

ASSESSMENT THE MOLLUSCICIDAL PROPERTIES OF AZADIRACHTIN AGAINST GOLDEN APPLE SNAIL, *POMACEA CANALICULATA*

(Penilaian Sifat-sifat Racun Siput Azadirachtin Terhadap Siput Gondang Emas, *Pomacea canaliculata*)

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Abstract

Concern with the negative impact of synthetic pesticide on environment and human health have led this study in order to evaluate the molluscicidal efficacies of azadirachtin in neem seed crude extract on golden apple snail. Azadirachtin was extracted by maceration technique using four different solvents and the quantity of azadirachtin in extracts was compared to select the best solvent. Then, bioassays were performed on adult of golden apple snail to compare the molluscicidal activity of azadirachtin. A comparison of the extractive yields of different solvents indicated that the polarity of the solvents tested not significantly influence in enhanced the azadirachtin yields. The result on mortality rate of golden apple snail subjected to various concentration and solvent extracts indicated that neem seed crude extracts significantly killed the golden apple snail. The LC_{50} values of the methanol extract (21.008mg/ml) were the lowest, indicating the highest potency, followed in order by ethanol extract (43.726mg/ml), acetone extract (48.110mg/ml) and water extract (53.654mg/ml). The mortality rate was correlated positively with the extract concentrations as the mortality of snail increased with the increase of extract concentration. Therefore, this study indicated that neem seed crude extracts possessed molluscicidal effect for controlling the golden apple snail.

Keywords: azadirachtin, golden apple snail, LC_{50} , molluscicide, neem extract

Abstrak

Kesan negatif racun perosak sintetik pada alam sekitar dan kesihatan manusia sangat membimbangkan menyebabkan kajian ini dijalankan bertujuan untuk mengkaji keberkesanan azadirachtin dalam ekstrak mentah biji semambu untuk kawalan siput gondang emas. Azadirachtin diekstrak secara teknik rendaman dalam empat pelarut yang berbeza dan jumlah azadirachtin dalam ekstrak dibandingkan untuk memilih pelarut yang terbaik. Seterusnya, ujian ketoksikan dijalankan ke atas siput gondang emas dewasa untuk membandingkan kebolehan azadirachtin dalam mengawal siput ini. Perbandingan hasil ekstrak bagi setiap pelarut menunjukkan bahawa kepolaran pelarut yang diuji tidak mempengaruhi dalam meningkatkan hasil azadirachtin. Keputusan bagi kadar kematian siput gondang emas yang diuji dengan pelbagai kepekatan berbeza dan ekstrak pelarut menunjukkan bahawa ekstrak mentah biji semambu secara nyata dapat membunuh siput gondang emas. Nilai LC_{50} (21.008mg/ml) bagi ekstrak metanol adalah yang paling rendah, menunjukkan potensi tertinggi, diikuti oleh ekstrak etanol (43.726mg/ml), ekstrak acetone (48.110mg/ml) dan ekstrak air (53.654mg/ml). Kadar kematian berhubung kait secara positif dengan kadar kepekatan ekstrak dimana kematian siput meningkat dengan meningkatnya kadar kepekatan ekstrak. Oleh itu, kajian ini menunjukkan bahawa ekstrak mentah biji semambu memiliki kesan positif dalam mengawal siput gondang emas.

Kata kunci: azadirachtin, siput gondang emas, LC_{50} , racun Siput, ekstrak semambu

Introduction

Concern with the negative impact of synthetic pesticide on environment and human health, numerous studies investigating botanical pesticide have been carried out in order to find alternative methods of pest control. In addition, the increasing amount of studies on plant-pest chemical interactions in the last few decades has unveiled the potential of utilising botanical pesticides in the form of secondary plant metabolites as pest control agents [1]. Pesticides of plant origin are widely used because of high selective toxicity, biodegradable, safe for human and low cost [2]. *Azadirachta indica* A. Juss (neem) is one of the most attractive plants and has emerged as the most important alternative source for plant-based pesticides. The pesticidal properties of neem have been recognized by the farmers for a long time. Every part of the neem tree; its seeds, fruit, flowers, leaves, twigs, barks and roots have all been examined and found to be the most promising phytochemicals for medicinal and natural pesticides.

