Heliyon 6 (2020) e04796

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Schistosomiasis prevention option: toxicological evaluation of *Vernonia amygdalina* on the tissues of *Bulinus truncatus* at different pH conditions

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ARTICLE INFO

Keywords:
Environmental science
Biological sciences
Biochemistry
Vernonia amygdalina
Tissues
Acetylcholinesterase
Acid phosphatase
Alkaline phosphatase
B. trucatus

ABSTRACT

Research into, and the use of plant products in the control of vectors of pathogens is being revived and seriously considered as an alternative or complete replacement for the classical synthetic agents. The study was designed to investigate toxicological assessment of the aqueous leaf extract of *Vernonia amygdalina* on mortality and tissue level damages of the freshwater snail *Bulinus truncatus* at different pH levels. The effects of the extract on total protein concentration and activities of acetylcholinesterase, acid phosphatase and alkaline phosphatase in the tissues of the snail were assayed using standard methods. Compared to the control (snail group not treated with the extract of *V. amygdalina*), there were significant ($p < 0.05$) reductions in the total protein concentrations and acetylcholinesterase activity in the snails' tissues of the treated groups (0.20 mg/L, 0.40 mg/L and 1.00 mg/L) at all the pH conditions (3.5, 7.0 and 10.5). The reverse of this trend followed in the case of acid and alkaline phosphatases' activities. The study provides a substantial possibility of exploiting local indigenous plant resources such as *V. amygdalina* for control of freshwater snails and monitor water pollution. The study also raised a possibility of the locals living around freshwater bodies prone to trematode borne diseases to reflexively control freshwater snail population by just squeeze-washing their *V. amygdalina* around the river banks.

1. Introduction

Populations all over the world are at the risk of infections with water and food-borne trematode parasites such as *Schistosoma hepatica, Schistosoma japonicum, Clonoorchis sinenses, Echinostoma spp*. and many others (Keiser and Utzinger, 1990). Most of such trematodes adopt freshwater snails as their intermediate host.

Enormous species of snails are of possible medical or veterinary importance (Rozendaal, 1997). *Schistosomiasis*, as a widespread human parasite disease, ranks second to malaria in terms of its socio-economic and public health importance in tropical and subtropical countries of Africa and other developing nations of the world (Inobaya et al., 2014). It is also considered the most prevalent of water-borne diseases and one of the greatest risks to health in rural areas of developing countries (Alavi and Salmanzadeh, 2016).

Snail population management through molluscicidal agents is a good tool for controlling these varieties of trematode diseases of man and livestock. Synthetic molluscicides have been widely used for the effective control of carrier snails (Singh and Singh, 2012) but recently, it has been realized that they are toxic to non-target animals and have a long term detrimental effect on the aquatic environment (Upadhyay and Singh, 2011). As safer and cheaper alternatives, molluscicides of plant origin are gaining importance than their synthetic counterparts. The search for herbal preparations that produce much less adverse effects in the non-target organisms and which are easily biodegradable remains a top research priority for scientists associated with alternative molluscicides.

Vernonia amygdalina, commonly known as the bitter leaf, family *Asteraceae* or *Compositae*, is a plant consumed locally as food and serves important ethnomedical uses. Parts of the plants, especially the leaves and stem are useful locally for treatment of fever, stomach disorder, jaundice, worm infestation, constipation, malaria, hiccups, kidney problems, amoebic dysentery, schistosomiasis, cough, wounds, diabetes, laxative and other bacterial and protozoal infections (Uchec, 2004).

Phytochemical screening on the ethanol leaf extract of *Vernonia amygdalina* revealed the presence of flavonoids, saponins, tannins, steroids, alkaloids, triterpenoids, reducing sugar and cardiac glycosides (Usunobun and Okolie, 2016). Shuaibu and Shuaibu (2017) screened the ethanol, hot water and cold water leaf extract of *V. amygdalina* for their

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https://doi.org/10.1016/j.heliyon.2020.e04796
Received 30 April 2020; Received in revised form 16 July 2020; Accepted 24 August 2020

phytochemical constituents and found out they contained alkaloids, cardiac glycosides, flavonoids, saponin glycosides, saponins, steroids, tannins and glycosides. Cardiac glycosides were not detected in not water extract.

The earliest form of medication known to man is the medicinal plants and lately has been tested by scientists for molluscicidal activities, since they are cheaper and more eco-friendly compared to their synthetic counterparts (WHO, 1985). Consequently, there is a rising interest in the potencies of these medicinal plants as molluscicidal agents for snail control in endemic poor nations of the world (Aladesanmi, 2007).

