Sains Malaysiana 53(5)(2024): 1081-1091
http://doi.org/10.17576/jsm-2024-5305-09

Molluscicidal Activity and Inhibition of Acetylcholinesterase Activity of *Azadirachta indica* Extract on *Pomacea canaliculata*

(Aktiviti Moluskisid dan Perencatan Aktiviti Asetilkolinesterase Ekstrak *Azadirachta indica* pada *Pomacea canaliculata*)

Nik Amalia Nasratiena Mat Zaib[1], Saiyidah Nafisah Hashim[2], Wan Rozianoor Mohd Hassan[1,3,*] & Chia Chay Tay[1]

[1]*Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*
[2]*Faculty of Applied Science, Universiti Teknologi MARA Cawangan Perak Kampus Tapah, 35400 Tapah, Perak, Malaysia*
[3]*Human Genetic and Biochemistry (HuGeB), Research Nexus UiTM (ReNeU), Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*

Received: 12 December 2023/Accepted: 5 April 2024

ABSTRACT

The Golden Apple Snail, *Pomacea canaliculata*, is a notorious pest in Malaysia, leading to substantial agricultural and economic losses, while, the neem tree, *Azadirachta indica* has been identified as alternative source for bio-pesticides. There is currently limited information on *A. indica* as natural molluscicide against *P. canaliculata.* This study aims to evaluate the effects of *A. indica* on *P. canaliculata* at various life stages (eggs, juvenile and adult). The bioactive compound, azadirachtin in *A. indica* extracts was quantified using high-performance liquid chromatography (HPLC). Egg hatchability was determined using spot spraying on egg clusters while the molluscicidal activity of *A. indica* on juvenile and adult specimens was evaluated using the immersion bioassay approach. The snail's acetylcholinesterase (AChE) inhibition was assessed using Ellman assay. The azadirachtin content in the ethanolic extract of *A. indica* leaves at a concentration of 1 µg/mL was found to be 45.42%. Increasing the concentration of *A. indica* extracts resulted in lower egg hatchability and increased mortality of juvenile and adult snails. *Azadirachta indica* extracts showed the highest effectiveness against adult *P. canaliculata* with an LC_{50} value of 0.52 mg/mL, compared to 0.92 mg/mL for juveniles and 0.74 mg/mL for eggs. Additionally, *A. indica* extract exhibited greater inhibition of AChE in adult specimens compared to juvenile specimens and eggs. The IC_{50} for adult is 0.60 µg/mL, which is the lowest while the IC_{50} for juvenile is 0.68 µg/mL, the highest. Taken together, *A. indica* shows immerse potential as natural pesticide for managing the population of *P. canaliculata*.

Keywords: *Azadirachta indica;* egg hatchability; molluscicides and inhibition of AChE*; Pomacea canaliculata*

ABSTRAK

Siput Gondang Emas (*Pomacea canaliculata*) adalah perosak utama yang menyebabkan kerugian dalam sektor pertanian dan ekonomi Malaysia, manakala pokok Mambu, *Azadirachta indica* telah ditemui sebagai sumber alternatif untuk racun serangga berasaskan bahan semula jadi dalam beberapa kajian, termasuklah sebagai racun moluska semula jadi terhadap *P. canaliculata*. Oleh itu, kajian ini bertujuan untuk menilai kesan ekstrak *A. indica* terhadap perosak *P. canaliculata* pada pelbagai peringkat kehidupan (telur, juvana dan dewasa). Sebatian bioaktif, azadiraktin daripada ekstrak *A. indica* ditentukan menggunakan kromatografi cecair prestasi tinggi (HPLC). Kebolehtetasan telur dinilai dengan semburan tompok pada kelompok telur manakala teknik rendaman digunakan untuk menilai aktiviti moluskisida *A. indica* pada peringkat juvana dan dewasa. Kemudian, perencatan asetilkolinesterase (AChE) pada siput ditentukan menggunakan ujian Ellman. Di sini, kami melaporkan bahawa terdapat 45.42% azadiraktin dijumpai di dalam 1 µg/mL ekstrak etanol daun *A. indica.* Keputusan kajian menunjukkan bahawa apabila kepekatan ekstrak *A. indica* meningkat, kebolehtetasan telur berkurangan manakala, kematian siput juvana dan dewasa didapati meningkat.

Ekstrak *A. indica* menunjukkan nilai LC_{50} terendah untuk siput dewasa (0.52 mg/mL) berbanding dengan juvana (0.92 mg/mL) dan telur (0.74 mg/mL). Ini menunjukkan potensi ekstrak yang tinggi terhadap *P. canaliculata* peringkat dewasa. Tambahan pula, ekstrak *A. indica* juga menunjukkan perencatan AChE yang lebih tinggi pada siput dewasa berbanding juvana dan telur. IC_{50} untuk dewasa adalah yang paling rendah (0.60 µg/mL) manakala IC_{50} untuk juvana adalah yang tertinggi (0.68 µg/mL). Secara keseluruhannya, *A. indica* didapati sangat berpotensi sebagai racun perosak semula jadi dalam mengawal populasi *P. canaliculata*.

Kata kunci: *Azadirachta indica*; kebolehtetasan telur; moluskisida dan perencatan AChE; *Pomacea canaliculata*

Introduction

Pomacea canaliculata or Golden apple snail (GAS), is a South American freshwater gastropod (Aromin et al. 2020). The snail was brought to Southeast Asia for consumption and as an export for the aquarium trade, but it has now escaped into agricultural wetlands and rice irrigation systems (Ji et al. 2018). *Pomacea canaliculata* was discovered for the first time in Malaysian mines in early September 1991 and is known as 'Siput Gondang Emas' by locals (Massaguni & Md Latip 2012; Yahaya et al. 2017). The invasive species are causing significant damage to nearly all paddy fields in Peninsular Malaysia, particularly in Selangor, Kedah, and Perak, due to the abandonment of snail farming initiatives (Massaguni & Md Latip 2012). The insatiable appetite of *Pomacea canaliculata* for rice crop results in them being a significant problem in paddy fields, as they can consume 7 to 24 young stems and leaves each day (Salleh et al. 2012). *Pomacea canaliculata* has rapid growth, high rate of reproduction, resilience to stress, and strong environmental adaptability, which complicates efforts to limit the snail invasion (Liu et al. 2018). An adult of *P. canaliculata* can lay a mass of eggs containing up to 500 eggs in a week with an 80% hatchability rate (Joshi 2007). In 2010 alone, *P. canaliculata* and their egg masses hindered the growth of rice plants in Malaysia, resulting in an estimated loss of RM 82 million (28 million USD) (Yahaya et al. 2017).