Some laboratory and field studies have found pesticidal properties of leaves and seeds extracts from neem tree on more than 400 species of phytophagous insects [3]. However, the seed is an important source of natural pesticidal compounds which consists of bioactive limonoid such as azadirachtin, azadiradione, salannin, nimbin, nimbidin, quercetin, and meliantriol [4]. Among all of the triterpenoid identified in that limonoid group, azadirachtin ($C_{35}H_{44}O_{16}$) has been reported as the most important and active component which possess pesticidal properties such as antifeedant, attractant, repellent, and growth disrupting against a large number of insect pests [5]. The evaluation on the pesticidal effect of azadirachtin have been carried out mostly on insects and there are only few works against the snail species. Reference [6] have tested azadirachtin at concentration of 1% on herbivorous land snail *Arianta arbustorum* and concluded that the treatment could control the snail as effective repellent. In trials conducted by [7], they found that bioactive component of azadirachtin incorporated in attractant food pellets at a concentration 1.25% are highly toxic to *Lymnaea acuminata* snail up to 96 hours. The methanolic extract [8] and acetone extract [9] of neem were reported as having molluscicidal effect against dangerous snail borne disease in India, *Lymnaea auricularia* and *Indoplanorbis exustus*. Thus, further research is needed on the evaluation of molluscicidal effects of azadirachtin in this plant on aquatic mollusc especially golden apple snail.

The golden apple snail, *Pomacea canaliculata* is an alien invasive species that causing economic damage on the rice cultivation in Malaysia [10]. The snails are herbivorous which feed on almost all types of plants, and they were prefer a softer young parts since they feed by scraping plant surface with the rough tongue [11]. Golden apple snail could consume the young rice seedling in a whole field overnight and the obvious signs of severe damage are characterized by missing hills and floating fragments of rice plants. Current strategies for controlling golden apple snail in paddy fields is relied heavily on synthetic molluscicides. Excessive use of synthetic molluscicides have been found to have numerous drawbacks on the environment and hazard and human health [12]. The hazardous nature of synthetic molluscicides has prompted the scientists to figure out the least disruptive options of pest control technologies.

Numerous studies investigating molluscicidal properties of neem on golden apple snail have been carried out due to concern with this problem. A report of [13] was evaluated the molluscicidal toxicity of aqueous neem seed and leaves extract on two different size of golden apple snail range within 20-40 mm at 96 hours exposure. The toxicity evaluation of aqueous neem crude extracts showed that the small size of golden apple snail were susceptible to the treatment than the large size of snail. A report by [14] indicated that organic extracts of neem leaves and seed oils exhibited high toxicity on golden apple snail, while the water extracts give low toxicity. They reported that the butanol extracts of neem leaves and seeds were the most toxic, causing 100% mortality of golden apple snail at 200ppm compared to neem oil, meanwhile neem water extracts were inactive at 10,000ppm.

Most of bioassay studies only investigating the potential toxicology effect and effectiveness of neem extract on golden apple snail, but insufficient study on quantifying the active constituent in extract that responsible caused mortalities on the snails. Thus, the main objective of this study was to quantify the concentration of azadirachtin extracted from neem seed crude extract and evaluate its molluscicidal efficacies on golden apple snail.

Materials and Methods

Test Organism

The golden apple snail, *Pomacea canaliculata* were collected from paddy field area in Federal Land Consolidation and Rehabilitation Authority (FELCRA) Seberang Perak, Perak. The snails were left to acclimatize to laboratory condition for seven days before being used in the experiments. Only the active golden apple snail were selected for the bioassay experiment.

Plant Materials

Neem, (*Azadirachta indica* A. Juss) samples were collected from FELCRA Seberang Perak, Perak. Authentication of the plant materials was carried out at the Forest Research Institute Malaysia (FRIM) Kepong, Selangor and voucher specimens were deposited at the Laboratory of Faculty of Plantation and Agrotechnology, UiTM Puncak Alam (Specimen code: SBID 026/12). The sample preparation were conducted by thoroughly washed neem seeds, and then dried in the conventional drier (Memmert, Germany), so as to bring down the initial large moisture content to enable its prolonged storage life [15]. The dried seed were pulverized with Waring blender (Waring product division, New Hartford), sieved and transferred into closed containers, to aid extraction. The maximum period for sample storage at room temperature was one month [16].