Apart from the ethnomedicinal use of *V. amygdalina,* it is also a very important food in tropical East and West Africa, where it is consumed by a large proportion of the population. The plant is mainly cultivated in the raining season and squeeze-washing is one of the predominantly means of processing the vegetable (Ejoh et al., 2005). People who earn their livelihood by trading on the processed *V. amygdalina* leaf vegetable usually squeeze-wash very large quantities around the rivers banks, streams and other available freshwater bodies leaving behind its chlorophyll which eventually flows into the water bodies. Naturally, the pH of these water bodies might be increased by limestone and other bicarbonate materials (Utah State University, 2013) while sulfate-reducing bacteria in Wetlands, airborne particulates from wildfires and even lighting can decrease the pH (NADP, 2012). The aim of the study, therefore, is to evaluate the effect of aqueous crude extract of *V. amygdalina* leaves on the freshwater snail (*B. truncatus*) tissues at different water pH level.

2. Materials and methods

2.1. Materials

2.1.1. Instruments

Rotary evaporator, Bench Centrifuge Model 800D (Microfield Instrument England, UK), SpectrumLab 752S Uv/Vis spectrophotometer.

2.1.2. Sample preparation and extraction of plant materials

Three months old fresh leaves of *Vernonia amygdalina* Delile, collected during the rainy season (May 2018) from Nsukka town in Nsukka Local Government of Enugu, Nigeria, were used for the study. It was identified and authenticated by Mr Nwafor Felix, a taxonomist in the Department of Pharmacognosy & Environmental Medicines, University of Nigeria, Nsukka, where the voucher specimen with the voucher number PCG/UNN/030 was deposited.

One kilogram (1kg) of the air-dried plant leaves were ground into coarse particles and placed in a stoppered container with water and allowed to stand for 3 days with frequent agitation until the soluble matter became dissolved. The mixture was then strained, the marc (the damp solid material) was pressed, and the combined liquid was clarified by filtration. The filtrate was then concentrated to dryness using a rotary evaporator.

2.1.3. pH of the medium

The pH of the medium was modified using NaOH and HCl to achieve the pH 3.5, 7 and 10.5 as used in the experiment. The pH of the extract was measured to be 4.8.

2.1.4. Freshwater snails

Non-infected, adult *Bulinus truncatus* with mean length 1.9 ± 0.7 cm and an average weight of 31 ± 8.0 g were collected at the banks of Ebenyi river in Eha-amufu in Isi Uzo Local Government of Enugu State. The snails were acclimatized in laboratory conditions for two weeks. They were kept in plastic aquaria containing stream water at 25 °C and fed with fresh lettuce leaves ad libitum.

Clearance and approval for conducive experimental conditions and humane use and handling of laboratory animals were given by the ethical committee of the Department of Zoology and Environmental Biology, University of Nigeria Nsukka.

2.2. Methods

2.2.1. Molluscicidal activity assay

2.2.1.1. Experimental setup. Three (3) stock solutions of de-chlorinated tap water and *Vernonia amygdalina* extract of 0.2 mg/L, 0.4 mg/L and 1.00 mg/L concentrations were prepared. Three plastic aquaria were provided for each concentration; for each aquarium, the pH was modified to pH 3.5, pH 7.0 and pH 10.5. Three (3) snails were put in each aquarium. This setup was prepared for the different concentrations of the stock solution. Setups of a particular pH condition and concentrations were replicated into 3. For the control, three aquaria with de-chlorinated tap water of pH 3.5 pH 7.0 and pH 10.5 without any *V. amygdalina* extract were provided and 3 snails were placed in each of them; this was also replicated 2 times for each pH condition. Period of 96 h exposure was allowed, followed by 48 h recovery period.

2.2.1.2. Collection and preparation of tissue homogenates. The homogenization of muscle, hepatopancreas and intestine was carried following the methods reported by Olagunju et al. (2000).

The shells of the active snails were broken after 96 h recovery period in freshwater. The muscle, hepatopancreas, haemolymph and intestine of each snail were collected separately and weighed. Each of the muscles (average weight of 1.9 g) in each group was homogenized using 10 ml of normal saline. The hepatopancreas per snail (average weight of 0.59 g) in each group was homogenized using 5.0 ml of normal saline. The intestine per snail (average weight of 0.9 g) in each group was homogenized using 10 ml of normal saline. The homogenization was carried out using mortar and pestle aided with washed fine sand. The homogenates were centrifuged at 3000 rpm for 10 min. The supernatant in each tube was collected into labelled vials and kept in the deep-freezer at 4 °C until analyzed.

