Molluscicidal Effects of Bambusa Blumeana (Bamboo) Leaf Extracts Against The Adult Stage of the Snail Vector Model Pomacea Canaliculata

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Abstract: Many aquatic snails are known to cause different parasitic diseases. Over the past decades, snail-transmitted diseases, most especially Schistosomiasis has become a major health problem, due to its resistance against the common chemotherapeutic agents to counter act it. With growing incidence of drug resistance, studies for vector control are being conducted nowadays. Thus, this study investigates the molluscicidal activity of *Bambusa blumeana* leaf extracts against the adult stages of the snail vector model *Pomacea canaliculata*. Extracts of plants were oven dried and powdered, and were tested for phytochemicals associated with molluscicidal effects. Methanolic crude extracts were partitioned with different organic solvents through modified Kupchan method. Snail subjects were exposed with the leaf extracts by immersion. Toxic effects of different preparations on the biochemical parameters present in them will also be studied against the snail. Different extracts proved to have molluscicidal activity against snail vector model, *Pomacea canaliculata snails*. Starting at 400mg/L concentration extracted using the solvent N-hexane, 200 mg/L ethyl acetate and 100 mg/L butanol was found out to exhibit the most potent molluscicidal effect against the tested snails and has the same efficacy in comparison with the synthetic and commonly used niclosamide. *B. blumeana* leaf extracts contain phytochemicals such as saponins and flavonoids that induced mortality of the snails. Molluscicidal property of *B. blumeana* leaf extract was supported by increased concentrations of total protein and ALT at higher concentrations of plant extracts.
Keywords: Schistosomiasis, mollusk-transmitted infections, Bambusa blumeana, Pomacea canaliculata, molluscicidal activity

INTRODUCTION

According to WHO, over 78 countries have been reported to have trematode infections particularly schistosomiasis, derived from such snail vectors. In recent years, Schistosomiasis problem in the Philippines is alarming (Olveda et al., 2014). The number of incidence is continuously increasing. The highest number of prevalence is found in Oriental Mindoro followed by Sorsogon and some came from NCR who are migrants from Cagayan Valley (Leonardo et al., 2012).

Schistosomiasis is a snail-borne tropical disease caused by the Schistosoma species namely *Schistosoma japonicum, S. mansoni* and *S. haematobium.* These three are considered to be the most pathogenic to humans, but in the Philippines, the endemic species is *S. japonicum* (Leonardo et al., 2016). This pathogen causes hematuria, dysuria, uremia, anemia, stunting, bladder cancer, urosepsis and human immunodeficiency virus coinfection (Rinaldi et al., 2015).

The lifecycle of this parasite begins when the adult male and female worms live within the veins of their human host, where they mate and produce fertilized eggs. The eggs are either shed into the environment through feces or urine, or are retained in human tissues where they induce inflammatory response and eventually die. The eggs that will reach freshwater will later on hatch; thus, releasing the free-living ciliated miracidia stage, which may then infect a mollusk host (Colley, Bustinduy, Secor, & King, 2014). Freshwater snails are the mollusk group that serve as the intermediate host of *Schistosoma spp* and are considered targets for Schistosoma control programs. Many researchers are investigating on plant molluscicides since it is cheaper and more obtainable than artificial molluscicides (Al-Zanbagi, 2013). It is also stated by Singh, Yadav, and Singh (2010) and Demetillo, Baguio, Limitares, Madjos, and Abrenica-Adamat (2015) that the use of plants with molluscicidal properties appears to be a more simple, inexpensive and safer alternative since they contain biodegradable compounds, which are less likely to cause environmental contamination.

Pomacea canaliculata, commonly known as the golden apple snail, is considered as the most common and ideal snail model and substitute used in molluscicidal laboratory tests. Such snails are easily cultured and raised under laboratory conditions, due to their longer lifespan and ability to adapt and survive on aquatic environments with room or controlled temperature (Seuffert & Martin, 2013). In relation to Schistosoma vectors

Oncomelania quadrasi, Biomphalaria glabrata, the golden apple snail has the same susceptibility against molluscicides, in which all of these species were classified under Order Gastropoda. The snails are susceptible against niclosamide, as well as on biochemical compounds such as saponins, flavonoids and glycosides (Faria et al., 2017).