The Malaysian government has initiated ongoing control, containment, and eradication programs for farmers to manage the spread of *P. canaliculata* in rice fields, recognizing it as a threat to national food security. These initiatives utilized chemical, biological, physical, cultural, and natural products for mitigating these pests (Ismail & Musa 2021). The farmers prefer to use synthetic molluscicides such as metaldehyde ($C_8H_{16}O_4$) and niclosamide ($C_{13}H_8Cl_2N_2O_4$) to swiftly and effectively eradicate the snails (Massaguni & Md Latip 2012). However, the overuse of synthetic molluscicide can lead to chemical reactions that pose a health risk to farmers due to acute and chronic poisoning nature of the substances (Yahaya et al. 2017). Minimal exposure to organophosphate insecticides may induce lymphoma in the human body (Vinotha Alex & Mukherjee 2021). Furthermore, exposure to organophosphate pesticides is very harmful to creatures that are not the intended targets, including other animals (earthworm, freshwater fish, birds), plants (rice plants, corn, sugarcane), and insects (bees, ladybirds beetle, parasitic wasp) (Sidhu et al. 2019).

More than 1400 plant species with molluscicidal properties have been reported by prior researchers, such as *Azadirachta indica* (neem), *Allium sativum* (garlic), *Piper nigrum* (black pepper), *Gliricidia sepium* (madre de cacao), *Zingiber officinale* (ginger) and *Carica papaya* (papaya) (Aromin et al. 2020; Ismail & Musa 2021; Teixeira et al. 2012). *Azadirachta indica* has been used as a natural source for insecticides, pesticides, and agrochemicals (Fernandes et al. 2019). Additionally, *A. indica* is renowned for having low toxicity to biocontrol agents, predators, and parasitoids (Kilani-Morakchi, Morakchi-Goudjil & Sifi 2021). Azadirachtin, salannin, meliantriol, and nimbin are active compounds found in *A. indica* (Zheng et al. 2018). Azadirachtin and nimbolide are two of the most potent limonoids identified in *A. indica* that exhibiting cytotoxic effects (Kumar & Priyadarsini 2010).

A study on the molluscicidal properties of *A. indica* extract showed that azadirachtin is the active ingredient responsible for acting as a feeding deterrent and inducing mortality in *P. canaliculata* (Massaguni & Md Latip 2015). There was a strong correlation between the content of extracts and the molluscicidal activity of *A. indica* seed extract on *P. canaliculata* (Massaguni & Md Latip 2015). Additionally, it has been acknowledged that *A. indica* is an effective antifeedant for *P. canaliculata*.

Azadirachta indica seed methanolic extract demonstrated strong antifeedant properties with an 81% Feeding Deterrent Index (FDI) (Md. Latip, Keni & Rosli 2017). Previous studies mainly focused on the molluscicidal impact of *A. indica* extract on adult of *P. canaliculata*, with insufficient research on the egg and juvenile stages of the snail. Furthermore, the active constituent in *A. indica* extract that is responsible for molluscicidal activity is still uncertain.

Many pesticides have the effect of inhibiting the acetylcholinesterase (AChE) neurotransmitter (Thanomsit et al. 2018). The mechanism of action of organophosphate and carbamate insecticides was led by the inhibition of the AChE activity in the pest (Kristoff et al. 2011). The inhibition of acetylcholinesterase (AChE) activity has been widely used as a biomarker for the presence of pesticides (Araújo et al. 2018). Senthil Nathan et al. (2008) reported that the main compound of *A. indica*, azadirachtin showed AChE inhibition in brown planthopper *Nilaparvata lugens*. Besides that, a study on the larval stage of *Tribolium castaneum* showed a 20% reduction in AChE activity as the concentration of azadirachtin used in the test increased (Sami et al. 2016). Unfortunately, the study of AChE inhibition of *A. indica* on *P. canaliculata* to date has been inadequate.

Hence, this study was conducted to investigate the molluscicidal activity of *A. indica* leaves ethanolic extract towards the hatchability of eggs, juvenile and adult of *P. canaliculata*. Additionally, high-performance liquid chromatography was used to identify the bioactive component in *A. indica* extract. This study also will determine the inhibition of AChE activity of *A. indica* in the different stages of *P. canaliculata.* We theorized that azadirachtin in *A. indica* leaves ethanolic extract inhibits the AChE activity of *P. canaliculata* snails, affecting the hatchability of eggs, juveniles, and adults. This research will benefit the management of agricultural pests and strengthen food security in Malaysia.

Materials and Methods

Preparation of Samples

Leaves of *A. indica* were collected from FELCRA Seberang Perak, Perak (4° 0' 44'' North, 101° 1' 4'' East) while *P. canaliculata* was collected from paddy field Changkat Lada, Perak (4° 7' 59'' North, 100° 55' 0'' East). The snails were then left to acclimatize in the laboratory at the Faculty of Applied Sciences, UiTM Perak Branch Tapah Campus. The snails were raised in a water tub and provided with fresh lettuce for food. Vertical sticks were placed in the water tub to help the mother snail lay big egg masses more effortlessly. The egg masses were gathered for additional examination.

Extraction of *A. indica* Leaves

The extraction of *A. indica* leaves was done using maceration method (Al-hashemi & Hossain 2016). The *A. indica* leaves were cleaned with water to eliminate dust and contaminants, then dried in an oven for 24 h, set between 50 °C and 60 °C (Memmert UN55, Federal Republic of Germany). The dried leaves were ground into powder using a blender (Panasonic MX-GM1011, Malaysia). The leaf powder was combined with 260 mL of ethanol solvent and immersed in a beaker containing 140 g for a duration of three days. The sample was further filtered with Whatman No. 1 filter paper to remove the liquid component from the mixture. The filtrate was evaporated to remove the ethanol solvent using a rotary evaporator (Buchi Rotavapor R-3, Switzerland) under decreased pressure and stored in a glass bottle.