2.2.2. Biochemical analyses of tissue homogenates

The following biochemical analyses were carried out on the snails' haemolymph and other tissue homogenates.

2.2.2.1. Total protein assay. The total protein concentration was determined by a simplified method for the quantitative assay of a small amount of protein in biological material as described by Shacterk and Pollack (1973).

Typically, 1.0 ml of each of the homogenates was pipetted into clean test-tubes in triplicates; 1.0 ml of alkaline copper reagent (10% Na_2CO_3, 0.1% K–Na tartarate and 0.05% $CuSO_4$. $5H_2O$) was added and mixed thoroughly. The mixtures were left to stand undisturbed for 10 min at 25 °C. Then, 4.0 ml of Folin-Ciocalteu's Phenol reagent (1:10 dilution) was added forcibly and rapidly. The blank was also prepared using distilled water instead of the homogenates. The mixture was then incubated at 55 °C for 5 min, removed and cooled under running water. The absorbance was read at 650 nm against the blank.

The protein concentration was calculated using the expression:

The concentration of protein (g/dl) = Abs. of sample/Abs. of standard x df x concentration of standard protein.

where

Abs is the absorbance at 650 nm and
df is the dilution factor.

2.2.2.2. Assay of acetylcholinesterase activity. This assay was carried out by a slightly modified method of Ellman and Courtney (1961).

The reaction mixtures were made up of 25 µl of haemolymph and 50 µl each of the other homogenates in triplicates. Then, 50 µl (5, 5′-dithiobis-2-nitrobenzoic acid (DNTB) in 5 mM sodium phosphate buffer,

Table 1. Changes in the acetylcholinesterase activity in different tissues of *B. truncatus* after 96 h exposure to different concentrations of *V. amygdalina* extract at different pH levels.

Acetylcholinesterase activity (μmole/min)												
	Hepatopancreas			Intestine			Muscle			Haemolymph		
Concentration	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5
Control 0.00 mg/L	2.44 ± 0.16^{a1}	2.58 ± 0.16^{b1}	2.59 ± 0.16^{b1}	1.89 ± 0.03^{a1}	2.13 ± 0.53^{b1}	2.30 ± 0.53^{b1}	1.89 ± 0.08^{a1}	2.13 ± 0.08^{b1}	2.26 ± 0.08^{c1}	1.85 ± 0.03^{a1}	2.12 ± 0.03^{b1}	2.77 ± 0.03^{c1}
0.02 mg/L	1.60 ± 0.05^{a2}	1.73 ± 0.05^{b2}	1.87 ± 0.05^{c2}	1.46 ± 0.08^{a2}	1.62 ± 0.08^{b2}	1.66 ± 0.08^{c2}	1.50 ± 0.16^{a2}	1.67 ± 0.16^{b2}	1.76 ± 0.16^{c2}	1.76 ± 0.01^{a2}	1.84 ± 0.01^{b2}	1.89 ± 0.01^{c2}
0.04 mg/L	1.24 ± 0.22^{a3}	1.57 ± 0.22^{b3}	1.64 ± 0.22^{c3}	1.62 ± 0.03^{a3}	1.63 ± 0.03^{a2}	1.66 ± 0.03^{c2}	1.45 ± 0.20^{a3}	1.61 ± 0.20^{b3}	1.76 ± 0.20^{c2}	1.46 ± 0.07^{a3}	1.60 ± 0.07^{b3}	1.61 ± 0.07^{b3}
1.00 mg/L	1.26 ± 0.09^{a3}	1.33 ± 0.09^{b4}	0.35 ± 0.09^{c4}	1.06 ± 0.10^{a4}	1.35 ± 0.10^{b3}	1.62 ± 0.10^{c2}	1.28 ± 0.07^{a4}	1.59 ± 0.07^{b3}	1.75 ± 0.02^{c2}	1.32 ± 0.02^{a4}	1.59 ± 0.02^{b3}	1.63 ± 0.02^{b3}

Values are expressed as ± Standard error of mean.
Down the columns, values with different numeric superscript are significantly different ($p \leq 0.05$) while values with the same numeric superscript are not significantly different ($p \leq 0.05$). Across the rows values with different alphabet superscript are significantly different ($p \leq 0.05$) while values with the same alphabet superscript are not significantly different ($p \leq 0.05$).

pH 8.0, containing 17.74 mM $NaHCO_3$) was added followed by 1.45 ml of 5 mM sodium phosphate buffer at pH value of 8.0. The reaction mixtures were mixed properly and transferred into a cuvette followed by the addition of 1.45 ml of 5 mM sodium phosphate buffer at pH of 8.0. The initial absorbance was read at 412 nm. The reaction was initiated by the addition of 50 μl substrate (12.5 mM acetylcholine iodide) and stirred. The absorbance was read at 30 s interval for 4 min. The change in absorbance per time (slope) for each experimental data was determined.