Extraction of Plant Materials

The defatting procedure was modified from [17] procedures. Then, the defatted neem seed marc was macerated with methanol under constant agitation at room temperature and left for 48 hours by following the procedure of reference [18]. The same procedure was used for another solvents; distilled water, acetone, and ethanol. All of the experiments were run in triplicate.

Quantification of Azadirachtin Content in Neem Crude Extract

A standard reference of azadirachtin powder (95% purity) was purchased from Sigma-Aldrich, Malaysia. The estimation of azadirachtin content in neem seed crude extract were carried out by following the procedure of [19] with some modifications using a Uv-vis spectrophotometer (Sastec UV-8000 Spectrophotometer, Malaysia) at a selected wavelength of 215 nm. The standard solution of azadirachtin, in concentrations ranging from 1.0-50.0µg/ml were used to construct the standard calibration curves. Then, 1.0 ml of each sample extracts were dissolved in HPLC grade acetonitrile prior to analyse. The analyses were performed in triplicate. The value of azadirachtin content in each neem crude extract was calculated based on external standard calibration curve.

Bioassay Procedure

The definitive test protocol was adapted and modified slightly from the [13] procedure. Ten snails were maintained in each aquarium with 30 snails total per each concentration for each treatment, including the control (treated with water) and check (treated with solvent only). The golden apple snail were exposed to a series of concentrations of acetone extract (from 45.465 to 52.814mg/ml), ethanolic extract (from 40.163 to 44.776mg/ml), methanolic extract (from 15.518 to 21.894mg/ml) and aqueous extract (from 48.751 to 61.911mg/ml) by treated paddy seedlings given as a food sources during test period. Mortality of snail was observed for 96 hours after exposure (HAE) with 24 hours interval. A snail was considered dead if it was remained immobile and either retracted well into or hanged out of the shell.

Statistical Analysis

The statistical significance between treatment values was determined by Analysis of variance (ANOVA) test using the statistical software package of Minitab[®] Release 14.1 (Minitab, Inc.). Mortality data was used to calculate LC₅₀ values, fiducial limits with 95% confidence limit, Chi-square (χ^2), coefficient of correlation (R^2) and also slope values through Probit analysis which performed using the software package of POLO-Plus (LeOra Software).

Results and Discussion

Quantification of Azadirachtin Content in Neem Crude Extract

The proposed spectrophotometric method allowed a rapid and accessible quantitation of azadirachtin in neem seed crude extracts without any time-consuming sample preparation. Moreover, the spectrophotometric method involved simple instrumentation compared with other instrumental techniques. A spectrophotometric scan of the absorbance

spectrum for standard azadirachtin indicated a maximum absorbance was 215nm. Therefore, 215nm was selected for the measurement of azadirachtin content in samples. The calibration curves for the response of azadirachtin at 215nm were obtained between the concentration range of 1.0 to 50.0 µg/ml (Figure 1). The linear regression analysis of the calibration curves yielded the following equations; $y=0.0366x - 0.0251$ with the excellent correlation coefficient (R^2) greater than 0.999.

As mentioned by [20] that azadirachtin is a highly polar substance, hence water, the most polar with a polarity index (PI) of 9.0 [21] and polar organic solvents were selected and used in this study. Generally, aqueous alcohols of polar solvents particularly acetone ($PI=5.1$), ethanol ($PI=5.2$) and methanol ($PI=5.1$) are most commonly employed in extraction of azadirachtin from neem for reasons of lack of toxicity and abundance. Thus, a comparative study was conducted in evaluating the influence of acetone, ethanol, methanol and distilled water on the extraction efficiency of neem seed extract with regard to quantity of azadirachtin in each extract. The concentration of azadirachtin yield in neem seed crude extracts were quantified using slopes of the external standard calibration curves. The yields recorded for azadirachtin concentration were 0.0502% (acetone), 0.0510% (ethanol), 0.0512% (methanol), and 0.0508% (water) in 100g defatted neem seed (Table 1). The quantity values of azadirachtin observed in this study were lower than the findings of [5] as they found that the methanol extraction of neem defatted marc afforded a concentrate of 125g/kg containing 2.2% of azadirachtin. In addition, the quantities of azadirachtin extracted were found to vary with solvents polarity which in contrast to the expectations as yield of azadirachtin increases with increasing the polarity of solvent. Reference [22] reported that the amount of azadirachtin A extractable from neem seeds with water and methanol were 0.35mg and 0.24mg of 100g dried seeds, respectively.

