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Development and Evaluation of Poly Herbal Molluscicidal Extracts for Control of Apple Snail (*Pomacea maculata*)

Guruswamy Prabhakaran *, Subhash Janardhan Bhore and Manikam Ravichandran

Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Bedong-Semeling Road, Semeling, 08100 Bedong, Kedah, Malaysia; subhashbhore@gmail.com (S.J.B.); mravichandran08@gmail.com (M.R.)
* Correspondence: prabhakaran@aimst.edu.my; Tel.: +60-166-740-205

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Abstract: Golden Apple Snail (GAS) is the most destructive invasive rice pest in Southeast Asia. The cost of synthetic molluscicides, their toxicity to non-target organisms, and their persistence in the environment have propelled the research of plant-derived molluscicides. Most research efforts have focused on individual plant extracts for their molluscicidal potency against GAS and have not been proven to be entirely effective in rice field conditions. Selective combination of synergistically acting molluscicidal compounds from various plant extracts might be an effective alternative. In this direction, ethanolic extracts from six different plants (Neem, Tobacco, Nerium, Pongamia, Zinger, and Piper) were evaluated against *Pomacea maculata* Perry. Of the various combinations studied, a binary extract (1:1) of nerium and tobacco (LC_{90} 177.71 mg/L, 48 h), and two tri-herbal extract formulations (1:1:1) of (nerium + tobacco + piper) and (nerium + tobacco + neem) were found to be most effective, with LC_{90} values of 180.35 mg/L and 191.52 mg/L, respectively, in laboratory conditions. The synergistic effect of combined herbal extracts resulted in significant reduction in LC_{90} values of the individual extracts. The findings of this study demonstrate that the selective combinations of potent molluscicidal herbal extracts are effective for management of *P. maculata* under laboratory conditions.

Keywords: golden apple snail; plant molluscicides; poly herbal extracts; *Pomacea maculata*

1. Introduction

Rice is a staple food and an essential crop grown worldwide, with Asia being the largest producer and consumer. Golden Apple Snail (GAS), a major rice pest, has a voracious appetite for the young rice seedlings of both transplanted and direct-seeded rice [1]. Infestations by GAS are responsible for a huge economic loss of $1.47 billion per annum in rice production [2]. *Pomacea canaliculata* Lamarck is the most pervasive; it is destructively invasive in most rice growing regions of the world and amongst the world's top 100 worst invasive alien species [3]. The distribution of apple snails is more pronounced in Peninsular Malaysia due to the method of paddy cultivation, which is predominately (90%) by wet paddy method. Among the two species of apple snails, *Pomacea maculata* is more abundant than *P. canaliculata* in the states of Kedah and Perlis, the two major paddy growing regions which produce more than 50% of rice in Malaysia [4,5]. Hence, the present study was focused on *P. maculata*. The Integrated Pest Management (IPM) strategies for the control of GAS are based on the use of cultural approaches, biological methods, snail predators (ducks and fish), by water management and chemical control measures [6,7].

The application of synthetic molluscicide formulations based on niclosamide and metaldehyde is widely practiced for effective control of snails. Niclosamide has become the mainstay of GAS control and eradication programs [8]. Synthetic molluscicides are known for their knock-down effect; however, their negative impact on the environment and the high costs of application have stimulated interest in the search for plant-derived molluscicides which are target specific, and environmentally and toxicologically safe [9–11]. Botanical pesticides have long been explored as effective alternatives to synthetic chemical counterparts due to their low toxicity to non-target organisms, their biodegradability, and due to them being less expensive in their crude form, in addition to the prevention of development of resistance against phytochemical mixtures [12–15]. Moreover, botanical pesticides are easily obtainable and more associated with indigenous self-sufficient pest snail control strategies than their imported synthetic pesticides.

Molluscicidal properties have been reported in more than 1400 plant species [16]. In the recent past, most research efforts were focused on individual plant extracts for their molluscicidal potency against GAS. The different plants (*Annona squamosa* seed, *Nerium indicum* leaves, *Stemona tuberose* root, *Cyperus rotundus* corm, and *Derris elliptica* root) were evaluated [17]. The results indicated that *D. elliptica* and *C. rotundus* (LC_{50} 23.68 mg/L and 133.20 mg/L, respectively) showed the highest toxicity on GAS, while Vulgarone B, a sesquiterpene isolated from *Artemisia douglasiana*, showed 100% mortality of GAS at a concentration on par with metaldehyde [18]. The molluscicidal potency of methanol extract from neem seed (*A. indica* A. Juss), oleoresin of *Zingiber officinale*, and cyclotide extracts of *Oldenlandia affinis* and *Viola odorata* on GAS was ascertained [19–22]. The extract of neem leaves and garlic (*Allium sativum*) recorded more than 90% mortality at 1000 mg/L on GAS [23]. The toxicity of different solvent extracts of *Agave sisalana* on *P. canaliculata* was established by Li et al. [11].

The extracts of *Cymbopogon citratus* induced alterations in growth and development of *P. canaliculata* and resulted in high mortality of snails [24,25]. The ethyl acetate soluble fraction of *Aglaia duperreana* was effective against GAS [26]. In rice field conditions, the molluscicidal efficacy of chemically modified quinoa (*Chenopodium quinoa*) saponins [27], methanol extract of *Camellia oleifera* seed meal [28], and *Sapindus saponaria* extract [29] were tested against GAS. The application of tobacco waste in field trials proved its efficacy with 100% mortality of GAS [30]. However, the vast majority of individual plant extracts were not proven to be entirely effective in rice field conditions. Therefore, to leverage the cumulative benefits of synergistically acting phytochemical mixtures, a selective combination of potent molluscicidal compounds from various plant extracts might be an effective alternative against apple snails.