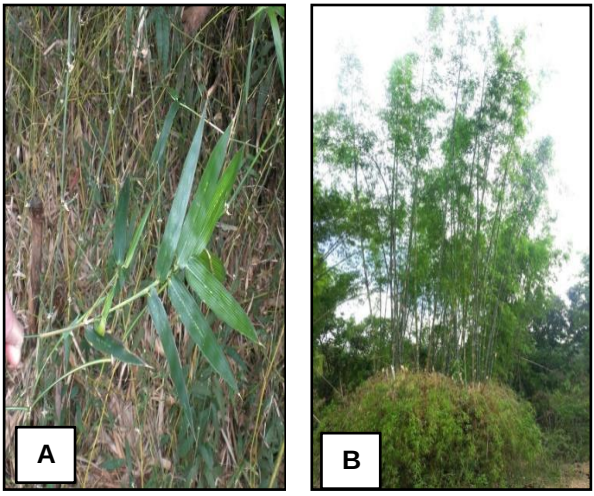


Figure 1. Bambusa blumeana (A) tree and (B) leaves

According to Musman, Kamaruzzaman, Karina, Rizqi and Arisca (2013) certain plants exhibit molluscicidal activity due to the presence of biochemicals, including antioxidants. Studies for *Bambusa blumeana* leaves revealed to have antioxidant properties.

***B. blumeana*,** is a specie of bamboo that grows in the tropical regions of Asia. It is known locally in Philippines as *Kawayang Tinik*. The plant's leaves are lance-shaped and on average 10-20 cm long and 12-25 mm wide. Recent studies proved that bamboo plants under genus *Bambusa* leaf extracts contain antioxidants which exhibit anti-inflammatory and anti-diabetic activities (Senthilkumar, Sivakumar, Faisal, Rajesh, & Perumal, 2011). According to Valentino, Ganado, Ganado, and Undan (2015), all ethanolic extracts of *Bambusa blumeana* indicated the presence of tannins, steroids, saponins, terpenoids, and flavonoids, and such chemical components are being investigated nowadays to have potentials of having molluscicidal effects (Al-Zanbagi, 2013).

The liver of the snail vector model *Pomacea canaliculata* is analogous to the hepatic system of higher animals, which is considered as the major metabolic organ (Rawi, Al-Hazmi, & Al-Nassr, 2011). In the present study, the molluscicidal activity test determined the toxic and lethal effects of *B. blumeana* leaf extracts by measuring the snails' hepatic profile, consisting of total protein and alanine aminotransferase level determinations. Aspartate aminotransferase was not included in the panel, since the hemolymph source from each mollusk was extracted within the animals' heart.

To date, there is no study on molluscicidal activity for *B. blumeana;* hence, the objective of the present study is to examine the individual effect of *B. blumeana* leaves extracted using different organic solvents against the adult stages of *P. canaliculata* through the leaves' phytochemistry, concentration, and to determine the effect of the plant in terms of hemolymph total protein concentration and alanine aminotransferase activity. By altering the development of *Schistosoma spp.* in its intermediate host, there might be a potential decrease of incidence.

MATERIALS AND METHODS

Collection and preparation of snail model

Snails were obtained from Barangay Magsaysay, San Antonio Quezon which were identified and authenticated at the Parasitology Department of the University of the Philippines – Manila, College of Public Health. The snail preparation followed the standard protocol of World Health Organization which states that snails should be provided with food, water, simulating its natural habitat. The snails were transported to the laboratory in plastic containers with filtered fresh water and fed three times a week with water lettuce (Angaye, Bassey, Ohimian, Izah, & Asaigbe, 2015; Pinto, Da Silva, Vargas & Baptista, 2013).

Collection and preparation of plant material

B. blumeana leaves were collected from Barangay Palanas, Lemery, Batangas. An acknowledged taxonomy from the Forestry Department of the University of the Philippines-Los Banos authenticated the botanical identity of the plants.

The leaves were placed in a wet black plastic bag while transferring it to the laboratory to avoid direct exposure to sunlight, which may cause its dehydration. Then, they were washed with

distilled water to remove sand, dust and other debris (Otarigho & Morenikeji, 2013).