However, comparing extraction efficiency of azadirachtin from neem seed extracts with the analysis of variance (F -test=2.13), there were no significant differences ($p>0.05$) between quantity of azadirachtin from the solvents employed (Table 1). It was afterwards concluded that extracted samples have only varied slightly between different solvents and the polarity of the solvents tested seem not significantly influence in enhanced the azadirachtin yields.

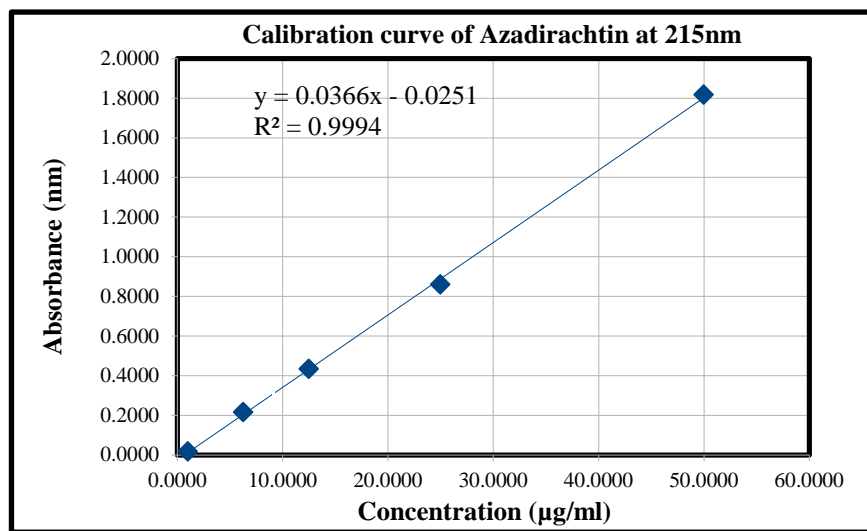


Figure 1. Calibration curve of azadirachtin (95% purity) as external standard at 215nm

Table 1. Quantity of azadirachtin in neem seed extract

Azadirachtin concentration (%)	Quantity in 100g defatted neem seed				F-test	P-value
	Acetone (PI=5.1)	Ethanol (PI=5.2)	Methanol (PI=5.1)	Water (PI=9.0)		
	0.0502	0.0510	0.0512	0.0508	2.13	0.174

*PI: Polarity Index; F-test: Analysis of variance (ANOVA); values significant at $p < 0.05$

Molluscicidal Efficacy of Neem Crude Extract

Molluscicidal efficacy of neem seed crude extracts using different organic solvents were tested against golden apple snail. Mortality of snails after 96 hours exposure were used for monitoring the molluscicidal efficacy. Our findings indicate that the exposure time and concentration of extracts are affected the toxicity of neem seed extract, as there was a significant correlation between the mortality rate with exposure time and concentrations. Figure 2 presented the mortality rate of snail after exposed to neem seed crude extracts for 24, 48, 72 and 96 hours. From the result, the mortality rate of golden apple snail were significantly increase ($p < 0.05$) as time increase for all extract. The mortality of snail were gradually increase from 24 hours till 72 hours of exposures, but after 96 hours, it was observed that the mortality rate had declined. Similar trend of toxicity was observed in case of acetone, ethanol and methanol extracts against golden apple snail except for aqueous extract which it was inconsistence.

Analysis of variance (ANOVA) indicated that the mortality of golden apple snail were significantly different between groups (between time (hour) = 53.89, $p < 0.05$; between solvent = 8.90, $p < 0.05$). However, the results showed no significant interaction between time and solvent on snail mortality at 96 hours after treatment (time*solvent = 1.24, $p > 0.05$) (Table 2).