The activity of acetylcholinesterase was estimated using the following expression:

Enzyme activity (μmol SH hydrolyzed min/mg protein) = ΔA × TV/ εDTNB × ℓ × SV,

where
ΔA = change in absorbance/min;
TV = total volume of the assay;
SV = sample volume (25 μl/50 μl);
ℓ = path length of cuvette (1 cm);
εDTNB = molar extinction co-efficient of DTNB (1.36 × 104 M -1cm-1).

2.2.2.3. Assay of alkaline and acid phosphatases. The assays of alkaline and acid phosphatases were carried out by the method described by Sanni and Van Etten (1978) as reported with slight modification by Oyedapo (1996).

The reaction mixture contained 50 μl haemolymph (or 50 μl of muscle/intestine/hepatopancreas homogenates) in triplicates and incubated at 37 °C for 3 min. The blank was similarly prepared using water instead of the liver homogenates or plasma samples. The reaction was initiated by the addition of 1.0 ml of the substrate (5 mM p-nitro phenyl phosphate, Na salt) in an appropriate buffer. The reaction mixtures were incubated for an additional 15 min, followed by the addition of 2.0 ml 0.02 M NaOH to terminate the reaction. The reaction mixtures were allowed to cool down to room temperature. The absorbance was read at 410 nm against the reagent blank.

The activities of the phosphatases were calculated using the following expression:

$$\frac{Absorbance}{\varepsilon \times l} \times 10^6 \times \frac{1}{t} \times \frac{TV}{SV}$$

where
ε = molar extinction co-efficient (1.88 × 104 $M^{-1}cm^{-1}$);
l = path length of cuvette (1.0 cm);
TV = total assay volume;
SV = sample volume (25 μl/50 μl) and
t = incubation time (15 min)

2.3. Statistical analysis

Data were analyzed using SPSS version 21. Two-Way Analysis of Variance (ANOVA) was used to compare differences between setups of different pH and different concentration. When effects are significant, in the ANOVA, Duncan Multiple Range Test was used to separate differences $p < 0.05$. Multiple regression analysis (MRA) using the stepwise approach was performed.

3. Results

The results (Tables 1, 2, 3, and 4) present the changes in acetylcholinesterase, acid phosphatase and alkaline phosphatase activities and total protein concentrations in various tissues of water snail at 96 h

Table 2. Changes in the total protein concentration in different tissues of *B. truncatus* after 96 h exposure to different concentrations of *V. amygdalina*extract at different pH levels.

Total protein concentration (mg/dl)												
	Hepatopancreas			Intestine			Muscle			Haemolymph		
Concentration	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5
Control 0.00 mg/L	1.66 ± 0.01^{a1}	1.64 ± 0.01^{a1}	1.70 ± 0.01^{b1}	1.47 ± 0.05^{a1}	1.47 ± 0.05^{a1}	1.6 ± 0.05^{b1}	1.69 ± 0.10^{a1}	1.74 ± 0.10^{b1}	1.91 ± 0.10^{c1}	1.58 ± 0.02^{a1}	1.60 ± 0.02^{a1}	1.79 ± 0.02^{b1}
0.02 mg/L	0.70 ± 0.02^{a2}	0.71 ± 0.02^{a2}	0.72 ± 0.02^{a2}	0.67 ± 0.10^{a2}	0.71 ± 0.10^{a2}	0.73 ± 0.10^{a2}	1.39 ± 0.07^{a2}	1.41 ± 0.07^{a2}	1.40 ± 0.07^{a2}	1.12 ± 0.11^{a2}	1.15 ± 0.11^{a2}	1.27 ± 0.11^{c2}
0.04 mg/L	0.55 ± 0.08^{a3}	0.56 ± 0.08^{a3}	0.56 ± 0.08^{a3}	0.59 ± 0.02^{a3}	0.57 ± 0.02^{a3}	0.57 ± 0.02^{a3}	0.54 ± 0.09^{a3}	0.57 ± 0.09^{a3}	0.55 ± 0.09^{a3}	0.70 ± 0.04^{a3}	0.69 ± 0.04^{a3}	0.72 ± 0.04^{a3}
1.00 mg/L	0.41 ± 0.01^{a4}	0.43 ± 0.01^{a4}	0.50 ± 0.01^{b4}	0.52 ± 0.09^{a4}	0.54 ± 0.09^{a3}	0.79 ± 0.09^{b4}	0.62 ± 0.01^{a5}	0.69 ± 0.01^{b4}	0.64 ± 0.01^{a4}	0.60 ± 0.06^{a4}	0.65 ± 0.06^{b3}	0.61 ± 0.06^{a4}