2. Materials and Methods

2.1. Collection of Snails (Pomacea maculata)

Snails with shell length of 10 mm to 30 mm were collected from a snail-infested rice field located in Semeling, Kedah, Malaysia. The snails were identified based on the conchological characters [31,32]. The identification of species was confirmed by officials at the District Agriculture Office, Sungai Petani, Kedah, Malaysia. The shell length of each snail was measured with a Vernier calliper. The snails were acclimated in 20 litre laboratory aquarium tank (60 × 30 × 30 cm) containing dechlorinated tap water at room temperature (25 ± 2 °C). The snails were fed with fresh lettuce (*Lactuca sativa)* which was maintained ad libitum by feeding them two to three times a week according to their consumption, in order to avoid bacterial growth and water fouling [33,34].

2.2. Collection of Plant Materials

A total of 5 kg fresh leaves of *Nerium indicum* and *Nicotiana tabacum* were collected from Sungai Petani and Jitra, Kedah, Malaysia. The plants were taxonomically identified and authenticated by the botanist at the Biotechnology Department of AIMST University, Malaysia. Three kilograms of fresh rhizome of *Zingiber officinale* and 0.5 kg of black piper seeds (*Piper nigrum*) were purchased from the local TESCO supermarket. One kilogram of pongamia oil (*Pongamia pinnata*) and neem oil (*Azadirachta indica)* were obtained from Spic Ltd., Chennai, Tamil Nadu, India. The leaves of *N. indicum* and *N. tabacum* were thoroughly cleaned with running tap water and shade dried for three weeks at room temperature (25 ± 2 °C). The zinger rhizome was chopped into small pieces and shade dried for four weeks. The dried materials were ground into coarse powder using a food blender and stored in air-tight containers. The details of plant materials evaluated for their molluscicidal properties are given below in Table 1.

Table 1. Plant materials evaluated for molluscicidal properties.

No.	Scientific Name	Family	Parts Used
1	*Azadirachta indica* A. Juss.	Meliaceae	Neem kernel oil (cold processed)
2	*Nicotiana tabacum* L.	Solanaceae	Leaves
3	*Nerium indicum* Mill.	Apocynaceae	Leaves
4	*Pongamia pinnata* L.	Fabaceae	Seed oil
5	*Zingiber officinale* L.	Zingiberaceae	Rhizome
6	*Piper nigrum* L.	Piperaceae	Seeds

2.3. Preparation of Plant Extracts

The 100 g of dried and coarse plant material was macerated in 1000 mL of 95% ethanol at room temperature (25 ± 2 °C) for three days. The 95% ethanol was used to maintain the homogeneity in the individual plant extracts and to enhance the compatibility among the plant extracts for development of poly herbal formulations. The crude extract was separated by filtering under vacuum using No. 1 Whatman filter paper (Camlab Limited, Cambridge, UK). The retentate was then added to 1000 mL of fresh 95% of ethanol, macerated for three days further at room temperature, and filtered. Both the filtrates were pooled together and concentrated to dryness using a rotary evaporator at 60 °C until the solvent was completely evaporated. The pongamia oil and neem oil were extracted twice with equal volume of 95% of ethanol, and ethanol layers were pooled and concentrated under vacuum in a rotary evaporator. The ethanolic extract of individual plants obtained was labelled and stored at 5 °C until evaluation of molluscicidal activities and analysis of phytochemicals.

2.4. Evaluation of the Molluscicidal Potency of Plant Extracts

Evaluation of molluscicidal activity of plant extracts against juvenile snails of *P. maculata* was performed as per the World Health Organization (WHO) guidelines [35]. The different concentrations of individual, binary, and poly plant extracts were prepared to test against the snails. A stock solution of individual crude plant extract was prepared by dissolving 1 g of each extract in 1000 mL of distilled water to make it a 0.1% concentration which constitutes 1000 mg/L. From this stock solution, a 0.01% (100 mg/L) test solution was made by tenfold dilution with dechlorinated tap water. Similarly, the required concentrations (200 mg/L to 500 mg/L) were prepared from the 0.1% stock solution.

2.5. Preparation of Binary, Tri, and Poly Herbal Combinations of Plant Extracts

The plants examined in this study were selected on the basis of ethnobotanical information and recognized molluscicidal activity on *P. canaliculata* as reported in previous studies [17,21,22,30]. The following six different binary combinations of plant extracts were evaluated (Table 2). The different concentrations of binary plant extracts (100 mg/L–500 mg/L) at the ratio of 1:1 *v*/*v* were prepared from 0.1% stock solutions of individual extracts with appropriate dilution with dechlorinated tap water.

Table 2. Binary combination of plant extracts.

No	Plant A	Plant B
1	Nerium (*Nerium indicum*)	Tobacco (*Nicotiana tabacum*)
2	Nerium (*Nerium indicum*)	Piper (*Piper nigrum*)
3	Nerium (*Nerium indicum*)	Neem (*Azadirachta indica*)
4	Tobacco (*Nicotiana tabacum*)	Piper (*Piper nigrum*)
5	Tobacco (*Nicotiana tabacum*)	Neem (*Azadirachta indica*)
6	Piper (*Piper nigrum*)	Neem (*Azadirachta indica*)

The selective combinations of tri-herbal extracts at 1:1:1 ratio (*v*/*v*) were prepared by combining individual plant extract test solutions for the evaluation against *P. maculata* (Table 3). Five different concentrations of tri-herbal extracts (50 mg/L to 250 mg/L) were prepared from 0.1% stock solution of individual extract with appropriate dilution with dechlorinated tap water. Similarly, different concentrations (50 mg/L to 250 mg/L) of poly herbal extract test solutions with nerium, tobacco, piper and neem were prepared at the ratio of 1:1:1:1 (*v*/*v*) by combining the 0.1% stock solution of appropriate plant extracts.

Table 3. Tri-herbal combinations of plant extracts.