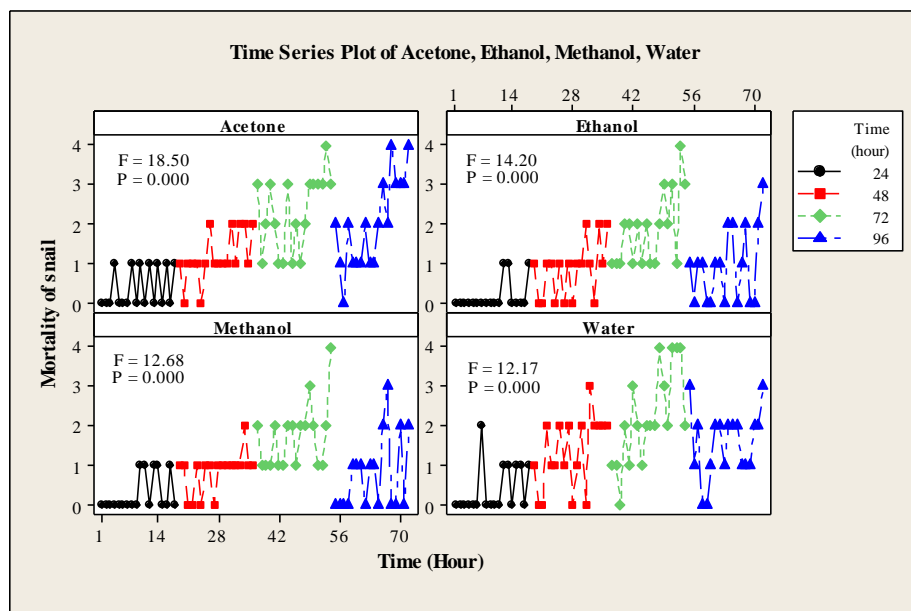


Figure 2. A mortality of golden apple snail after exposed to neem seed crude extract at different time

Table 2. ANOVA for mortality of golden apple snail versus time and solvent

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Hour	3	107.8715	107.8715	35.9572	53.89	0.000
Solvent	3	17.8160	17.8160	5.9387	8.90	0.000
Hour*Solvent	9	7.4757	7.4757	0.8306	1.24	0.268
Error	272	181.5000	181.5000	0.6673		
Total	287	314.6632				

*DF: Degree of freedom; SS: Sum of squares; MS: Mean square, F: Analysis of variance value; P: Probability value

The results demonstrated that the molluscicidal activities of neem seed crude extract on golden apple snail were highly correlated with the concentration of extracts. All of the treatments were resulted in significantly ($p < 0.05$) killed the golden apple snail and the mortality rate was positively correlated with the extract concentrations. In Figure 3, the sets treated with acetone extracts had significantly higher snail mortality (90%) followed by aqueous extract (83%), ethanol extract (63%) and methanol extract (53%) at all tested concentration. The present findings are in conformation with earlier works of [25] who reported that the efficacy of three different plant extracts tested against *Oncomelania hupensis* snail increase with increasing concentration of the plant extracts.

Comparison of lethal concentration (LC_{50} and LC_{90}) values, 95% confidence limit, slopes (β), chi square (χ^2), and heterogeneity of varying solvent used in neem seed extract neem against golden apple snail after 96 hour of exposure are set out in the Table 3. The lowest LC_{50} values were observed in methanol extract (21.008mg/ml) that indicating the highest potency, followed in order by ethanol extract (43.726mg/ml), acetone extract (48.110mg/ml) and water (53.654mg/ml). These values of results were higher if compared to those reported by [26] and [14] which 100ppm of benzene neem seeds extracts and 200ppm butanol neem seeds extract causing 100% mortality of golden apple snail after 48 hours. From the bioassay results, it indicated that the organic solvent extract of neem seed have a significant molluscicidal activities against golden apple snail. Molluscicidal efficacy of neem seed crude extract were considered significantly different as their 95% confidence limit of the LC_{50} did not overlap.

Table 3. Molluscicidal efficacies of different solvent neem seed crude extract on golden apple snail

Crude Extract	LC_{50} (mg/ml)	95% Limit (mg/ml)		LC_{90} (mg/ml)	95% Limit (mg/ml)		Chi-square (χ^2)	Slope value (β)	Heterogeneity
		LCL	UCL		LCL	UCL			
Acetone	48.110	45.740	49.600	54.280	51.935	62.690	4.123	24.455±4.998	1.03
Ethanol	43.726	42.909	45.407	49.445	46.907	57.798	0.550	24.006±6.195	0.14
Methanol	21.008	19.545	25.467	32.822	26.496	71.690	0.356	6.613±1.949	0.09
Pure water	53.654	51.334	55.543	67.487	1.757	62.999	79.415	12.865±2.817	0.44