Quantification of Azadirachtin Content in *A. indica* Leaves Extract

High-performance liquid chromatography (HPLC) (Agilent 1260, India) was used to determine azadirachtin content in ethanol extract of *A. indica.* The percentage of azadirachtin was calculated according to the following formula (Soni et al. 2012).

$$\text{Azadirachtin content \%} = \frac{A_1 \times W_2 \times P}{A_2 \times W_1}$$

where, A_1 is the peak area of azadirachtin in sample solution; A_2 is the peak area of azadirachtin in standard solution; W_1 is the weight in g of sample; W_2 is the weight in g of standard; and P is the purity of standard azadirachtin.

A standard azadirachtin powder (95% purity) from Sigma-Aldrich was used as a reference. The mobile phase consists of solvent A (acetonitrile): B (water) with a ratio of 30:70. The separation was performed using isocratic elution (0-10 min) with a flow rate of 1.0 mL/min and a column temperature at 25 °C. The injection volume was 25 µL, and UV detection was at 219 nm. Separation was performed with a reversed-phase Spherisorb C-18 ODS 5 µm column with an acetonitrile/water gradient system.

MOLLUSCICIDAL ACTIVITY

The assessment of the molluscicidal activity of *A. indica* crude extracts on the eggs of *P. canaliculata* was assessed using a spot spraying method (Musman et al. 2013; Sisa et al. 2016). The crude extracts of *A. indica* leaves were stored at room temperature before evaluating egg hatchability. The extracts were then diluted with distilled water to create five different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL, respectively). The fertilised egg masses were placed in Petri dishes and treated with 2 mL of the plant extracts. All egg surfaces must remain in contact with the extraction liquid, and no surplus liquid is permitted to drip off. The spraying was continued the following day until all 2 mL extracts utilized. Distilled water was used as an experimental control by spraying it on a set of trials. The experiment was conducted with each unit set up in triplicates. The egg hatchability was assessed through the number of successfully hatching eggs (% of hatching success). Observations were conducted every day for 30 days. Any eggs that failed to develop thereafter, were considered unsuccessful in terms of hatchability.

The molluscicidal activity was tested on *P. canaliculata* as recommended by WHO (Joshi 2007). The snails were categorized into two groups based on size: juvenile for snails under 20 mm and adult for snails over 20 mm. 10 healthy juvenile and adult of *P. canaliculata* were immersed in different concentration of aqueous solution of *A. indica* extract for each concentration of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL, respectively, for 24 h at temperature of 24 °C to 26 °C. After 24 h of exposure, the snails were rinsed three times with dechlorinated water and then reared in dechlorinated water. The mortality of *P. canaliculata* was observed and recorded at 24, 48 and 72 h, respectively. The deceased snails were recognized by provoking the *P. canaliculata* with a needle and noting the absence of movement. The dead snails were removed immediately to avoid contamination. The test was conducted three times for each group. The percentage of mortality were calculated by using the following formula (Massaguni & Md Latip 2015),

$$\% \text{ mortality} = \frac{\text{Mean mortality treatment} - \text{Mean mortality control}}{\text{Mean mortality in treatment}} \times 100$$

INHIBITION OF AChE ACTIVITY

The inhibition of AChE activity was determined using Ellman's colorimetric method (Ellman et al. 1961). The protein of the snails and the eggs were extracted by adding 100 µL of homogenate sample (snail or egg) with 2.9 mL of 0.1 M phosphate buffer (pH 8) for 50 min at 30 ℃ in a set of test tube. The enzyme was pre-incubated with 20 µL of *A. indica* extract (ug/mL) at 37 °C for 30 min. After that, 100 µL of 0.01 M of the 5-dithiobis-2-nitrobenzoic acid (DTNB) and 20 µL 0.075 M acetylthiocholine (ATC) were added into the test tube. The reaction mixture was incubated at 30 ℃ for 20 min. A blank was prepared by adding sample solution to all reaction reagents without an AChE solution. The absorbance of the mixture was taken at 412 nm using a UV-visible spectrophotometer (Thermo Scientific Genesys 20, United States). The percentage inhibition of the enzyme was calculated based on the mean optical density of the enzyme as follows:

$$\text{Inhibition \%} = \frac{(1 - \text{Absorption of sample})}{\text{Absorption of blank}} \times 100$$

STATISTICAL ANALYSIS

Data of molluscicidal activity and inhibition of AChE activity were analyzed using the statistical software SPSS (IBM SPSS Statistics 28) and calculated with Tukey test and one-way analysis of variance (ANOVA) test.

RESULTS AND DISCUSSION

QUANTIFICATION OF AZADIRACHTIN CONTENT IN *A. indica* LEAVES EXTRACT

Azadirachtin is one of four main biologically active compounds in *A. indica* extract besides salannin, meliantriol, and nimbin which are highly effective as pesticides (Manikanta & Dokuparthi 2014). Previous studies have proved that the five compounds such as azadirachtin A, azadirachtin B, azadirachtin H, desacetylnimbin and salannin, exhibited sharp peaks by using HPLC analysis (Silva et al. 2007). A calibration curve plotted absorbance values versus respective concentrations of standard azadirachtin (95%) purity is shown in Figure 1. Our findings determined 45.42% azadirachtin content in ethanolic extract of *A. indica* leaves.