No.	Plant A	Plant B	Plant C
1	Nerium	Tobacco	Piper
2	Nerium	Piper	Neem
3	Tobacco	Piper	Neem
4	Nerium	Neem	Tobacco

2.6. Preparation of Niclosamide Test Solutions

The synthetic chemical molluscicide niclosamide 70% wettable powder (WP) was used as the reference standard for comparison. The Bayluscide® WP 70 (Bayer, AG, Leverkusen, Germany) was purchased from the local market at Sungai Petani (Kedah, Malaysia) and was used as positive control in the experiments. Bayluscide consists of 81.4% *w*/*w* active ingredient (niclosamide ethanolamine), as indicated on the label. To prepare different concentrations of niclosamide (0.25 mg/L, 0.50 mg/L, 0.75 mg/L, and 1 mg/L), a stock solution of 10 mg/L was prepared and then diluted appropriately. Since 1 g of Bayluscide contains 0.814 g of niclosamide as active ingredient, 12.3 mg was dissolved in one litre of distilled water to obtain 10 mg/L stock solution. Tenfold dilution of the stock solution with dechlorinated tap water was prepared to obtain 1 mg/L stock solution. From this stock solution, two-fold dilutions were made to prepare 0.5 mg/L test solution.

2.7. Evaluation of Molluscicidal Potency of Plant Extracts

Groups of 10 uninfected juvenile snails (20 to 30 mm of shell length) were placed in plastic trays with 1000 mL of different concentrations of individual, binary, and poly herbal extracts test solutions separately. Five different concentrations of each test solution of the plant extracts were tested, each with three replicates of 10 snails. Three replicates were prepared with 0.5 mg/L niclosamide in dechlorinated water and used as positive control. Control experiments were performed with dechlorinated tap water alone (negative control). A total of 210 snails were used for the evaluation of an extract. All of the molluscicidal evaluations were carried out at room temperature (25 ± 2 °C) under normal diurnal lighting. The plastic trays were individually covered with a fine plastic mesh to prevent the snails from crawling out. Snails exposed to different concentrations of the plant extract were left for observation for 24 h and 48 h. After 48 h, the plant extract suspension was decanted; the snails were rinsed twice with dechlorinated tap water and transferred to a new container filled with dechlorinated tap water and observed for another 24 h, which served as the recovery period, following which the mortality rates were determined. The snails were not fed during the exposure and recovery periods. Upon observation, the dead snails were removed from the containers. Snails were considered dead if they did not move and either had retracted well into their shells or were hanging out of the shells. The death of each snail was further ascertained by the complete opening of operculum and if the head did not respond when pricked with a sharp needle. The mortality of snails was recorded for both exposure periods and the recovery period. The LC_{50} and LC_{90} values with their associated 95% confidence intervals (95% CI) were calculated by probit analysis using SPSS (Version 22, IBM Software Group, Chicago, IL, USA). The mortality of snails exposed to different concentrations of six selected plant extracts was analysed using a one-way analysis of variance (ANOVA) and mean comparisons were performed by Duncan's multiple comparison tests using SPSS (Version 22) at the 5% significant level.

3. Results

3.1. Molluscicidal Activity of Six Individual Plant Extracts

The molluscicidal activities of the six plants at different concentrations (100 mg/L to 500 mg/L) against *P. maculata* were evaluated. Neither behavioural symptoms observed nor death occurred in control groups with water, indicating that no factor other than plant moieties were responsible for the altered behaviour and mortality of snails. An analysis of the molluscicidal activities of the six plant extracts on *P. maculata* showed that there was a linear relationship between the concentrations of the individual extracts and the mortality of snails (Figure 1).

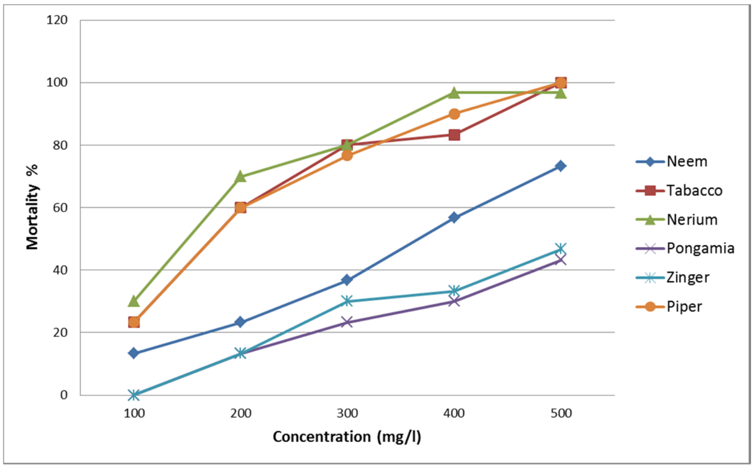

Figure 1. Molluscicidal activity of six plant extracts at different concentrations on *P. maculata*.

This observation was in agreement with the findings of Chauhan and Singh [36] and EI-Din et al. [37] wherein increases in the concentration of plant extracts resulted in high mortality rates of various pest snails. A similar positive correlation between the concentration of plant extracts (*Sandoricum vidalii, Harpulia arborea*, and *Parkia* sp.) and the mortality of *P. canaliculata* was reported by Taguiling et al. [38]. Likewise, a positive correlation between the neem seed crude extract concentrations and the mortality of *P. canaliculata* was observed [22]. In the present study, the LC_{50} and LC_{90} of *N. indicum*, *P. nigrum*, *N. tabacum*, and *A. indica* extracts were 179.36 and 341.57 mg/L, 202.01 and 359.89 mg/L, 205.70 and 375.84 mg/L, and 365.10 and 624.67 mg/L, respectively. Compared to the above four plants, *Z. officinale* and *P. pinnata* recorded higher LC_{50} and LC_{90} values (485.48 and 767.63 mg/L, 512.62 and 804.49 mg/L, respectively) and hence were not effective against snails. The results indicate that the snails were more sensitive to *N. indicum*, *P. nigrum*, and *N. tabacum* extracts, as evidenced by their lower LC_{50} and LC_{90} values indicating the highest potency. Eventually, the crude ethanol extract of *N. indicum* was found to be the most effective, with a LC_{90} value (341.57 mg/L, 48 h), whereas Dai et al. [39] recorded the lowest LC_{50} value (3.71 mg/L, 96 h) with purified cardiac glycosides of *N. indicum* for the control of *P. canaliculata.*