*LCL and UCL: Lower and Upper Confidence Limit, Values significant at $p < 0.05$

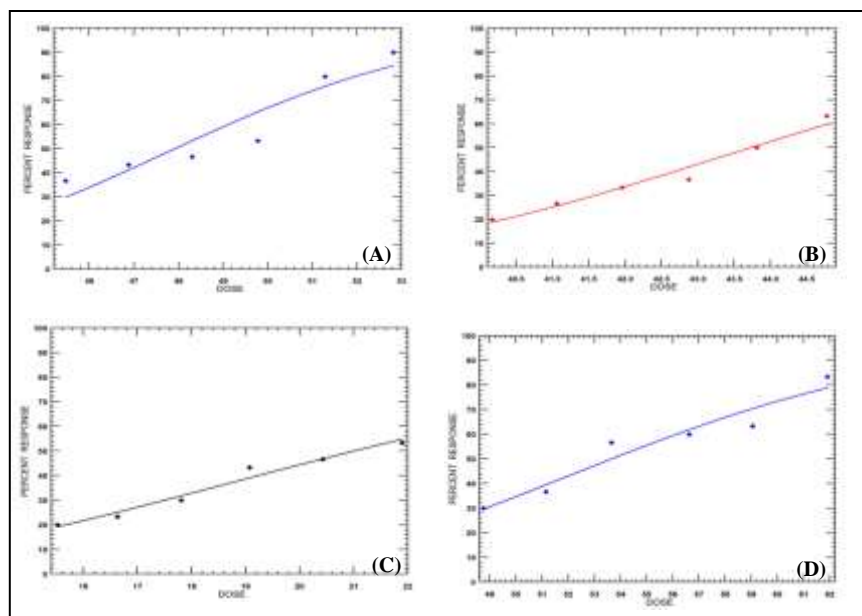


Figure 3. Effect of acetone (A), ethanol (B), methanol (C) and pure water (D) extracts at different concentration on mortality of golden apple snail

The slope values (β) indicated the steepness of the slope was high in mortality of snails with relative increment in the concentration of the neem seed crude extracts. The slopes of the concentration-mortality curves for neem extracts of varying organic solvent used were 24.455, 24.006, 6.613 and 12.865 for acetone, ethanol, methanol and water, respectively. Higher slope value was observed in acetone crude extract, followed by ethanol and aqueous to methanolic extract. The Chi-square (χ^2) test for goodness of fit values ranged from 0.356-79.415 of all solvents were significant at $p < 0.05$ level, showing the heterogenous response of golden apple snail towards tested neem extracts. The heterogeneity range from 0.14-0.44 which demonstrated that the data fit the model, significant at heterogeneity < 1.0 level. A heterogeneity of acetone which > 1.0 level revealed that the data did not fit the model due to outliers.

A comparison of the molluscicidal efficacy of different solvent extract suggests a possible relationship between bioactivity of varying azadirachtin content in neem extract and golden apple snail mortality. It is generally indicated that the LC_{50} values decrease with increasing azadirachtin concentration. The results clearly demonstrated that the highest azadirachtin yield (0.0512%) in methanol extract has the lowest LC_{50} values, which indicating the highest potency. As reported in previous study, azadirachtin was known as a feeding deterrent which does not kill the test organism directly but inhibits feeding, the test organism then possibly dying through starvation [3,4,27]. Incorporation of the neem extracts in the diet (paddy seedlings) significantly effective against golden apple snail as neem works as a systemic pesticide which it absorbed into the plant and carried throughout the tissues to be ingested by snails when they feed on the plant [1].

Conclusion

The result shows that a comparison of the extractive capacity of different solvents indicated that there were no significant differences ($p > 0.05$) between azadirachtin mean yields from the solvents employed; acetone, ethanol, methanol and water. However, the result on mortality rate of golden apple snail subjected to various concentration and solvent extracts at 96 hours exposures indicated that seed extracts significantly killed the golden apple snail. The exposure time and concentration of extracts have a significant correlation with the mortality rate of golden apple snail, as the mortality of golden apple snail were increase as time and concentration of extract increase. It

suggests that the toxicity evaluation of neem seed extract in 72 hours was sufficient to cause high mortality rate of the golden apple snail. Among different solvent fractions of this extract, methanol extract significantly gives the lowest LC₅₀ values at 95% confidence interval, which indicating the highest potency. Molluscicidal evaluation of these neem extract suggested that azadirachtin plays an important role as a feeding deterrent and caused mortality of golden apple snail. Thus, further purification and isolation of the azadirachtin from the crude extract is needed which able to enhance the toxicity efficacy.

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