Preparation of plant extracts

Fresh leaves of *B. blumeana* were dried according to the method of Srivastava and Dwivedi (2016) using a drying oven set in the temperature of 40^0C for 2 hours. The leaves were powdered using an electric blender (Upadhay, Singh, & Singh, 2013).

The dried and grounded plant powder weighing 500 grams were extracted with 2.5 liters of methanol in an air tight, clean flat bottomed container for 7 days at room temperature with occasional stirring and shaking. The extracts were then filtered first through a fresh cotton plug and finally with a Whatman filter paper. The filtrate was concentrated using a rotary evaporator at low temperature (30ºC) and pressure. The weight of the crude extract was measured (Muhit, Tareq, Apu, Basak, & Islam, 2010).

Solvent-solvent partitioning using Kupchan's Method

B. blumeana leaf extract using methanol were undertaken solvent-solvent partitioning by using modified Kupchan method. The 5g crude extract were triturated by dissolving in 90% aqueous methanol (methanol: water; 9:1 v/v) to make the mother solution. The prepared solution was then fractionated successfully using increasing polarity such as N-hexane, ethyl acetate and then butanol. All these fractions were evaporated to dryness using rotary evaporators at low temperature of 45ºC and kept in air tight containers for further analysis (Muhit et al., 2010; Chaity, Khatun, & Rahman, 2016).

Phytochemical Screening

The chemical test was performed in different solvent extracted fractions of *Bambusa blumeana* leaves using standard procedure.

Test for Saponins: A volume of 0.5mL of *B. blumeana* leaf extracts was added to 10mL distilled water and is shaken vigorously to obtain a stable persistent froth. Persistent frothing indicates the presence of saponin (Valentino et al., 2015).

Test for Glycosides: Each *B. blumeana* leaf extracts was hydrolyzed with HCl and neutralized with NaOH solution. A Few drops of Fehling's solution A and B will be added to each mixture. Formation of red precipitate indicates the presence of glycosides (Rauf et al., 2013).

Test for Cardiac Glycoside: In a 2ml of *B. blumeana* leaf extracts, 1ml of glacial acetic acid and 5% ferric chloride was added. Then few drops of concentrated H_2SO_4 were added. Presence of greenish blue color indicates the presence of cardiac glycosides (Rauf et al., 2013).

Test for Flavonoids: Few drops of 1% NH_3 solution was added to 5 mL *B. blumeana leaf* extracts in a test tube. Yellow coloration indicates the presence of flavonoids (Valentino et al., 2015).

Molluscicidal Activity Tests

The plants were evaluated for molluscicidal activity on adult snails as follows: 5 groups of 10 snails were placed in plastic containers holding 500 ml of distilled water. The set ups were left for 24 h and snails were fed on dried lettuce. Different concentrations; 50 mg/liter 100 mg/liter, 200 mg/liter, 400 mg/liter and 800 mg/liter of all organic extracts were prepared (Musman, 2010). Positive control was prepared as 1 mg/liter of niclosamide and the negative control was 500 ml of distilled water. After 24 hours distilled water was discarded from the containers holding 10 snails each and replaced with 500 ml of the different extracts concentrations, positive control and a negative control preparation after which mortality was assessed (Taquiling, 2015).

Bioassays. Death of snails was determined by pricking the snails to elicit typical withdrawal movements. Absence of movements or reaction indicates that the snails were dead. (Taquiling, 2015). Mortality is confirmed by immersion two or three at a time in 15-20% sodium hydroxide in petri-dish was used, if bubbles and blood come out of the shell, it is recorded as alive, if not it is recorded as dead snails (Rawi et al., 2011).

Biochemistry Tests

After every 24 hours of exposure, live snails of each group were sampled for hemolymph.

Sample preparation. The snails were gently washed in clean saline. A piece of cotton wool was dipped in 70% ethanol and the shell of the snail was thoroughly cleaned. The snails were carefully and gently immobilized using one hand. A pair of sterile scissors was flamed and the shell of the snail was meticulously removed to expose the hemolymph (blue fluid). A sterile syringe was then used to siphon the fluid by pulling the plunger. The collected hemolymph was then transferred into sterile test tubes.