The result of the probit analysis on the mortality data of individual plant extracts on snails at LC_{50} and LC_{90} and fiducial limits are summarized in Table 4. Chi-square tests for probit analysis of Pearson Goodness-Fit test were carried out. A significant molluscicidal potency ($p < 0.001$) of nerium coincides with its LC_{90} value. A similar trend was observed with piper and tobacco. The heterogeneity value of less than 1 indicates that all the plant extracts exhibited a significant effect on the mortality of snails. Similarly, the heterogeneity value of less than 1 was correlated to the chi-squared values by Massaguni and Latip [22]. On comparative analysis, nerium extract was found to be the most effective, followed by piper and tobacco extracts. Niclosamide (0.5 mg/L) was used as a positive control, where 100% mortality was recorded in 24 h.

Table 4. Summary of probit analysis on the mortality data of six different plant extracts on *P. maculata.*

Plant Name	Hetero-Geneity	Chi Square	*p*	LC_{50}	Fiducial Limits (mg/L)		LC_{90}	Fiducial Limits (mg/L)	
					LL	UL		LL	UL
Azadirachta indica	0.999	3.863	0.920	365	321	420	624	541	768
Nicotiana tabacum	0.765	11.68	0.232	205	172	237	375	334	436
Nerium indicum	0.052	26.114	0.001	179	130	222	341	289	430
Pongamia pinnata	0.996	4.904	0.842	512	445	641	804	666	1111
Zingiber officinale	0.973	7.024	0.634	485	425	592	767	643	1028
Piper nigrum	0.704	12.575	0.183	202	170	232	359	320	417

LC: Lethal concentration. LL, UL: Lower and Upper Confidence Limit. Degree of freedom (df) = 9 for each concentration of individual plant extracts tested.

3.2. Molluscicidal Activity of Binary Combinations of Plant Extracts

A plant extract is considered to be effective at a concentration below 100 mg/L [40], whereas, the six different plant extracts investigated in this study were in the order of 341.57 to 804.50 mg/L for 90% mortality of the target snails. Therefore, in order to ascertain potent combinations of plant extracts which could bring down the concentration, different binary combinations of these plant extracts were formulated at 1:1 ratio by *v/v* and tested against *P. maculata*. The molluscicidal activity of six different binary combinations of plant extracts was compared at their LC_{90} values, and the results are depicted in Figure 2.

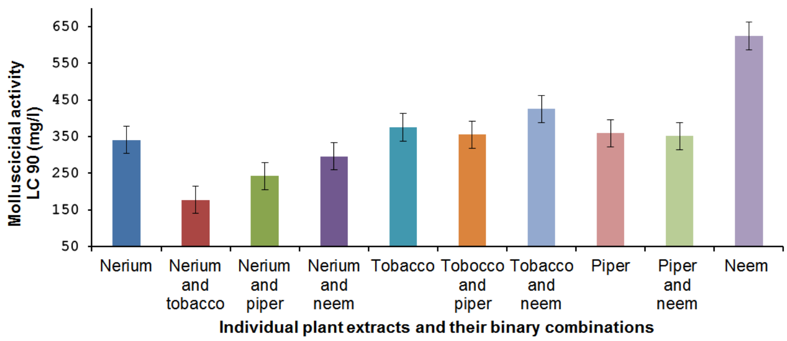

Figure 2. Comparison of molluscicidal activity (LC_{90}) of individual and binary combinations of plant extracts against *P. maculata*.

When two plant extracts were combined at a ratio of 1:1 (*w*/*w*), the individual plant extract's concentration would reduce to 50%. Therefore, it is expected that in the diluted form their combination would be needed in a high concentration for achieving 90% mortality of the snails. In this aspect, when LC_{90} values of individual extracts were compared with LC_{90} values of their binary combinations, the synergistic effect among four extracts such as (nerium + tobacco), (nerium + piper), (nerium + neem), and (tobacco + neem) was observed. This resulted in significant reductions in the LC_{90} values on the mortality of *P. maculata* in comparison to the individual extracts. Similarly, Chauhan and Singh [36] reported that the binary combination (1:1 ratio) of taraxerol from *Codiaeum variegatum* and acetone extract of *Euphorbia tirucalli* on *L. acuminata* increased thetoxicity by 9.51 times relative to the individual treatments. Furthermore, the binary combination of latex powder of *Euphorbia pulcherima* and *Jatropha gossypifolia* significantly reduced the fecundity, hatchability, and survival of young snails of *L. acuminata* [41]. The molluscicidal activity of six different binary combination of plant extracts on *P. maculata* is shown in Table 5.

Table 5. Molluscicidal activity of six different binary combinations of plant extracts on *P. maculata*.

Concentration (mg/L)	Percentage Mortality of *Pomacea maculata*					
	Binary Combination of Plant Extracts					
	Nerium and Tobacco	Nerium and Piper	Nerium and Neem	Tobacco and Piper	Tobacco and Neem	Piper and Neem
100	63.33 ± 5.7	63.33 ± 5.7	63.33 ± 5.7	36.60 ± 5.7	36.60 ± 5.7	36.60 ± 5.7
200	90.00 ± 10	76.60 ± 15.2	70.00 ± 10	66.60 ± 5.7	56.60 ± 5.7	56.60 ± 5.7
300	100 ± 0.00	93.30 ± 5.7	80.00 ± 10	80.00 ± 10	73.30 ± 5.7	76.60 ± 5.7
400	100 ± 0.00	100 ± 0.00	100 ± 0.00	93.30 ± 5.7	83.30 ± 5.7	93.30 ± 5.7
500	100 ± 0.00	100 ± 0.00	100 ± 0.00	96.60 ± 5.7	93.30 ± 5.7	100 ± 0.00

Each value is Mean ± standard deviation (SD) of three replicates (n = 10).

