppm extract, whereas no snail mortality was observed in all negative controls (water only). The initial reactions of snails to the extract were loss of muscular control and excessive production of mucus. At high *tagpo* extract concentration, snails had difficulty adhering to the container walls and they immediately closed their operculum. To assess the effect of soil on the bioactivity of *tagpo* extract, the molluscicidal efficacy of *tagpo* extract was also evaluated under simulated field conditions.

Table 1. Molluscicidal efficacy of *tagpo* extract against golden apple snails (GAS) under laboratory conditions (*N* = 1 140 snails).

Concentration, ppm	Mortality rate			Mean mortality rate
	Trial 1[a]	Trial 2[b]	Trial 3[b]	
13.2	26.0	20.0	20.0	22.0
17.6	76.0	65.0	52.5	64.5
22.0	76.0	70.0	67.5	71.2
26.4	84.0	82.5	90.0	85.5
35.0	98.0	97.5	95.0	96.8
43.6	100.0	100.0	100.0	100.0
Negative control[c]	0	0	0	0
Positive control[d]	100.0	100.0	100.0	100.0

[a]five replicates per trial, 10 snails per replicate
[b]four replicates per trial, 10 snails per replicate
[c]water only, six replicates per trial, 10 snails per replicate
[d]2.0 ppm niclosamide, six replicates per trial, 10 snails per replicate

Mimicking the field conditions during the simulated field assay reduced the efficacy of *tagpo* extract (Table 2). The dose-dependent snail mortality was still evident, although the maximal molluscicidal activity of the extract was observed at a higher concentration (56.3 ppm) compared to that of the laboratory assay (43.6 ppm). These results were reproduced in all three trials.

Apart from molluscicidal efficacy, the toxicity of *tagpo* extract was also assessed using tilapia fries (*Oreochromis niloticus*) (Table 3). The data revealed that *tagpo* extract had a maximal fish-killing effect at 9 ppm. For comparison, the modelled dose-response curves for *tagpo* extract in the laboratory, simulated field, and toxicity assays are presented in Fig. 3. Tilapia fries were shown

Table 2. Molluscicidal efficacy of *tagpo* extract against GAS under simulated field conditions (*N* = 1 440 snails).

Concentration, ppm	Mortality rate			Mean mortality rate
	Trial 1	Trial 2	Trial 3	
13.2	3.3	5.0	5.0	4.4
22.0	13.3	36.7	6.7	18.9
30.7	58.3	71.7	66.7	65.6
39.3	76.7	86.7	86.7	83.3
47.8	86.7	93.3	95.0	91.7
56.3	100.0	100.0	100.0	100.0
Negative control[a]	0	0	0	0
Positive control[b]	98.3	100.0	100.0	99.4

[a]water only, six replicates per trial, 10 snails per replicate.
[b]2.0 ppm niclosamide, six replicates per trial, 10 snails per replicate.

Table 3. Toxicity of *tagpo* extract against tilapia fries under laboratory conditions (*N* = 1 050 tilapia fries).

Concentration, ppm	Mortality rate			Mean mortality rate
	Trial 1	Trial 2	Trial 3	
1	30.0	6.7	10.0	15.6
3	23.3	10.0	6.7	13.3
5	26.7	33.3	20.0	26.7
6	43.3	43.3	36.7	41.1
7	50.0	46.7	53.3	50.0
8	90.0	80.0	66.7	78.9
9	100.0	100.0	100.0	100.0
11	100.0	100.0	100.0	100.0
Negative control[a]	1.3	5.0	0	2.1
Positive control[b]	100.0	100.0	100.0	100.0

[a]water only, eight replicates per trial, 10 tilapia fries per replicate
[b]2.0 ppm niclosamide, eight replicates per trial, 10 tilapia fries per replicate

to be more susceptible than GAS to the activity of *tagpo* extract (green curve). The farther the curve is to the left, the more potent the effect of the extract to that organism or in that assay type. The molluscicidal efficacy of *tagpo* extract was greater under laboratory conditions (blue curve) compared to simulated field conditions (red curve).

To compare the quantitative effect of *tagpo* extract on GAS and tilapia fries, lethal concentrations of *tagpo* extract at 50% and 90% mortality were calculated using Probit analysis. The LC_{50} values of *tagpo* extract, presented with their standard error, were 5.36 ± 0.21, 16.85 ± 0.41, and 27.83 ± 0.53 ppm for toxicity assay, molluscicidal lab assay, and molluscicidal simulated field assay, respectively. The LC_{90} values, in the same assay sequence, were 13.17 ± 1.01, 27.39 ± 0.98, and 42.04 ± 1.17 ppm, respectively (Table 4).