The tubes were tightly capped and stored in a refrigerator at 4°C until testing for alanine amino transaminase (ALT) and total protein (TP), and to prevent the proteins in the hemolymph from disintegrating. The extraction process was undertaken separately for each snail and was performed aseptically. Hemolymph extract from one of the snails were properly labeled by its corresponding group number (Donkor et al., 2017). The hemolymph collected was centrifuged at 1500 g for 10 min to obtain the plasma prior to testing of total proteins and alanine aminotransferase (Cueto, Giraud-Billaud, Vega, & Castro-Vasquez, 2011).

Determination of Total Protein

This assay was performed in accordance with the Biuret reaction, using reagents for total protein by New Tech. A mixture of 10 µL of centrifuged hemolymph and 1000 µL of the Biuret reagent were read in a spectrophotometer at 550 nm. Results were calculated by dividing the absorbance of the sample at 550nm with the absorbance of the standard, and multiplied by standard concentration.

The results were expressed as g/L (Bonfim et al., 2014). Total protein control values for *P. canaliculata* hemolymph were obtained from the normal group of snails that were exposed on distilled water only. A value of 10 g/L was obtained after spectrophotometry, and this result was in accordance with the measurement done by Cueto et al. (2011) in their study.

Determination of Alanine Aminotransferase activities

The assay was performed using the alanine aminotransferase reagent kit and protocol by HUMAN Diagnostics. According to the protocol of HUMAN Diagnostics for ALT/SGPT, 100 µL of the supernatant of the centrifuged hemolymph was added with the buffer solution (composed of 125 mmol/L TRI buffer with pH 7.4, 625mmol/L L-alanine, 1.5kU/l LDH and 0.095% sodium azide). The mixture was incubated at 37 C for 5 minutes, and 250µL of the substrate solution (composed of 75mmol/L 2-oxoglutarate, 0.9mmol/L NADH, and 0.095% sodium azide) was added. Absorbance at 334nm was measured using the spectrophotometer after 1, 2 and 3 minutes. Results (U/l) were calculated by multiplying the absorbance /minute with the factor 2184 (substrate start at 37C).

Statistical Analysis

For statistical analysis, ANOVA was used in determining the molluscicidal effects of *B. blumeana* leaf extracts with varying concentrations in comparison with the positive and negative control. For the mortality test, ANOVA was also employed in the determination and correlation of the extracts effects on the total protein and alanine aminotransferase activities.

RESULTS AND DISCUSSION

I. Plant Extract

Figure 2*. Bambusa blumeana* crude extract

From 500g powdered leaves of *Bambusa blumeana*, 1500ml of methanolic extract was obtained. After subjecting the extract to rotary evaporator, 15.392g of dark green and oily crude extract. The obtained methanolic extract had underwent Kupchan method of ethyl acetate and butanol and the obtained extracts were 1.85 g, 1.525 g and 1.480 g, respectively. B. blumeana leaf extracts yield were the same as the extracts obtained by Rawi et al. (2011) as well as the bamboo leaf extracts produced by Valentino et al. (2015).

II. Phytochemical Screening

Table 1.Phytochemical Screening

Component	N-hexane	Ethyl Acetate	Butanol
Cardiac Glycosides	-	-	-
Flavonoids	+	+	+
Glycosides	-	-	-
Saponins	+	+	+

Legend: (+) Present; (-) Absent

Table 1 shows the phytochemical screening result of *B. blumeana* leaf extract. The presence of persistent frothing was indicated on the n-hexane, ethyl acetate and butanolic extracts of *B. blumeana*; thus, confirming the presence of saponins.

The presence of flavonoids from *B. blumeana leaf* extracts was determined by the yellow coloration that was visible after adding a few drops of 1% NH_3 solution to each organic extract. Based on the result, the presence of flavonoids in each organic extract was confirmed. Glycosides and cardiac glycosides on the other hand, were both absent on the organic extracts. The present study regarding such phytochemical findings were in agreement with the works of Rawi, et al (2011) and Souza, da Silva, Chicarino, and Bessa (2013), which obtained the same phytochemistry results for their molluscicidal activity tests.