The binary combination of nerium and tobacco resulted in 90% mortality of snails, even at 200 mg/L. In comparison, nerium extract administered on an individual basis at 200 mg/L concentration recorded 70% mortality. At the same concentration, the tobacco extract recorded 60% mortality (Figure 1). The higher kill rate of the binary combination at the same concentration level suggests a synergistic effect. The binary combination of nerium and piper resulted in 93.3% mortality at 300 mg/L, whereas, the individual plant extracts of nerium and piper showed 80% and 76.7% mortality, respectively, at 300 mg/L. Nerium combined with neem at higher concentration (400 mg/L) exhibited an increase in mortality up to 100%. It was observed that in all cases where nerium was one of the components of binary combination, a higher mortality was recorded compared to any of the other combinations.

3.3. Molluscicidal Activity of Tri and Poly Herbal Extracts

Combining different plant species with effective molluscicidal properties is advantageous for the development of molluscicides. In the present study, among the six plant extracts evaluated individually as well as in binary combinations, *N. indicum, N. tabacum, P. nigrum*, and *A. indica* were found to be effective on the snails in laboratory conditions. When these four plant extracts were tested individually the LC_{90} values ranged from 341.57 mg/L to 624.67 mg/L, whereas, in their binary combinations significant reductions in the LC_{90} values (171.71 to 296.17 mg/L) were recorded. It is evident that the binary combinations of plant extracts resulted in approximately 45% reductions in LC_{90} values based on the mortality of *P. maculata* as well as demonstrating the synergistic effect among these plant extracts. Therefore, combining different plant species with effective molluscicidal properties is advantageous for further reducing the lethal concentrations against snails. In light of this, the selective consortiums of tri-herbal and poly herbal extracts were prepared by combining individual plant extracts at 1:1:1 to 1:1:1:1 ratio (*v*/*v*) and evaluated at various concentrations on *P. maculata.* The molluscicidal activity of tri and poly herbal combinations of plant extracts on *P. maculata* is shown in Table 6. The results indicate that the combination of three plant extracts (nerium + neem + tobacco) showed the highest mortality rate (93.3%) with the lowest LC_{50} and LC_{90} (62.60 mg/L and 191.52 mg/L, respectively) even at a lower concentration of 50 mg /L. In comparison, at the same concentration, the combined extracts of (nerium+ tobacco + piper) had 86.7% mortality and with LC_{50} (73.91 mg/L) and LC_{90} (180.35 mg/L) (Table 7).

Table 6. Molluscicidal activity of tri and poly herbal combination of plant extracts on *P. maculata*.

Concentration (mg/L)	Percentage Mortality of *Pomacea maculata*				
	Tri and Poly Herbal Combination of Plant Extracts				
	Nerium, Tobacco, and Piper	Nerium, Piper, and Neem	Tobacco, Piper, and Neem	Nerium, Neem, and Tobacco	Nerium, Tobacco, Piper and Neem
50	86.60 ± 5.7	46.66 ± 5.7	36.70 ± 5.7	93.30 ± 5.7	66.60 ± 15.2
100	96.60 ± 5.7	73.30 ± 5.7	70.00 ± 10	96.60 ± 5.7	83.30 ± 5.7
150	100 ± 0.00	86.60 ± 5.7	80.00 ± 10	100 ± 0.00	90.00 ± 10
200	96.60 ± 5.7	96.60 ± 5.7	86.70 ± 5.7	100 ± 0.00	100 ± 0.00
250	100 ± 0.00	96.60 ± 5.7	93.30 ± 5.7	96.60 ± 5.7	96.60 ± 5.7

Each value is Mean ± SD of three replicates (n = 10).

Table 7. Comparison of LC_{90} values of individual, binary, and tri-herbal extracts for their synergistic molluscicidal effects on *P. maculata.*

LC (mg/L) 48 h	Nerium	Tobacco	Piper	Neem	Nerium and Tobacco	Nerium, Tobacco, and Piper	Nerium, Tobacco, and Neem
LC_{50}	179.36	205.71	202.02	365.10	100.18	73.91	62.60
LC_{90}	341.57	375.84	359.90	624.67	177.71	180.35	191.52

LC: Lethal concentration.

All the tri-herbal combinations exhibited a significant ($p < 0.05$) increase in mortality from 86.7% to 100% at 200 mg/L concentration. The (nerium + neem + tobacco) and (nerium + tobacco + piper) emerged as the most effective tri-herbal combinations in terms of mortality of snails with LC_{90} 191.52 mg/L and 180.35 mg/L, respectively. The results of this study supported the findings of Taguiling [38] who reported that the activities of plant extracts vary significantly according to the species combinations and dosages against GAS. Further, the variations in the molluscicidal efficacy of plant extracts might be attributed to three major factors: species-tolerance, concentrations used, and the phytochemical constituents [42].

The piper was more effective with LC_{50} (202 mg/L) compared to neem (LC_{50} 365 mg/L) (Table 4). The binary combination of nerium and tobacco was found to be more effective with a LC_{50} value of 100.18 mg/L (Figure 2). Hence, it is obviously expected that a tri-herbal combination containing nerium, tobacco, and piper should be most effective. Therefore, the molluscicidal activities of individual, binary, tri-herbal, and poly herbal extract combinations were compared (Table 7). Notably, it was found that the tri-herbal combination made with nerium, tobacco, and neem proved to be the most effective, with a LC_{50} value of 62.60 mg/L. It is inferred that synergism among nerium, tobacco, and neem is higher than the nerium, tobacco, and piper combination.