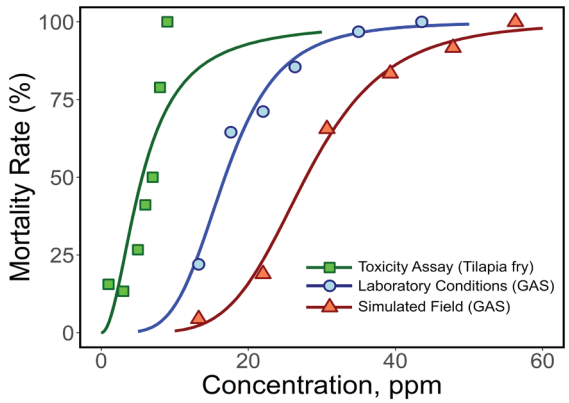

Fig. 3. Dose-response curves for *tagpo* extract in different bioassays. Green curve: toxicity assay against tilapia fries (*Oreochromis niloticus*); blue curve: molluscicidal assay under laboratory conditions against GAS; red curve: molluscicidal assay under simulated field conditions against GAS.

Table 4. Lethal concentrations in parts per million of *tagpo* extract against GAS and tilapia fries.

Organism	Assay conditions	LC_{50} ± SE	LC_{90} ± SE	*p*-value
GAS	Laboratory	16.85 ± 0.41	27.39 ± 0.98	0.236[a]
GAS	Simulated field	27.83 ± 0.53	43.04 ± 1.17	< 0.05[b]
Tilapia fry	Laboratory	5.36 ± 0.21	13.17 ± 1.01	< 0.05[c]

LC_{50} – Half-maximal lethal concentration; LC_{90} – Maximal lethal concentration; SE – Standard error
[a] χ^2 = 16.668; df = 16
[b] χ^2 = 40.312; df = 16
[c] χ^2 = 254.51; df = 22

Table 5 presents the results of lethal ratio tests among pairs of LC_{50} values of *tagpo* extract in different assays. This statistical test assesses if the difference between two lethal concentrations can be attributed to random errors by looking at the confidence interval of the lethal ratio (Robertson et al. 2017; Gomez and Anacta 2020). If the value 1.000 is included in the confidence interval, then the difference in paired LC_{50}s in question is interpreted as not significant. The ratio tests revealed that the difference in LC_{50} values of *tagpo* extract in any pair of assays was significant, meaning that the extract concentration that can effectively kill 50% of tilapia fries is lower compared to that needed for 50% snail mortality.

The results of molluscicidal assays and lethal ratio tests support *tagpo* extract's potent bioactivity against GAS. Since its LC_{90} is less than 100 ppm, the extract's active components may be isolated and characterized further. According to WHO, plant-based molluscicides with LC_{90} lower than 100 ppm may be further developed by field testing and active component identification (WHO 1983). In this study, the LC_{90} values of *tagpo* extract in the laboratory and simulated field conditions were shown to be significantly different. The decrease in efficacy under simulated field conditions may be attributed to

Table 5. Lethal concentration ratios of *tagpo* extract against GAS (laboratory and simulated field conditions) and against tilapia fries.

Assay pair	Lethal ratio (LC_{50}s)	Confidence interval[a]	Interpretation[a]
Sim : Lab	1.653	1.553–1.753	Significant
Sim : Tox	5.193	4.750–5.635	Significant
Lab : Tox	3.142	2.859–3.426	Significant

Sim – Simulated field conditions; Lab – Laboratory conditions; Tox – Toxicity assay
[a] Evaluated at 95% confidence level

the microbial degradation and adsorption of active component onto the soil. Slow-release delivery vehicles that can protect the active ingredient from microorganisms or serve as a physical barrier to mud may be developed in the future. The results underscore the importance of evaluating the efficacy of plant extracts under field conditions as candidate substances can have very different activities in the laboratory and in the field.

Tagpo fruits are small which may be limiting since more will be required for actual use. However, this limitation may be compensated by the number of fruits borne by the plant. *Tagpo* bears 2000–5000 fruits per plant which are available from December to May (Muñoz and Ackerman 2011). This plant can be reproduced by stem propagation, bearing fruits after 2 yr (Roh et al. 2005). For every kilogram of fresh *tagpo* fruits (about 4500 fruits), 86 g of *tagpo* powder can be obtained.