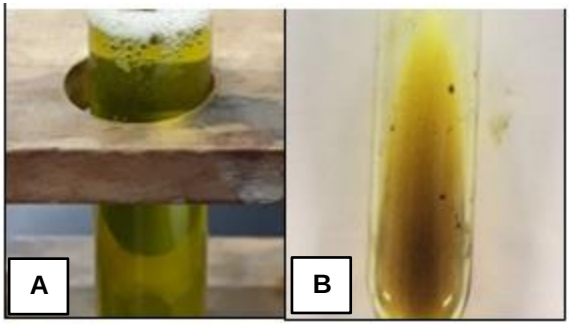


Figure 3. Positive result for the presence of saponin and (B) flavonoids

III. Bioassay

Table 2 shows the average number of dead snails from three trials with varying organic solvents. In the negative control, which contains distilled water, no death was observed. In the positive control, with 1 mg/L of Niclomax, all of the 10 snails died. This clearly shows that distilled water has no molluscicidal activity, whereas Niclomax is proven to be an effective molluscide. For N-hexane, at 50 mg/L concentration, only 1 snail from the 3 trials died, resulting to an average of 0.33. For 100mg/L and 200mg/L, the resulting number of dead snails was 6.33 and 8.33 respectively, moreover, at 400mg/L and 800mg/L, the resulting average of death were both 10.

Table 2. Average number of dead snails from trial 1 to trial 3 with varying organic solvents

	***Concentration of B. blumeana* leaf extract**	**Average No. of Dead Snails N=10**
Negative Control	Distilled water	0
Positive Control	1mg/L	10
N-hexane	50mg/L	0.33
	100mg/L	6.33
	200mg/L	8.33
	400mg/L	10
	800mg/L	10
Ethyl Acetate	50mg/L	0
	100mg/L	5
	200mg/L	6.33
	400mg/L	6.67
	800mg/L	6.67
Butanol	50mg/L	0
	100mg/L	6.33
	200mg/L	7.00
	400mg/L	7.67
	800mg/L	10

With ethyl acetate, all of the 10 snails were not killed at 50 mg/L; however, at 100 mg/L, 200 mg/L, 400 mg/L and 800 mg/L concentrations, the resulting average number of dead snails were 5, 6.33, 6.67 and 6.67, respectively. Furthermore, with butanol, all snails were also not killed at 50mg/L; whereas, at 100mg/l, 200mg/L, 400mg/L and 800mg/L concentrations, the resulting average number of dead snails were 6.33, 7.00, 7.67 and 10, respectively. It can be seen on the table that the lowest concentration of 50 mg/L was not able to kill the snails showing that such concentration cannot be used in killing snails. On the other hand, it is evident that increasing the concentration of *B. blumeana* leaf extract can increase the number of dead snails; thus, showing its molluscidal activity in increasing concentration. Such can be attributed to the phytochemical contents of the *B. blumeana* leaf extract, such as saponins and flavonoids, against the golden apple snails. Previous studies regarding plant saponins revealed that such substances are capable of inducing mass dehydration within the mollusk's body when exposed to high concentrations (Chaieb & Tayeb, 2009). The molluscicidal effect of the phytochemical saponin was also supported by the findings of Musman (2010), in which saponins have the ability to induce

the animals' mortality by damaging the snails' hepatic cell membrane through alterations of the surface tension of water.

In the present study, flavonoids are one of the phytochemicals detected. However, in a study conducted by Hamed et al. (2015), it was observed that most flavonoid groups alone in a leaf extract cannot induce molluscicidal and larvicidal properties. In relation to our study, *B. blumeana* leaf flavonoids may have induced its effect along with the saponin content.

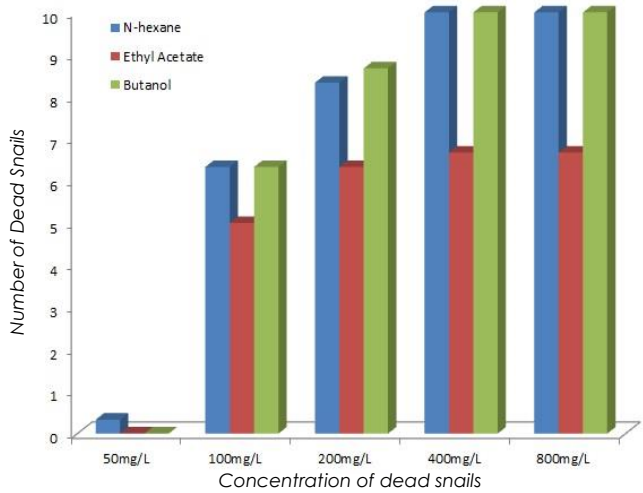


Figure 4. Effects of the *B. blumeana* leaf extracts using different organic solvent on adult stages of *P. canaliculata*