Values are expressed as ± Standard error of mean.
Down the columns, values with different numeric superscript are significantly different ($p \leq 0.05$) while values with the same numeric superscript are not significantly different ($p \leq 0.05$). Across the rows values with different alphabet superscript are significantly different ($p \leq 0.05$) while values with the same alphabet superscript are not significantly different ($p \leq 0.05$).

Table 3. Changes in the acid phosphatase activity in different tissues of *B. truncatus* after 96 h exposure to different concentrations of *V.amygdalina*extract at different pH levels.

Acid phosphatase activity (μmole/min/mg protein)												
	Hepatopancreas			Intestine			Muscle			Haemolymph		
Concentration	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5
Control 0.00 mg/L	0.50 ± 0.05^{a1}	0.46 ± 0.05^{a1}	0.45 ± 0.05^{a1}	0.49 ± 0.03^{a1}	0.42 ± 0.03^{b1}	0.37 ± 0.03^{c1}	0.43 ± 0.01^{a1}	0.43 ± 0.01^{a1}	0.42 ± 0.01^{a1}	0.50 ± 0.03^{a1}	0.43 ± 0.03^{b1}	0.44 ± 0.03^{b1}
0.02 mg/L	0.53 ± 0.01^{a1}	0.58 ± 0.01^{b2}	0.74 ± 0.01^{c2}	0.54 ± 0.01^{a1}	0.65 ± 0.01^{b2}	0.69 ± 0.01^{b2}	0.72 ± 0.04^{a2}	0.81 ± 0.04^{a2}	0.77 ± 0.04^{c2}	0.54 ± 0.7^{a1}	0.60 ± 0.07^{b2}	0.64 ± 0.07^{b2}
0.04 mg/L	0.57 ± 0.07^{a2}	0.56 ± 0.07^{a2}	0.66 ± 0.07^{b3}	0.60 ± 0.08^{a2}	0.65 ± 0.08^{b2}	0.76 ± 0.08^{c3}	0.67 ± 0.07^{a3}	0.65 ± 0.07^{a3}	0.75 ± 0.07^{b2}	0.68 ± 0.01^{a2}	0.68 ± 0.01^{a3}	0.77 ± 0.01^{b3}
1.00 mg/L	0.67 ± 0.02^{a3}	0.75 ± 0.02^{b3}	0.76 ± 0.02^{b2}	0.65 ± 0.01^{a2}	0.75 ± 0.01^{b3}	0.78 ± 0.01^{b3}	0.77 ± 0.01^{a4}	0.80 ± 0.01^{a2}	0.79 ± 0.01^{a2}	0.77 ± 0.04^{a3}	0.78 ± 0.04^{a4}	0.81 ± 0.04^{a3}

Values are expressed as ± Standard error of mean.
Down the columns, values with different numeric superscript are significantly different ($p \leq 0.05$) while values with the same numeric superscript are not significantly different ($p \leq 0.05$). Across the rows values with different alphabet superscript are significantly different ($p \leq 0.05$) while values with the same alphabet superscript are not significantly different ($p \leq 0.05$).

Table 4. Changes in the alkaline phosphatase activity in different tissues of *B. truncatus* after 96 h of exposure to different concentrations of *V.amygdalina*extract at different pH level.