4. Discussion

When the stock solutions of three plant extracts are combined at a ratio of 1:1:1 (v/v), there will be a dilution effect in the concentration of individual plant extracts. Therefore, such combinations should result in higher concentration requirement to achieve 90% mortality of snails. Even under the dilution effect if the plant extracts are synergistic, then the concentration of the plant extract required to achieve 90% mortality will be reduced. In the present study, the two tri-herbal extract combinations, namely (nerium + tobacco + neem) and (nerium + tobacco + piper), recorded low LC_{90} values, demonstrating a synergistic effect among the extracts of these plants. It is interesting to note that nerium and tobacco plant extracts are found to be common to all the effective tri-herbal extract combinations. The synergistic effect of these two extracts was also exhibited in their binary combination with LC_{90} of 177.71 mg/L and a similar trend was observed in their LC_{50} values (100.18 mg/L). Our results are in agreement with the research findings reported by Taguiling [38] who reported that the combination of extracts of three species (*S. vidalii* fruit and barks of *H. arborea* and *Parkia* sp.) at a ratio of 1:1:1 (w/w) was the most effective, with a mean mortality time of 6.56 min against adult GAS under laboratory and field trials. Furthermore, the binary combination of (*S. vidalii* + *Parkia* sp.) and (*H. arborea* + *Parkia* sp.)

recorded a mean mortality time of 8.11 min and 10.11 min, respectively [38]. A similar synergistic effect was reported with the binary combination of ferulic acid and azadirachtin against *Fasciola* larva in the snail *Lymnaea acuminata*, which was 64.28 times more effective than a single treatment with ferulic acid [43]. Rao and Singh [44] reported that the synergistic action in binary and tri-herbal combinations of *A. indica* and *Cedrus deodara* oil (1:7 ratio) and *A. indica*, piperonyl butoxide, and *C. deodara* at a ratio of 1:5:7 were found to be more toxic to *L. acuminata* than single treatment. Similarly, the binary (1:1) and tri-herbal (1:1:1) combinations of *Euphorbia pulcherima* latex powder, botulin, and taraxerol decreased significantly the LC_{50} dosages against snail *L. acuminata* [41].

Within five poly extracts studied only two combinations consisting of (nerium + neem + tobacco) and (nerium + tobacco + piper) were effective due to synergistic effects. This synergism may be attributed to the phytochemical constituents of individual plant extracts. Among the binary combinations, the nerium and tobacco extracts (LC_{90} 177.71 mg/L) was found to be the most effective against *P. maculate*, and this may be due to both the nicotine and cardiac glycosides content in these extracts. Glycogen is the primary and intermediate source of energy. The cardiac glycosides of *N. indicum* decreased acetylcholinesterase (AChE) activities and impaired the hepatopancreas tissues of *P. canaliculate*, resulting in the fatal inhibition of the activity of digestive enzymes as well as the feeding rate [39,45]. Nicotine acts by mimicking acetylcholine and it exerts a toxic effect in both vertebrates and invertebrates as a neurotoxin by binding to nicotinic acetylcholine receptors and inhibiting their penetration into the synapse [46]. The combination of lethal action in the active compounds of both nerium and tobacco possibly increased the snail mortality rate.

In summary, our study showed that of all the combinations of plant extracts evaluated, one binary combination (nerium and tobacco) and two tri-herbal combinations (nerium, tobacco, and neem) and (nerium, tobacco, and piper) were found to be the most effective against *P. maculata*. The study demonstrated the synergistic molluscicidal effect among the selectively combined crude extracts of *Nerium indicum*, *Nicotiana tabacum*, *Piper nigrum*, and *Azadirachta indica* for the effective control of *P. maculata* under laboratory conditions.

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References

1. International Rice Research Institute (IRRI), Rice Knowledge Bank. Available online: http://www.knowledgebank.irri.org/step-by-step-production/growth/pests-and-diseases/golden-apple-snails (accessed on 14 November 2016).
2. Nghiem, L.T.P.; Soliman, T.; Yeo, D.C.J.; Tan, H.T.W.; Evans, T.A.; Mumford, J.D.; Keller, R.P.; Baker, R.H.A.; Corlett, R.T.; Carrasco, L.R. Economic and Environmental Impacts of Harmful Non-Indigenous Species in Southeast Asia. *PLoS ONE* **2013**, *8*, e71255. [CrossRef] [PubMed]
3. GISD (Global Invasive Species Database); Invasive Species Specialist Group ISSG. The Global Invasive Species Database. Version 2015.1. Available online: http://www.issg.org/database (accessed on 1 June 2016).
4. Salleh, N.H.M.; Arbain, D.; Daud, M.Z.M.; Pilus, N.; Nawi, R. Distribution and Management of *Pomecae canaliculata* in the Northern Region of Malaysia: Mini Review. *APCBE Procedia* **2012**, *2*, 129–134. [CrossRef]
5. Arfan, A.G.; Muhamad, R.; Omar, D.; Nor Azwady, A.A.; Manjeri, G. Distribution of two *Pomacea* spp. in rice fields of Peninsular Malaysia. *Annu. Res. Rev. Biol.* **2014**, *4*, 4123–4136. [CrossRef]
6. Zhang, J.; Zhao, B.; Chen, X.; Luo, S. Insect Damage Reduction while Maintaining Rice Yield in Duck-Rice Farming Compared with Mono Rice Farming. *J. Sustain. Agric.* **2009**, *33*, 801–809. [CrossRef]