Since this study has also established that *tagpo* extract is toxic to tilapia fries, the extract if used in rice fields should not be drained immediately but should be allowed to degrade naturally. Notable at high *tagpo* extract concentration, snails immediately closed their operculum upon contact with the extract. This is a favorable property for a molluscicide as it could protect rice seedlings, especially in direct seeded rice farming. Joshi et al. (2008) observed that the ability of quinoa saponins to protect 5-d-old rice seedlings was correlated with the snails' response to the extract. They found that the extract caused the immediate closing of snail operculum upon contact and that the maximal snail mortality was observed after 48 h. Clearly, more studies are needed, such as determining the active components in the extract, assessing the effect of *tagpo* extract on the growth of rice seedlings, studying the histopathological effect of the extract on GAS and tilapia, and decreasing its toxicity to non-target organisms.

The active component in *tagpo* extract is suggestive of a saponin since the extract produces froth when shaken with water. Thus, the saponin content of the extract was determined spectrophotometrically. Based on three trials, the mean saponin content of crude fruit powder was 91.64 mg aescin equivalent per gram powder (Table 6). Saponins are believed to lower the surface tension of water and block the breathing process of snails (Musman 2010).

Table 6. Spectrophotometric determination of saponin in *tagpo* extract as milligram aescin equivalent per gram powder.

Trial 1	Trial 2	Trial 3	Mean
102.41	82.79	89.73	91.64

Calibration curve R^2 = 0.99

This study assessed the synergism between *tagpo* extract and niclosamide to show that the lethal concentration of either substance can be decreased in the presence of the sublethal dose of the other. First, the LC_{50} values of *tagpo* extract and niclosamide were determined, which were then used as the basis of concentration of molluscicide mixtures. This was followed by the determination of the effect of different concentrations of *tagpo* extract supplemented with a fixed sublethal concentration (at or near LC_{50} value) of niclosamide, and vice versa (Table 7). Three of these data sets are plotted in Fig. 4. The dose-response data for niclosamide, *tagpo* extract, and *tagpo* extract in 1.0 ppm niclosamide are dose-dependent, while for niclosamide in 24 ppm *tagpo* extract, a maximal effect was observed even at the lowest concentration prepared (0.41 ppm, 97%). As seen in Fig. 4B, the dose-response curve for *tagpo* extract in 1.0 ppm niclosamide has shifted to the left relative to the curve for *tagpo* extract, implying that the mortality rate of snails has increased in the presence of sublethal concentration of niclosamide.

Table 7. Dose-response data for synergy experiments.

Niclosamide		*Tagpo* extract		Niclosamide in 24 ppm TE		*Tagpo* extract in 1.0 ppm NIC	
Conc.	MR	Conc.	MR	Conc.	MR	Conc.	MR
0.40	10.0	13.3	10.0	0.41	96.7	4.4	16.7
0.60	16.7	17.8	5.0	0.62	96.7	8.9	40.0
0.81	26.7	22.2	18.3	0.83	100.0	11.1	50.0
1.01	50.0	26.7	56.7	1.03	100.0	13.3	63.3
1.26	73.3	31.1	70.0	1.29	100.0	15.5	80.0
1.51	86.7	35.6	83.3	1.55	100.0	17.8	83.3
2.02	93.3	40.0	81.7	2.07	100.0	22.2	96.7
2.52	100.0	44.4	90.0	-	-	-	-
Neg.	0	Neg.	0	Neg.	0	Neg.	0

Conc. – concentration in ppm; MR – mortality rate in %; Neg. – negative control
TE – *tagpo* extract; NIC – niclosamide

Table 8 presents the lethal concentrations of substances used in synergy experiments. The LC_{50} values for niclosamide, *tagpo* extract, and *tagpo* extract in 1.0 ppm niclosamide were 0.96 ± 0.05, 27.00 ± 0.71, and 9.87 ± 0.68 ppm, respectively. The corresponding LC_{90} values in the same substance sequence were 1.77 ± 0.15, 42.94 ± 2.00, and 22.39 ± 2.61 ppm, respectively. In this study, the lethal concentrations calculated for niclosamide against GAS are consistent with the results in some published

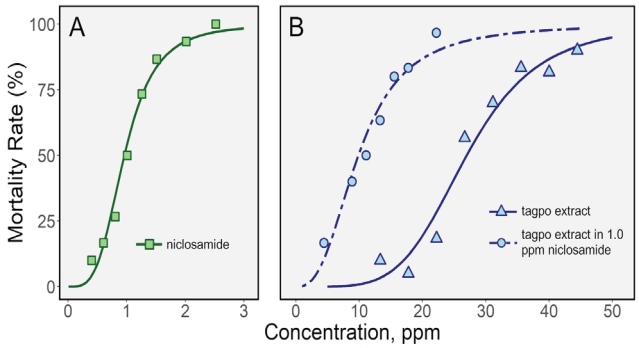

Fig 4. A: Dose-response curve for niclosamide under laboratory conditions against GAS; and B: Dose-response curves for *tagpo* extract and *tagpo* extract in 1.0 ppm niclosamide under laboratory conditions against GAS.