Alkaline phosphatase activities (μmole/min/mg protein)												
	Hepatopancreas			Intestine			Muscle			Haemolymph		
Concentration	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5	pH 3.5	pH 7.0	pH 10.5
Control 0.00 mg/L	0.39 ± 0.03^{a1}	0.45 ± 0.03^{b1}	0.51 ± 0.03^{c1}	0.42 ± 0.02^{a1}	0.45 ± 0.02^{a1}	0.44 ± 0.02^{a1}	0.42 ± 0.01^{a1}	0.50 ± 0.01^{b1}	0.50 ± 0.01^{b1}	0.41 ± 0.02^{a1}	0.44 ± 0.02^{a1}	0.51 ± 0.02^{b1}
0.02 mg/L	0.42 ± 0.01^{a1}	0.45 ± 0.01^{ac1}	0.48 ± 0.01^{c1}	0.53 ± 0.03^{a2}	0.54 ± 0.03^{a2}	0.58 ± 0.03^{a2}	0.61 ± 0.04^{a2}	0.63 ± 0.04^{a2}	0.66 ± 0.04^{a2}	0.48 ± 0.08^{a2}	0.49 ± 0.08^{a2}	0.59 ± 0.08^{b2}
0.04 mg/L	0.51 ± 0.01^{a2}	0.58 ± 0.01^{b2}	0.57 ± 0.01^{b3}	0.47 ± 0.05^{a3}	0.52 ± 0.05^{b2}	0.52 ± 0.05^{b3}	0.51 ± 0.01^{a3}	0.61 ± 0.01^{b2}	0.60 ± 0.01^{b3}	0.44 ± 0.01^{a2}	0.49 ± 0.01^{b2}	0.51 ± 0.01^{b1}
1.00 mg/L	0.68 ± 0.06^{a3}	0.65 ± 0.06^{a3}	0.72 ± 0.08^{c4}	0.75 ± 0.01^{a4}	0.71 ± 0.01^{a3}	0.71 ± 0.01^{a4}	0.67 ± 0.05^{a4}	0.73 ± 0.05^{b3}	0.75 ± 0.05^{b4}	0.69 ± 0.06^{a3}	0.71 ± 0.06^{a3}	0.76 ± 0.06^{b3}

Values are expressed as ± Standard error of mean.
Down the columns, values with different numeric superscript are significantly different ($p \leq 0.05$) while values with the same numeric superscript are not significantly different ($p \leq 0.05$). Across the rows values with different alphabet superscript are significantly different ($p \leq 0.05$) while values with the same alphabet superscript are not significantly different ($p \leq 0.05$).

exposure of different concentrations of the crude extract at different pH conditions (3.5, 7.0 and 10.5).

The treatment of *Bulinus truncatus* with various concentrations of leaf extract of *Vernonia amygdalina* at varying pH conditions caused reductions in the activity of acetylcholinesterase in the haemolymph, muscle, hepatopancreas and intestine of the snails. The highest reductions in the acetylcholinesterase activity were observed in hepatopancrease (pH values of 7.0 and 10.5), intestine (at all pH levels), muscle (pH levels of 3.5 and 7.0) and haemolymph (pH of 3.5 and 7.0) at 1.00 mg/L of the extract. There was significant ($p < 0.05$) reduction of the enzyme's activity at all the extract's concentrations compared to the control. In the control, there was an increase in the enzyme's activity in all the tissues as the pH increased.

There were reductions in the total protein concentration in the snails' tissues as a result of treatment with various concentrations of the extract of *V. amygdalina*. Highest reductions were recorded in the hepatopancreas and haemolymph (at all pH conditions) at 1.00 mg/L of the extract. There were significant ($p < 0.05$) reductions in the total protein concentrations in all the tissues at different concentrations of the extract at different pH conditions compared to the control.

Treatment with different concentrations of *V. amygdalina* leaf extract at various pH values exhibited significant elevations in the acid phosphatase activity in the water snails' tissues. Highest elevations were observed in the intestine, hepatopancreas and haemolymph at the extract concentration of 1.00 mg/L at the pH value of 10.5. Treatment with the extract at various concentrations with varying pH values produced significant ($p < 0.05$) elevations in the enzyme's activity compared to the control.

When compared to the extract treatment concentrations, the alkaline phosphate activity was significantly ($p < 0.05$) lower in the control except in the hepatopancreas (pH values of 7.0 and 10.5) and the haemolymph (pH value of 10.5). There were significant ($p < 0.05$) increases in the enzyme's activity in the *B. truncatus*' tissues at the extract concentration of 1.00 mg/L (at all pH conditions) compared to the other extract concentrations (0.20 mg/L and 0.40 mg/L) and the control (0.00 mg/L).

The result shows that the varying concentrations of the leaf extract of *V. amygdalina* were responsible for the changes in the concentration in total protein in haemolymph, intestine and hepatopancrease. Different pH conditions and varying concentrations the extract of *V. amygdalina*

Table 5. Step-wise multiple regression result of significant setup condition responsible for the changes in total protein concentrations in the different tissues of *B. truncatus*.