Based on the chromatograms, the retention time of the standard was 4.622 min while the sample was 5.081 min. This is in line with a previous study that stated the retention time of azadirachtin A was found between 5.8 and 6.1 min (Sinha et al. 1999). Besides that, a previous study by Silva et al. (2007), reported that the retention

time of azadirachtin H, A, and B by using methanol: water (50:50) were 6.76 min, 11.24 min and 13.76 min, respectively. The differences in elution time were probably due to the proportion of acetonitrile present in the mobile phase (Kaushik 2002). A previous study by Soni et al. (2012) reported the azadirachtin content in the ethanolic extract of leaves as 73.62% with the retention time of standard and sample found to be 3.6 min and 2.9 min, respectively. The differences in the findings are probably because of the varied azadirachtin content in the different parts of the *A. indica* and the locality of the plant (Massaguni & Md Latip 2015). The amount of azadirachtin could be influenced by weather, soil, habitat, or genetic factors (Zheng et al. 2018). Besides that, the determination of azadirachtin that showed better peaks can be enhanced by using suitable solvents such as ethanol, methanol, hexane and water (Fernandes et al. 2019). Hence, the differences in physical parameters thus affect the azadirachtin content.

MOLLUSCICIDAL ACTIVITY

Research examining the molluscicidal activity of *A. indica* against juveniles and eggs of *P. canaliculata* was limited; therefore, this experiment marked the initial attempt to determine the potential of extracts derived from *A. indica* leaves in controlling *P. canaliculata* adults, juveniles, and eggs. Data presented in Table 1 shows the percentage of mortality after molluscicidal test of *A. indica* ethanolic leaves extracts on eggs, juvenile and adult of *P. canaliculata*. Based on the observations, the ethanolic extract of *A. indica* leaves produce a strong molluscicidal effect on eggs, juvenile and adults of *P. canaliculata*. The trend shows that with increasing concentrations of *A. indica* leaves extract (0.2 mg/mL to 1.0 mg/mL), the percentages of mortality were increased significantly. Thus, the study has confirmed that *A. indica* leaves extract have a significant molluscicidal activity toward *P. canaliculata.*

The study found a correlation between the proportion of inhibitions and the toxicity of *A. indica* leaves extract on eggs, juveniles, and adults of *P. canaliculata*. The lowest inhibition value for 0.2 mg/mL of ethanol *A. indica* leaves extracts were 8.88% for eggs, 13.33% for juveniles and 16.67% for adults of *P. canaliculata*. The ethanolic leaf extracts of *A. indica* at a dosage of 1.0 mg/mL showed inhibitory percentages of 64.35% for eggs, 56.67% for juveniles, and 93.33% for adult of *P. canaliculata*. The ANOVA analysis showed a substantial increase in the inhibition percentage of *P. canaliculata* (egg, juvenile, and adult stages) compared to the control at a significance level of $p<0.05$.

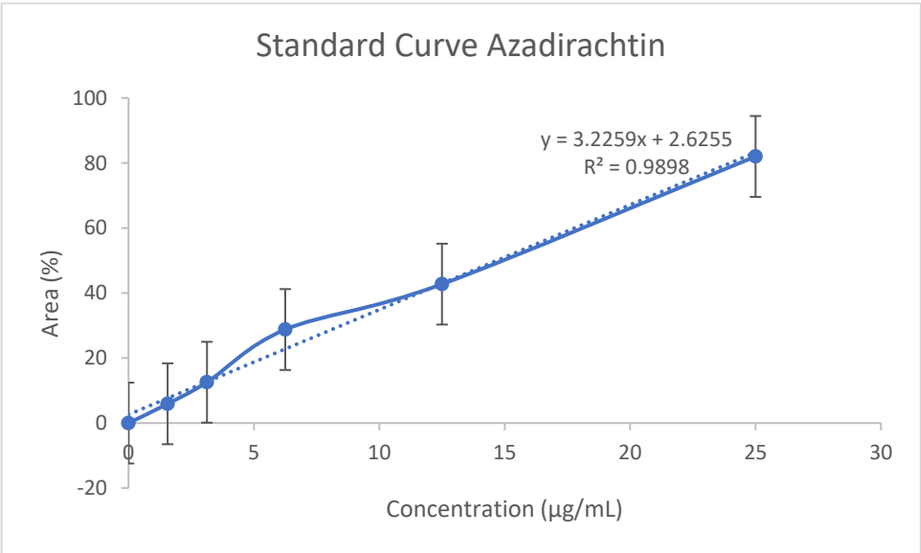

FIGURE 1. Calibration curve of azadirachtin at 219 nm

The Lethal Concentration 50 (LC_{50}) value of the ethanolic extract from *A. indica* leaves was determined to find the concentration needed to kill 50% of *P. canaliculata*. Figure 2 presents the comparison of LC_{50} values of ethanolic leaf extract of *A. indica* against different stages (egg, juvenile and adult) of *P. canaliculata*. The LC_{50} value against egg snail was 0.74 mg/mL. Previous studies showed the egg hatchability rate and that the offspring development is related to the availability of oxygen (O_2) and carbon dioxide (CO_2) concentration. During incubation, the exchange of O_2 and CO_2 is fundamental importance for the development of eggs embryonic and effects the hatchability of the eggs (Decuypere et al. 2006). Based on our findings, the hatchability of *P. canaliculata* egg decreased due to the *A. indica* extract affecting the availability of O_2 and CO_2 during the incubation of the eggs. The present study is in agreement with the previous study which reported that the number of embryos of Neo Gastropod mollusks decreased when the air supply decreased (Bigatti et al. 2010). Azadirachtin is the active molluscicidal constituent that also caused a significant reduction in egg viability of the aquatic snail *Lymnaea acuminata* (Singh & Singh 2001). The decrease in egg hatchability is likely caused by the presence of azadirachtin, the primary active compound found in *A. indica* extracts.

The *A. indica* leaves ethanolic extract exhibited a significant molluscicidal activity against *P. canaliculata*. In this investigation, the LC_{50} value of *A. indica* extracts on adult snails was 0.52 mg/mL, which was lower than the LC_{50} value on juveniles (0.92 mg/mL). The lowest LC_{50} value suggested that *A. indica* leaves extract was most effective as a molluscicide against adult snails. The result showed that the LC_{50} value was lower compared to those previous study in which the LC_{50} value observed in ethanol extract was 43.73 mg/mL (Massaguni & Md Latip 2015). The surface area to body ratio of adult snails exposed to the plant extract is larger than that of smaller snails (Abubakar, Bala & Singh 2017), which caused adult snails to be more susceptible to snail fatalities compared to juvenile snails due to the significant influence of size on the absorption of plant extracts (Rawi, Al-Hazmi & Al Nassr 2011). The molluscicidal effects of *A. indica* towards *P. canaliculata* are likely attributed by the presence of azadirachtin.