7. Liang, K.; Zhang, J.; Song, C.; Luo, M.; Zhao, B.; Quan, G.; An, M. Integrated Management to Control Golden Apple Snails (*Pomacea canaliculata*) in Direct Seeding Rice Fields: An Approach Combining Water Management and Rice-Duck Farming. *Agroecol. Sustain. Food Syst.* **2014**, *38*, 264–282. [CrossRef]
8. Plant Health Australia. *Contingency Plan: Golden Apple Snail*; Plant Health Australia: Deakin, Australia, 2009; p. 15.
9. Duke, S.O.; Cantrell, C.L.; Meepagala, K.M.; Wedge, D.E.; Tabanca, N.; Schrader, K.K. Natural Toxins for Use in Pest Management. *Toxins* **2010**, *2*, 1943–1962. [CrossRef] [PubMed]
10. Singh, S.K.; Singh, A. Metabolic Changes in Freshwater Harmful Snail *Lymnaea acuminata* Due to Aqueous Extract of Bark and Leaf of *Euphorbia pulcherima* Plant. *Am.-Eurasian J. Toxicol. Sci.* **2010**, *2*, 13–19.
11. Li, X.; Deng, F.; Shan, X.; Pan, J.; Yu, P.; Mao, Z. Effects of the molluscicidal agent GA-C13:0, a natural occurring ginkgolic acid, on snail mitochondria. *Pestic. Biochem. Physiol.* **2012**, *103*, 115–120. [CrossRef]
12. Prakash, A.; Rao, J.; Nandagopal, V. Managing resistance to insecticides is a key for the future of crop protection. *J. Biopestic.* **2008**, *1*, 154–169.
13. El-Wakeil, N.E. Botanical pesticides and their mode of action. *Gesunde Pflanz.* **2013**, *65*, 125–149. [CrossRef]
14. Isman, M.B.; Grieneisen, M.L. Botanical insecticide research: Many publications, limited useful data. *Trends Plant Sci.* **2014**, *19*, 140–145. [CrossRef] [PubMed]
15. Pavela, R. Essential oils for the development of eco-friendly mosquito larvicides: A review. *Ind. Crops Prod.* **2015**, *76*, 174–187. [CrossRef]
16. Teixeira, T.; Rosa, J.S.; Rainha, N.; Baptista, J.; Rodrigues, A. Assessment of molluscicidal activity of essential oils from five Azorean plants against Radix peregra (Müller, 1774). *Chemosphere* **2012**, *87*, 1–6. [CrossRef] [PubMed]
17. Ruamthum, W.; Visetson, S.; Milne, J.R.; Bullangpoti, V. Toxicity of botanical insecticides on golden apple snail (*Pomacea canaliculata*). *Commun. Agric. Appl. Biol. Sci.* **2010**, *75*, 191–197. [PubMed]
18. Joshi, R.C.; Meepagala, K.M.; Sturtz, G.; Cagauan, A.G.; Mendoza, C.O.; Dayan, F.E.; Duke, S.O. Molluscicidal activity of vulgarone B from *Artemisia douglasiana* (Besser) against the invasive, alien, mollusc pest, *Pomacea canaliculata* (Lamarck). *Int. J. Pest Manag.* **2005**, *51*, 175–180. [CrossRef]
19. Shukla, S.; Singh, V.K.; Singh, D.K. The effect of single, binary, and tertiary combination of few plant derived molluscicides alone or in combination with synergist on different enzymes in the nervous tissues of the freshwater snail *Lymnaea* (Radix) *acuminata* (Lamark). *Pestic. Biochem. Physiol.* **2006**, *85*, 167–173. [CrossRef]
20. Plan, M.R.R.; Saska, I.; Cagauan, A.G.; Craik, D.J. Backbone Cyclised Peptides from Plants Show Molluscicidal Activity against the Rice Pest *Pomacea canaliculata* (Golden Apple Snail). *J. Agric. Food Chem.* **2008**, *56*, 5237–5241. [CrossRef] [PubMed]
21. Keni, M.F.; Latip, S.N.H.M. *Azadirachta indica* seed as potential biopesticides for controlling golden apple snail, *Pomacea canaliculata* in rice cultivation. In Proceedings of the 2013 IEEE Business Engineering and Industrial Applications Colloquium (BEIAC), Langkawi, Malaysia, 7–9 April 2013; pp. 251–256.
22. Massaguni, R.; Latip, S.N.H.M. Assesssment the Molluscicidal Properties of Azadirachtin against Golden Apple Snail, *Pomacea Canaliculata*. *Malays. J. Anal. Sci.* **2015**, *19*, 781–789.
23. Benchawattananon, R.; Boonkong, U. The toxicity of leave crude extract from neem tree (Azadirachta indica Juss.) and Garlic (*Allium sativom* L.) on mortality rate of golden apple snail (*Pomacea* sp.). In Proceedings of the 32nd Congress on Science and Technology of Thailand, Bangkok, Thailand, 10–12 October 2006.
24. Musman, M.; Kamaruzzaman, S.; Karina, S.; Rizqi, R.; Arisca, F. A preliminary study on the anti hatching of freshwater golden apple snail *Pomacea canaliculata* (*Gastropoda: Ampullariidae*) eggs from *Barringtonia racemosa* (*Magnoliopsida: Lecythidaceae*) seeds extract. *Aquac. Aquar. Conserv. Legis. Int. J. Bioflux Soc.* **2013**, *6*, 394–398.
25. Demetillo, M.T.; Baguio, M.L.; Limitares, D.E.; Madjos, G.G.; Abrenica-Adamat, L.R. Effect of *Cymbopogon citratus* (lemon grass) crude leaf extracts on the developmental stages of *Pomacea canaliculata* (golden apple snail). *Adv. Environ. Sci.* **2015**, *7*, 460–467.
26. Zhang, H.; Xu, H.H.; Song, Z.J.; Chen, L.Y.; Wen, H.J. Molluscicidal activity of Aglaia duperreana and the constituents of its twigs and leaves. *Fitoterapia* **2012**, *83*, 1081–1086. [CrossRef] [PubMed]
27. Martín, R.S.; Ndjoko, K.; Hostettmann, K. Novel molluscicide against *Pomacea canaliculata* based on quinoa (*Chenopodium quinoa*) saponins. *Crop Prot.* **2008**, *27*, 310–319. [CrossRef]
28. Kijprayoona, S.; Toliengb, V.; Petsoma, A.; Chaicharoenpongb, C. Molluscicidal activity of *Camellia oleifera* seed meal. *Sci. Asia* **2014**, *40*, 393–399. [CrossRef]