Setup conditions	Tissues	*R*	R^2	*F-Values*	*P-Value*	Regression equation
V. amygdalina extract	hepatopancrease	0.633	0.401	22.739	0.00	Y = 0.154 + 0.276(*V. amygdalina* extract)
V. amygdalina extract	Intestine	0.701	0.491	32.783	0.00	Y = 0.239 + 0.246 (*V. amygdalina* extract)
V. amygdalina extract[a] and pH	Muscle	0.895	0.801	68.221	0.00	Y = 0.243(*V. amygdalina* extract) + 0.213 (pH)
V. amygdalina extract	haemolymph	0.358	0.128	4.992	0.032	Y = 0.700 + 0.136 (*V. amygdalina* extract)

p significant at 5% alpha level. a = setup condition with a stronger effect.

Table 6. Step-wise multiple regression result of significant setup condition responsible for the changes in acetyl cholinesterase activities in different tissues of *B. truncatus*.

Setup conditions	Tissues	*R*	R^2	*F-Values*	*P-Value*	Regression equation
V. amygdalina extract[a] and pH	hepatopancrease	0.916	0.839	85.976	0.000	Y = 0.554 + 0.393 (*V. amygdalina* extract) + 0.115 (pH)
V. amygdalina extract[a] and pH	Intestine	0.875	0.765	53.814	0.000	Y = 0.769 + 0.228(*V. amygdalina* extract) + 0.170 (pH)
V. amygdalina extract[a] and pH	Muscle	0.838	0.702	38.820	0.000	Y = 0.800 + 0.219(*V. amygdalina* extract) + 0.200 (pH)
V. amygdalina extract[a] and pH	Haemolymph	0.836	0.745	48.125	0.000	Y = 0.937 + 0.247(*V. amygdalina* extract) + 0.189 (pH)

p significant at 5% alpha level. a = setup condition with a stronger effect.

Table 7. Step-wise multiple regression result of significant setup condition responsible for the changes in acid phosphatase activities in different tissues of *B. truncatus*.

Setup conditions	Tissues	*R*	R^2	*F-Values*	*P-Value*	Regression equation
V. amygdalina extract[a] and pH	hepatopancrease	0.759	0.576	22.388	0.000	Y = 0.403 + 0.076(*V. amygdalina* extract) +0.044 (pH)
V. amygdalina extract[a] and pH	Intestine	0.806	0.650	30.614	0.000	Y = 0.393 + 0.094(*V. amygdalina* extract) + 0.039 (pH)
V. amygdalina extract	Muscle	0.729	0.532	38.596	0.000	Y = 0.518 + 0.099(*V. amygdalina* extract)
V. amygdalina extract	Haemolymph	0.887	0.787	125.259	0.000	Y = 0.469 + 0.110(*V. amygdalina* extract)

p significant at 5% alpha level. a = setup condition with a stronger effect.

Table 8. Step-wise multiple regression result of significant setup condition responsible for the changes in alkaline phosphatase activities in different tissues of*B. truncatus*.

Setup conditions	Tissues	*R*	R^2	*F-Values*	*P-Value*	Regression equation
V. amygdalina extract and pH[a]	Hepatopancrease	0.937	0.878	122.004	0.000	Y = 0.150 (pH) + 0.082 (*V. amygdalina* extract)
V. amygdalina extract and pH[a]	Intestine	0.955	0.911	174.685	0.000	Y = 0.142 (pH) + 0.086(*V. amygdalina* extract)
V. amygdalina extract	Muscle	0.369	0.136	5.351	0.027	Y = 0.684–0.034 (*V. amygdalina* extract)
V. amygdalina extract and pH[a]	Haemolymph	0.953	0.907	166.356	0.000	Y = 0.151 (pH) + 0.081(*V. amygdalina* extract)

p significant at 5% alpha level. a = setup condition with a stronger effect.

were responsible for the changes in the concentration of total protein in the muscle though the extract has a stronger effect than pH.

From the regression analysis carried out on the data, the result (Table 5) shows that the varying concentrations of *V. amygdalina* leaf extract concentration were responsible for the changes in the total protein concentration in haemolymph, intestine and hepatopancrease. Both the varying pH conditions and varying concentrations of the leaf extract were responsible for the concentration of total protein in the muscle; the varying concentrations of the leaf extract had a stronger effect. Changes in acetyl cholinesterase activity were caused by the varying concentrations of leaf extract of *V. Amygdalina* and different pH values (Table 6) but the extract had a stronger effect.