INHIBITION OF AChE ACTIVITY

Inhibition of AChE of *P. canaliculata* was measured by a colorimetric method where a chemical reaction with enzyme acetylcholinesterase, a substrate (acetylthiocoline iodide), the Ellman's reagent (DTNB) and *A. indica* extract as an inhibitor was carried out in this experiment. The DTNB is light-sensitive, thus the reaction should be carried out in a dark condition and the system should be prevented from light disturbance. The thiol or oxime containing compounds can cause color changes that can be erroneously measured as an AChE activity (Vinotha Alex & Mukherjee 2021).

TABLE 1. The mortality percentage of the *P. canaliculata*

Concentration mg/mL	% Mortality		
	Snail's egg	Juvenile	Adult
0.2	$8.88^{b} \pm 3.57$	$13.33^{b} \pm 5.773$	$16.67^{b} \pm 5.77$
0.4	$30.88^{ab} \pm 13.25$	$30.00^{ab} \pm 0$	$23.33^{ab} \pm 5.77$
0.6	$37.09^{ab} \pm 16.86$	$33.33^{ab} \pm 5.77$	$66.67^{ab} \pm 11.55$
0.8	$56.12^{ab} \pm 6.50$	$40.00^{ab} \pm 0$	$73.33^{ab} \pm 5.77$
1.0	$64.35^{a} \pm 8.11$	$56.67^{a} \pm 5.77$	$93.33^{a} \pm 11.55$

Result expressed as mean ± standard error. a: The highest average value that are significant were symbolized as alphabet (a) and the lowest average value that are significant were symbolized by subsequent alphabet. The value that was labelled with the same alphabet showed no significant difference at $p < 0.05$ Tukey test

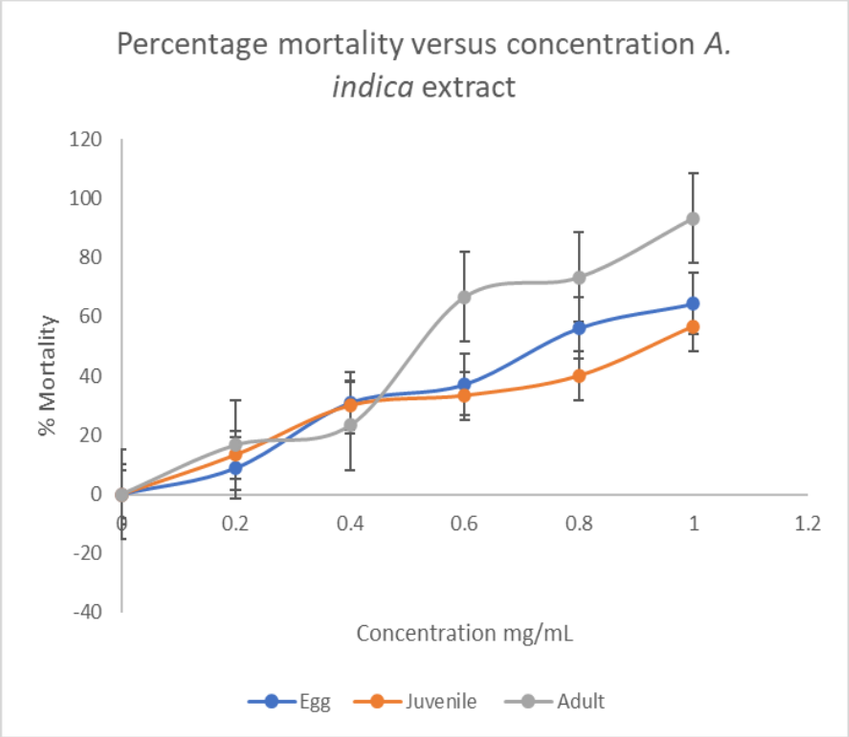

FIGURE 2. Graph of LC_{50} of *A. indica* leaves extract against different stages of the *P. canaliculata*

Generally, Table 2 determined the leaf extract of *A. indica* that has efficacy against AChE activity in adult, juvenile and eggs of *P. canaliculata*. Results indicated the percentages of inhibition increased as the extract concentration increased. The *A. indica* extract shows higher AChE inhibition towards adult of *P. canaliculata* than juvenile and egg. Maximum inhibition of the AChE activity was observed in adult of *P. canaliculata* (55.65%) at the highest concentration of tested extract. On the other hand, the *A. indica* extract exhibited the lowest AChE activity of juvenile *P. canaliculata* (5.59%) at concentrations of 0.2 ug/mL of *A. indica* extract.

The inhibitor potency is assessed in terms of the quantity necessary to give 50% inhibition (the IC_{50} value) (Ramsay & Tipton 2017). Inhibition of AChE by *A. indica* extracts is represented in Figure 3, IC_{50} values were calculated from the graph percentage of inhibition versus concentration of the extract, then the concentration (x) at which 50% inhibition was observed. The AChE activity exhibited significant variability throughout different stages of *P. canaliculata*. The adult exhibited the lowest IC_{50} at 0.60 µg/mL in this investigation. The IC_{50} value for eggs is 0.64 µg/mL, but juveniles had the highest IC_{50} value of 0.68 µg/mL. A low IC_{50} value suggests strong enzyme inhibition since it indicates that only a little quantity is needed for maximal inhibition.

Acetylcholinesterase is an enzyme that regulates the neurotransmitter acetylcholine and stops nerve impulses (Narayanan et al. 2020). AChE is a crucial enzyme found in nervous systems that breaks down or hydrolyzes acetylcholine (ACh) into acetic acid and choline (Chen 2012). AChE inhibition causes ACh to accumulate at nerve synapses, resulting in overstimulation of muscarinic and nicotinic receptors (Vinotha Alex & Mukherjee 2021). Thus, the inhibition of AChE will lead to paralysis, ataxia, lack of coordination in the neuromuscular system, and eventually death (Kumar, Singh & Singh 2012). Prolonged stimulation of cholinergic receptors by excess acetylcholine is known to be lethal (Lushchak et al. 2018).