29. Quijano, M.; Riera-Ruiz, C.; Barragan, A.; Miranda, M.; Orellana, T.; Manzano, P. Molluscicidal activity of the aqueous extracts from *Solanum mammosum* L., *Sapindus saponaria* L. and *Jatropha curcas* L. against *Pomacea canaliculata*. *Emir. J. Food Agric.* **2014**, *26*, 871–877. [CrossRef]
30. Tangkoonboribun, R.S.S. Molluscicide from Tobacco Waste. *J. Agric. Sci.* **2009**, *1*, 76. [CrossRef]
31. Cowie, R.H.; Hayes, K.A. Invasive ampullariid snails: Taxonomic confusion and some preliminary resolution based on DNA sequences. In Proceedings of the APEC Symposium on the Management of the Golden Apple Snail, Pingtung, Taiwan, 6–11 September 2005.
32. Hayes, K.A.; Cowie, R.H.; Thiengo, S.C.; Strong, E.E. Comparing apples with apples: Clarifying the identities of two highly invasive Neotropical Ampullariidae (Caenogastropoda). *Zool. J. Linn. Soc.* **2012**, *166*, 723–753. [CrossRef]
33. Burela, S.; Martín, P.R. Evolutionary and functional significance of lengthy copulations in a promiscuous apple snail, *Pomacea canaliculata* (Caenogastropoda: Ampullariidae). *J. Molluscan Stud.* **2011**, *77*, 54–64. [CrossRef]
34. Seuffert, M.E.; Martín, P.R. Juvenile growth and survival of the apple snail *Pomacea canaliculata* (Caenogastropoda: Ampullariidae) reared at different constant temperatures. *SpringerPlus* **2013**, *2*, 312. [CrossRef] [PubMed]
35. World Health Organization. *Snail Control in Prevention of Bihariasis Monograph*; World Health Organization: Geneva, Switzerland, 1965; Volume 50, pp. 124–138.
36. Chauhan, S.; Singh, A. Impact of Taraxerol in combination with extract of Euphorbia tirucalli plant on biological parameters of *Lymnaea acuminata*. *Rev. Inst. Med. Trop. São Paulo* **2011**, *53*, 265–270. [CrossRef] [PubMed]
37. El-Din, A.S.; El-Sayed, K.; Mahmoud, M. Effect of ethanolic extract of Dalbergia sissoo plant parts on Biomphalaria alexandrina snail, the intermediate host of *Schistosoma mansoni*. Medical Malacology Laboratory. *J. Evolut. Biol. Res.* **2011**, *3*, 95–100.
38. Taguiling, N.K. Effect of Combined Plant Extracts on Golden Apple Snail (*Pomacea canaliculata* (Lam.)) and Giant Earthworm (*Pheretima* sp). *Int. J. Agric. Crop Sci.* **2015**, *8*, 55–60.
39. Dai, L.; Wang, W.; Dong, X.; Hu, R.; Nan, X. Molluscicidal activity of cardiac glycosides from Nerium indicum against *Pomacea canaliculata* and its implications for the mechanisms of toxicity. *Environ. Toxicol. Pharmacol.* **2011**, *32*, 226–232. [CrossRef] [PubMed]
40. World Health Organisation. Guidelines for evaluation of plant molluscicides. In *Phytolacca Dodecandra (Endod) Dublin*; Lemma, A., Heyneman, D., Silangwa, S., Eds.; Tycooly International Publishing Limited: Dublin, Ireland, 1983; pp. 121–124.
41. Yadav, R.; Singh, A. Combinations of binary and tertiary toxic effects of extracts of *Euphorbia pulcherima* latex powder with other plant derived molluscicides against freshwater vector snails. *Internet J. Toxicol.* **2008**, *7*, 2.
42. Olofintoye, L.K. Comparative evaluation of Molluscicidal effects of *Securidaca longepedunculata* (Fres.) and *Tephrosia bracteolata* (*Guilland Perr*) on *Bulinus globosus*. *J. Parasitol. Vector Biol.* **2010**, *2*, 44–47.
43. Sunita, K.; Kumar, P.; Singh, V.K.; Singh, D.K. In vitro phytotherapy of vector snails by binary combinations of larvicidal active components in effective control of fascioliasis. *Rev. Inst. Med. Trop. Sao Paulo* **2013**, *55*, 303–308. [CrossRef] [PubMed]
44. Rao, I.G.; Singh, D.K. Combinations of *Azadirachta indica* and *Cedrus deodara* oil with *piperonyl butoxide*, MGK-264 and *Embelia ribes* against *Lymnaea acuminata*. *Chemosphere* **2001**, *44*, 1691–1695. [CrossRef]
45. Dai, L.; Qian, X.; Nan, X.; Zhang, Y. Effect of cardiac glycosides from *Nerium indicum* on feeding rate, digestive enzymes activity and ultrastructural alterations of hepatopancreas in *Pomacea canaliculata*. *Environ. Toxicol. Pharmacol.* **2014**, *37*, 220–227. [CrossRef] [PubMed]
46. Okwute, S.K. Plants as Potential Sources of Pesticidal Agents: A Review. In *Pesticides—Advances in Chemical and Botanical Pesticides*; Soundararajan, R.P., Ed.; InTech: Rijeka, Croatia, 2012.