Table 8. Lethal concentrations in ppm against GAS.

Test substances	LC_{50} ± SE	LC_{90} ± SE	*p*-value
Niclosamide	0.96 ± 0.05	1.77 ± 0.15	0.8291[a]
Tagpo extract	27.00 ± 0.71	42.94 ± 2.00	0.2111[b]
Niclosamide in 24 ppm *tagpo* extract	*nd*	*nd*	*nd*
Tagpo extract in 1.0 ppm niclosamide	9.87 ± 0.68	22.39 ± 2.61	0.1267[c]

LC_{50} – Half-maximal lethal concentration; LC_{90} – Maximal lethal concentration
SE – Standard error; *nd* – not determined
[a] χ^2 = 15.729; df = 22
[b] χ^2 = 23.62; df = 19
[c] χ^2 = 50.337; df = 40

articles, such as Palis et al. (1997). The LC_{50} lethal ratio and its confidence interval, between *tagpo* extract and *tagpo* extract in 1.00 ppm niclosamide, were 2.74 (2.34 – 3.13) ppm, respectively (Table 9). This means that the LC_{50} of *tagpo* extract is 2.70 times higher than that of the *tagpo*-niclosamide mixture. The efficacy of the mixture was more than doubled which means that the combined effect of its components is more than the sum of their individual activity, which is the definition of synergism. For niclosamide in 24 ppm *tagpo* extract, a maximal effect was already observed at the lowest niclosamide concentration, providing another evidence of potential synergism.

The results of these experiments revealed that combining *tagpo* extract and niclosamide can result in a synergistic effect in killing GAS under laboratory conditions. This means that the lethal concentration of both substances could be lowered when applied as a mixture, which may lead to lower toxicity to non-target organisms and a lower biomass requirement for *tagpo*. Although the LC_{50} value of *tagpo* extract in 1.00 ppm niclosamide against GAS (9.87 ± 0.68 ppm) is still higher than the LC_{50} value of *tagpo* extract against tilapia fries (5.36 ± 0.21 ppm), the results of this study support that development of synergistic molluscicide formulations can be a viable strategy

Table 9. Lethal concentration ratios of *tagpo* extract and niclosamide against GAS under laboratory conditions.

Assay pair	Lethal ratio (LC_{50}s)	Confidence interval[a]	Interpretation[a]
Tag : Tag-Nic	2.735	2.340–3.130	Significant
Tag : Nic	28.137	25.044–31.229	Significant
Tag-Nic : Nic	10.288	8.579–11.997	Significant

Tag – *Tagpo* extract; Tag-Nic – *Tagpo* extract in 1.00 ppm niclosamide; Nic – Niclosamide
[a] Evaluated at 95% confidence level

towards decreasing the lethal concentration of potentially toxic substances. Taguiling (2015) showed that the fruit extract of *Sandoricum vidalii* and the bark extract of *Harpulia arborea* and a *Parkia* species can be combined to make a more potent molluscicidal mixture. Another study revealed that mixed nerium and tobacco plant extracts and two ternary mixtures (nerium-tobacco-piper and nerium-tobacco-neem formulations) were found to have the best molluscicidal activity against GAS after screening different combinations of six plant extracts, namely neem (*Azadirachta indica* essential oil), tobacco (*Nicotiana tabacum* leaves), nerium (*Nerium indicum* leaves), pongamia (*Pongamia pinnata* essential oil), zinger (*Zingiber officinale* rhizome), and piper (*Piper nigrum* seeds) (Prabhakaran et al. 2017). The results of this study and those published on synergistic formulations support the notion that mixing different substances can be a viable strategy in molluscicide development.

CONCLUSION

This study assessed the potential of *tagpo* (*Ardisia tomentosa*) crude fruit extract as molluscicides against golden apple snails (GAS). Toxicity testing using tilapia fries and probing of synergism between the extract and niclosamide were also performed. Results showed that *tagpo* extract had a maximal snail-killing effect at less than 45 ppm in both the laboratory and simulated field conditions; however, it showed acute toxicity to tilapia fries. Moreover, mixing *tagpo* extract and niclosamide resulted in improved efficacy as molluscicides compared to when each substance was used alone. Taken together, these results imply that *tagpo* extract is a novel and potent molluscicide and that combining established and experimental molluscicides can have a synergistic effect to kill GAS. Thus, isolation and structure determination of active components in *tagpo* extract are warranted and further studies on the molluscicidal synergism of other plant extracts are encouraged.

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