TABLE 2. The AChE inhibition activities of *A. indica* extract on *P. canaliculata*

Concentration µg/mL	% Inhibition		
	Snail's egg	Juvenile	Adult
0.2	$6.33^{b} \pm 2.39$	$5.59^{b} \pm 1.24$	$10.28^{b} \pm 8.27$
0.4	$20.18^{ab} \pm 12.01$	$13.74^{ab} \pm 11.86$	$16..57^{ab} \pm 6.31$
0.6	$28.48^{ab} \pm 8.98$	$23.04^{ab} \pm 15.53$	$30.48^{ab} \pm 3.18$
0.8	$36.41^{ab} \pm 3.79$	$41.50^{ab} \pm 7.07$	$40.58^{ab} \pm 14.69$
1.0	$50.22^{a} \pm 1.76$	$51.49^{a} \pm 8.08$	$55.65^{a} \pm 11.94$

Result expressed as mean ± standard error. a: The highest average value that are significant were symbolized as alphabet (a) and the lowest average value that are significant were symbolized by subsequent alphabet. The value that was labelled with the same alphabet showed no significant difference at $p < 0.05$ Tukey test

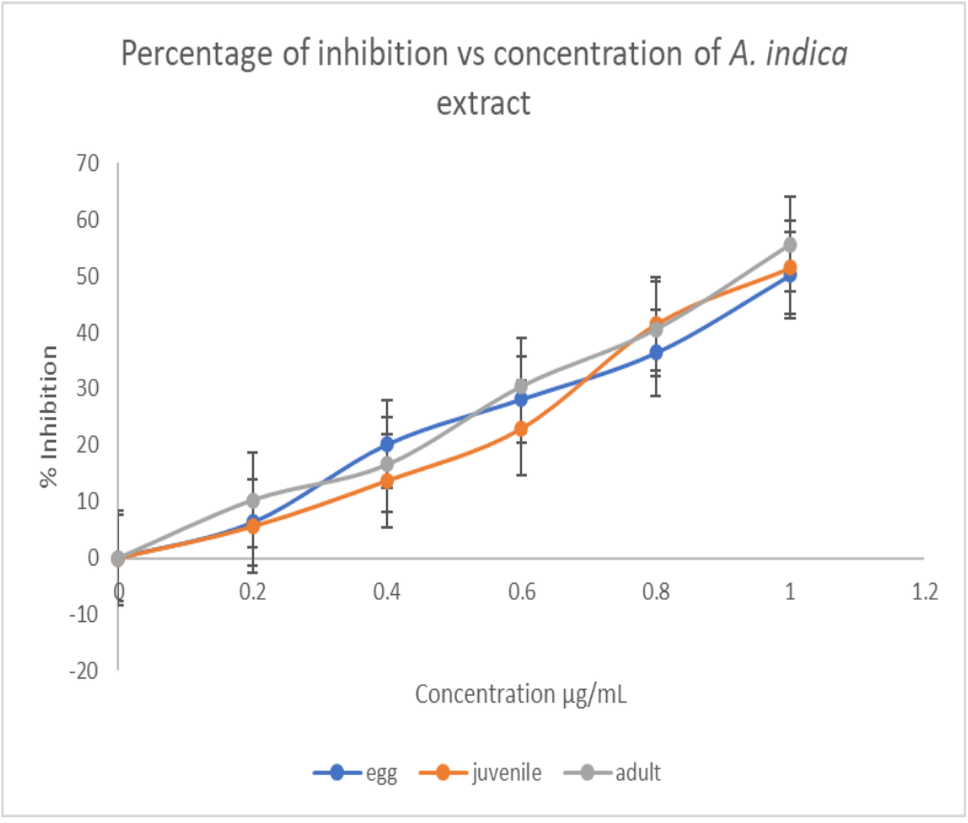

FIGURE 3. Graph of IC_{50} of *A. indica* leaves extract against different stages of the *P. canaliculata*

However, each pesticide possesses a unique inhibition effect towards AChE, depending on the chemical structure, binding affinity, charge, exposure dose, reaction conditions, phosphorylation rate, and the degree of enzyme regeneration (Sparling & Fellers 2007). The azadirachtin in the *A. indica* extracts probably played a role in inhibiting AChE activity. The results are consistent with a prior study that found a positive association between azadirachtin and acetylcholinesterase inhibitory actions (Kumar, Singh & Singh 2012). A previous study observed that 4 ppm of azadirachtin led to the alteration of AChE of cockroach, *Periplaneta americana L.* (Shafeek et al. 2004). Similarly, a study conducted by Singh and Singh (2000) that involved snails, *Limnaea acuminate Lamarck* also proved the inhibition of AChE by 40% and 80% concentrations of *A. indica* oil. Therefore, the mortality of *P. canaliculata* is in correlation with the AChE inhibition by *A. indica* extract.

Conclusion

The study concludes that the extract from *A. indica* leaves has strong molluscicidal and AChE inhibition effects on eggs, juvenile, and adult of *P. canaliculata*. The mortality rate and inhibition of acetylcholinesterase in *P. canaliculata* were correlated with the concentrations of *A. indica* leaf extract used in the treatment. Increased treatment concentrations result in greater inhibition of the snail. There is a direct positive relationship between mortality and the inhibition of AChE, likely caused by the azadirachtin component found in *A. indica* extract. Additional research is required to ascertain the mechanism of action and physiological impacts of *A. indica* leaf extract on *P. canaliculata*. This would enhance the management of *P. canaliculata* incursions by promptly and effectively utilizing insecticides derived from natural sources.

Acknowledgments

This work is part of a research project, FRGS grant, FRGS/1/2018/STG04/UITM/03/1. We would like to thank the Faculty of Applied Sciences, Universiti Teknologi MARA, Perak Branch, for providing us with the facilities and equipment.

References

Abubakar, A., Bala, A.Y. & Singh, K. 2017. Plant molluscicides and their modes of action: A review. *International Journal of Scientific Research in Technology, Applied Sciences & Health Studies* | IJSRTASHS 2(1): 37-49.