The result also shows (Table 7) that the changes in acid phosphatase activity in the hepatopancrease and intestine were caused by the varying concentrations of *V. amygdalina* leaf extract and varying pH conditions. The varying concentrations of the extract were largely responsible for the changes in acid phosphatase activity in the muscle and haemolymph. Varying pH values and varying concentrations of *V. amygdalina* leaf extract were responsible for the changes in alkaline phosphatase activities (Table 8) in the hepatopancrease, intestine and haemolymph with varying pH values having a stronger effect. Nevertheless, varying concentrations of *V. amygdalina* aqueous leaf crude extract was exclusively responsible for the changes in alkaline phosphatase activities in the muscle.

4. Discussion

The subjection of *Bulinus truncatus* to varying pH conditions produced an inhibition of the acetylcholinesterase activity in the haemolymph, muscle, hepatopancreas and intestine of the snails with the highest inhibition occurring at the pH value of 3.5. This might be as a result of the acidification of the heamolymph. Ewald et al. (2009) reported that *Elimia flava* subjected to a pH condition of 4.0 were inactive, experienced more dramatic ionic disturbances and reduced haemolymph than snails subjected to higher pH conditions of 5, 6, and 7.

Acetylcholinesterase (AchE) is an enzyme of importance in the nervous system of animals; the enzyme occurs in the outer basal lamina of nerve synapses, neuromuscular junction and other tissues (Guyton and Hall, 2006). AchE terminates cholinergic impulses by the hydrolysis of acetylcholine released during synaptic transmission, thus inhibiting the acetylcholinesterase which then leads to accumulation of the synapses and when concentration rises beyond normal level, the result will be paralysis before the imminent death of the organism (Singh and Singh, 2003). Exposure of *B. truncatus* to varying concentrations of crude aqueous leaf extract of *V. amygdalina* at different pH conditions produced a significant inhibition in AChE activity of some tissues in the snail. This is in agreement with the report of Singh and Agarwal (1983) who suggested that a dose-dependent inhibition of AchE in freshwater snail might be due to its conversion to more toxic metabolites in the body.

There were constant reductions in total protein level with increasing concentrations of *V. amygdalina* crude extract in the haemolymph, muscle, hepatopancreas and intestine of the snails and could be attributed to the inability of protein-synthesizing machinery to function properly. This conforms to similar research done by Akinpelu et al. (2012).

Acid phosphatase plays an important role in catabolism, pathological necrosis, autolysis and phagocytosis (Aruna et al., 1979). The increase in acid phosphatase activity in intoxicated animals as observed in the present investigation may be due to the damaging of the lysosomal membrane which resulted in the release of the enzyme. The obvious increase in the enzyme's activity may be due to the damage caused to the lysosomal membrane, thus allowing the seepage of lysosomal enzyme into cytosol (Sherekar and Kulkarni, 1987).

Alkaline phosphatase plays a critical role in protein synthesis, shell formation and other secretary activities (Timmerman, 1969; Ibrahim

et al., 1974). Alkaline phosphatase breaks down ester compounds of orthophosphate acids under alkaline conditions (Adolph and Lorenz, 1981). The dip in the alkaline phospahtase activity to 0.40 mg/L in haemolymph, muscle and intestine could be due to inability of the enzyme to carry out the functions afore-stated as a result of toxicity elicited by the *V. amygdalina* crude extract and it may result in altered transport and also have an inhibitory effect on cell growth and multiplication, reduction in protein level and severe acidosis (Shaikila et al., 1993). The synthesis of special enzymes might have been involved in detoxification of activated materials and other waste metabolic products, which was used in getting rid of the molluscicide from snail's body (Akinpelu et al., 2012). The constant increase in the hepatic alkaline phosphatase activity with increasing concentration of the *V. amygdalina* crude extract observed in this study could be attributed to cellular damages or a response to overcome toxicity due to the crude extract.

In conclusion, for a plant to be considered as molluscicidal it should be effective at a concentration of about 100 μg/L. Thus the molluscicidal potencies of *Vernonia amygdalina* crude extract towards freshwater snail (*B. truncatus*) has been ascertained in this study. This investigation has shown that *V. amygdalina* crude extract meets all standards which support its potential use as a potent natural candidate molluscicide. It can be used for the control of freshwater snails and pollution. The study also raised a possibility of the locals living around freshwater bodies prone to trematode borne diseases to reflexively control freshwater snail population by just squeeze-washing their *Vernonia amygdalina* (Bitter leaves) around the river banks.

Declarations

Author contribution statement

F. Okafor: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

J. Eze: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

N. Nwankwo: Performed the experiments; Contributed reagents, materials, analysis tools or data.

E. Okeke and N. Onwudiwe: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We wish to acknowledge the staff members (both academic and non-academic) of the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, for their assistance during the period of this study.

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