Al-hashemi, Z.S.S. & Hossain, M.A. 2016. Biological activities of different neem leaf crude extracts used locally in Ayurvedic medicine. *Pacific Science Review A: Natural Science and Engineering* 18(2): 128-131.

Araújo, M.C., Assis, C.R.D., Silva, K.C.C., Souza, K.S., Azevedo, R.S., Alves, M.H.M.E., Silva, L.C., Silva, V.L., Adam, M.L., Carvalho Junior, L.B., Souza Bezerra, R. & Oliveira, M.B.M. 2018. Characterization of brain acetylcholinesterase of bentonic fish *Hoplosternum littorale*: Perspectives of application in pesticides and metal ions biomonitoring. *Aquatic Toxicology* 205: 213-226.

Aromin, E.J.F., Akol, G.B.D., Soriano, Z.M.L. & Lucero, J.A. 2020. The molluscicidal potential of selected plants against *Pomacea canaliculata*. *IAR Journal of Agriculture Research and Life Sciences* 1(1): 26-34.

Bigatti, G., Maximiliano, G.B., Israel, A.V., Pablo E.P. & Alfredo, C.V. 2010. The calcareous egg capsule of the patagonian neogastropod *Odontocymbiola Magellanica*: Morphology, secretion and mineralogy. *Journal of Molluscan Studies* 76(3): 279-288.

Chen, Y. 2012. Organophosphate-induced brain damage: Mechanisms, neuropsychiatric and neurological consequences, and potential therapeutic strategies. *NeuroToxicology* 33(3): 391-400.

Decuypere, E., Onagbesan, O., De Smit, L., Tona, K., Everaert, N., Witters, A., Debonne, M., Verhoelst, E., Buyse, J., Hassanzadeh, M., de Baerdemaeker, J., Arckens, L. & Bruggeman, V. 2006. Hypoxia and hypercapnia during incubation of chicken eggs: Effects on development and subsequent performance. *World's Poultry Science Journal XII* Europe (June): 486-487.

Ellman, G.L., Diane Courtney, K., Valentino, A. & Robert, M.F. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7(2): 88-95.

Fernandes, S.R., Barreiros, L., Oliveira, R.F., Cruz, A., Prudêncio, C., Oliveira, A.I., Pinho, C., Santos, N. & Morgado, J. 2019. Chemistry, bioactivities, extraction and analysis of azadirachtin: State-of-the-art. *Fitoterapia* 134: 141-150.

Ismail, H.N. & Musa N.N. 2021. Molluscicidal activity of *Zingiber officinale* and *Carica papaya* on the egg hatchability of the golden apple snail, *Pomacea canaliculata* (Gastropoda: Ampuliriidae). *Malaysian Journal of Science* 40(2): 40-50.

Ji, M., Cheng, P., Huai, E.H., Suganiya, R.R. & Yoon, Y.Y. 2018. Invasive apple snails in wetlands of Selangor, Malaysia: Species, distribution, and ecological associations. *Journal of Tropical Biology and Conservation* 15(1): 43-60.

Joshi, R.C. 2007. Problems with the management of the golden apple snail *Pomacea canaliculata*: An important exotic pest of rice in Asia. In *Area-Wide Control of Insect Pests: From Research to Field Implementation*, edited by Vreysen, M.J.B., Robinson, A.S. & Hendrichs, J. Dordrecht: Springer. pp. 257-264.

Kaushik, N. 2002. Determination of azadirachtin and fatty acid methyl esters of *Azadirachta indica* seeds by HPLC and GLC. *Analytical and Bioanalytical Chemistry* 374(7-8): 1199-1204.

Kilani-Morakchi, S., Morakchi-Goudjil, H. & Sifi, K. 2021. Azadirachtin-based insecticide: Overview, risk assessments, and future directions. *Frontiers in Agronomy* 3: 676208.

Kristoff, G., Luis, C.C., Noemí, R.V.G. & Adriana, C.C. 2011. Effects of the organophosphate insecticide azinphos-methyl on the reproduction and cholinesterase activity of *Biomphalaria glabrata*. *Chemosphere* 84(5): 585-591.

Kumar, G.H. & Priyadarsini, V.R. 2010. The neem limonoids azadirachtin and nimbolide inhibit cell proliferation and induce apoptosis in an animal model of oral oncogenesis. *Investigational New Drugs.* 28: 392-401.

Kumar, P., Singh, V.K. & Singh, D.K. 2012. Feeding of bait to snail *Lymnaea acuminata* and their effect on certain enzyme in the nervous tissue. *ISRN Biochemistry* 2012: 343047.

Liu, C., Zhang, Y., Ren, Y., Wang, H., Li, S., Jiang, F., Yin, L., Qiao, X., Zhang, G., Qian, W., Liu, B. & Fan, W. 2018. The genome of the golden apple snail *Pomacea canaliculata* provides insight into stress tolerance and invasive adaptation. *Gigascience* 7(9): giy101.

Lushchak, V.I., Matviishyn, T.M., Husak, V.V., Storey, J.M. & Storey, K.B. 2018. Pesticide toxicity: A mechanistic approach. *EXCLI J.* 17: 1101-1136.

Manikanta, P. & Dokuparthi, S.K. 2014. A review on role of *Azadirachta indica* A. Juss as a biopesticide. *International Journal of Universal Pharmacy and Bio Sciences* 3(2): 10.

Massaguni, R. & Md Latip, S.N.H. 2015. Assesssment the molluscicidal properties of azadirachtin against golden apple snail, *Pomacea canaliculata*. *Malaysian Journal of Analytical Sciences* 19(4): 781-789.

Massaguni, R. & Md Latip, S.N.H. 2012. Neem crude extract as potential biopesticide for controlling golden apple snail, *Pomacea canaliculata*. *Pesticides - Advances in Chemical and Botanical Pesticides*. InTech. doi:10.5772/48626.

Md. Latip, S.N.H., Keni, M.F. & Rosli, R. 2017. Antifeedant activities of neem seed extracts for controlling golden appple snail, *Pomacea canaliculata*. *The Social Sciences* 12(2): 294-298.

Musman, M., Kamaruzzaman, S., Karina, S., Rizqi, R. & Arisca, F. 2013. A preliminary study on the anti hatching of freshwater golden apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae) eggs from *Barringtonia racemosa* (Magnoliopsida: Lecythidaceae) seeds extract. *AACL Bioflux* 6(4): 394-398.

Narayanan, S.E., Abdul Rehuman, N., Harilal, S., Vincent, A., Rajamma, R.G., Behl, T., Uddin, M.S., Md Ashraf, G. & Mathew, B. 2020. Molecular mechanism of zinc neurotoxicity in Alzheimer's disease. *Environmental Science and Pollution Research* 27(35): 43542-43552.

Ramsay, R.R. & Tipton, K.F. 2017. Assessment of enzyme inhibition: A review with examples from the development of monoamine oxidase and cholinesterase inhibitory drugs. *Molecules* 22(7): 1192.

Rawi, S.M., Al-Hazmi, M. & Al Nassr, S.F. 2011. Comparative study of the molluscicidal activity of some plant extracts on the snail vector of *Schistosoma mansoni*, *Biomphalaria alexandrina*. *International Journal of Zoological Research* 7(2): 169-189.

Salleh, N.H.M., Dachyar, A., Daud, M.Z.M., Pilus, N. & Nawi, R. 2012. Distribution and management of *Pomacea canaliculata* in the Northern Region of Malaysia: Mini review. *APCBEE Procedia* 2: 129-134.

Sami, A.J., Sehrish, B., Khalid, M., Shakoori, F.R., Rehman, F. & Shakoori, A.R. 2016. Effect of crude neem (*Azadirachta indica*) powder and azadirachtin on the growth and acetylcholinesterase activity of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Pakistan Journal of Zoology* 48(3): 881-886.

Senthil Nathan, S., Choi, M.Y., Seo, H.Y., Paik, C.H., Kalaivani, K. & Kim, J.K. 2008. Effect of azadirachtin on acetylcholinesterase (AChE) activity and histology of the brown planthopper *Nilaparvata lugens* (Stål). *Ecotoxicology and Environmental Safety* 70(2): 244-250.

Shafeek, A., Jaya Prasanthi, R.P., Hariprasad Reddy, G., Chetty, C.S. & Rajarami Reddy, G. 2004. Alterations in acetylcholinesterase and electrical activity in the nervous system of cockroach exposed to the neem derivative, azadirachtin. *Ecotoxicology and Environmental Safety* 59(2): 205-208.

Sidhu, G.K., Singh, S., Kumar, V., Dhanjal, D.S., Datta, S. & Singh, J. 2019. Technology toxicity, monitoring and biodegradation of organophosphate pesticides: A review. *Critical Reviews in Environmental Science and Technology* 49(13): 1135-1187.

Silva, J.C.T., Gulab, N.J., Rosângela, D.L.O. & Leslie, B. 2007. Purification of the seven tetranortriterpenoids in neem (*Azadirachta indica*) seed by counter-current chromatography sequentially followed by isocratic preparative reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* 1151(1-2): 203-210.

Singh, A. & Singh, D.K. 2001. Molluscicidal activity of the custard apple (*Annona squamosa* L.) alone and in combination with other plant derived molluscicides. *Journal of Herbs, Spices and Medicinal Plants* 8(1): 23-29.

Singh, K. & Singh, D.K. 2000. Toxicity to the snail *Limnaea acuminata* of plant-derived molluscicides in combination with synergists. *Pest Management Science* 56(10): 889-898.

Sinha, S., Murthy, P.S.N., Rao, C.V.N., Ramaprasad, G., Sitaramaiah, S., Kumar, D.G. & Suresh, K.S. 1999. Simple method for enrichment of azadirachtin from neem seeds. *Journal of Scientific and Industrial Research* 58(12): 990-994.

Sisa, M.H., Aspani, F., Massaguni, R., Awang Damit, H. & Joseph, H. 2016. Inhibition of egg hatching of the golden apple snail by synthetic molluscicides. In *Regional Conference on Science, Technology and Social Sciences (RCSTSS 2014)*, edited by Yacob, N., Mohamed, M. & Megat Hanafiah, M. Singapore: Springer.

Soni, H., Mishra, K., Sharma, S. & Singhai, A.K. 2012. Characterization of azadirachtin from ethanolic extract of leaves of *Azadirachta indica*. *Journal of Pharmacy Research* 5(1): 199-201.

Sparling, D.W. & Fellers, G. 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii. Environmental Pollution* 147(3): 535-539.

Teixeira, T., José, S.R., Nuno, R., José, B. & Armindo, R. 2012. Assessment of molluscicidal activity of essential oils from five Azorean plants against *Radix peregra* (Müller 1774). *Chemosphere* 87(1): 1-6.

Thanomsit, C., Maprajuab, A., Saowakoon, S., Prasatkaew, W., Ocharoen, Y., Wattakornsiri, A., Nanuam, J. & Nanthanawat, P. 2018. Acetylcholinesterase (AChE): Potential biomarker for evaluating pesticide exposure on egg and tissue of golden apple snail (*Pomacea canaliculata*) from Huai−Saneng Reservoir, Surin Province, Thailand. *Thai J. Agric. Sci.* 51(3): 104-117.

Vinotha Alex, A. & Mukherjee, A. 2021. Review of recent developments (2018–2020) on acetylcholinesterase inhibition based biosensors for organophosphorus pesticides detection. *Microchemical Journal* 161: 105779.

Yahaya, H., Badrulhadza, A., Sivapragasam, A., Nordin, M., Muhamad Hisham, M.N. & Misrudin, H. 2017. Invasive apple snails in Malaysia. In *Biology and Management of Invasive Apple Snails*, 2nd ed., edited by Joshi, R.C., Cowie, R.H. & Sebastian, L.S. Philippine: Philippine Rice Research Institute. pp. 187-613.

Zheng, Y., Wu, J., Wang, Y., Peng, X. & Zhang, Y. 2018. Seed yield and azadirachtin content of *Azadirachta indica* in four ecosystems of Southwest China. *Industrial Crops and Products* 122: 23-27.

*Corresponding author; email: rozianoor@uitm.edu.my