Saponins and flavonoids may have act synergistically, since flavonoids are capable of increasing the rate of saponin absorption of the snail's hepatic cells, therefore intensifying the lethal effects to the snails. This is in agreement with the findings of Souza, da Silva, Chicarino, and Bessa (2013), which revealed that flavonoid compounds are capable of inducing molluscicidal properties by inhibiting the detoxification system of the snails, allowing saponins to induce a greater toxicity and fatal effect. The same results can be seen in figure 4.

Of the three solvents used in the extraction of the leaf extracts, the use of n-hexane as solvent revealed to have the most number of deaths. It may be implied that the use of such organic solvent may have contributed to the further release and extraction of phytochemicals, leading to the increased mortality of snails. Based on the experimental data obtained, it may also be implied

that ethyl acetate and butanol solvents may have induced less phytochemicals present compared to n-hexane, since such extracts with these two solvents produced fewer number of dead snails when compared with n-hexane.

Table 3. Comparison of Dead Snails on Different Concentration against Positive Control

Concentration of *B. blumeana* leaf extracts	p-value	Interpretation
N-hexane	50 mg/L	0.000	HS
	100 mg/L	0.001	S
	200 mg/L	0.011	S
	400 mg/L	1.000	NS
	800 mg/L	1.000	NS
Ethyl Acetate	50 mg/L	0.000	HS
	100 mg/L	0.005	S
	200 mg/L	0.072	NS
	400 mg/L	0.125	NS
	800 mg/L	0.125	NS
Butanol	50 mg/L	0.000	HS
	100 mg/L	0.125	NS
	200 mg/L	0.209	NS

Legend: Significant at p-value < 0.05 HS- Highly Significant S- Significant NS- Not Significant

Table 3 presents the comparison of the molluscicidal effect of the prepared concentrations of each of the different organic extracts used. The positive control used in the 3 trials consists of snail groups exposed with 1mg/L niclosamide (Niclomax). The use of negative control (distilled water) resulted to a p value of 0.000 which indicates that there is a highly significant difference when compared with positive control. This confirms that Niclomax is molluscicidal while distilled water is not. With n-hexane extracts, concentrations at 50 mg/L, 100 mg/L and 200 mg/L showed p-values that were less than 0.05; thus, interpreted to have significant difference against the positive control. Moreover, at concentrations 400 mg/L and 800mg/L, p-values were 1.000, which shows that the molluscicidal activity at such concentrations is similar with the positive control. For ethyl acetate extracts, at 50 mg/L and 100 mg/L concentrations, the obtained p-values were less than 0.05; thus, interpreted as having significant difference against the positive control while concentrations starting at 200 mg/L obtained results that were not significantly different against the positive control. For butanol, only 50 mg/L obtained a p-value of 0.000; thus, is highly significant which is comparable with the

negative control. Statistically no significant difference against the positive control was observed starting at the concentration of 100 mg/L.

According to statistical results, concentration of 50mg/L using N-hexane, ethyl acetate and butanol, has similar non-molluscicidal effect as distilled water. It is also evident that at concentrations of 400mg/L of n-hexane, 200 mg/L of ethyl acetate and 100 mg/L of butanol, the same molluscicidal effect with the positive control niclosamide was noted. Based on the results, it was observed that as the concentration of *B. blumeana* leaf extract increases, the molluscicidal activity increases as well; thus it may be implied that the greater the concentration of the leaf extract, the greater the amount of phytochemicals being exposed against the snails. This finding is in agreement with the works done by Rawi et al. (2011) as well as Chaieb and Tayeb, (2009), wherein the number of mortalities of snails increases as the concentration of leaf extract increases.

Table 4 shows multiple comparison of different concentration of *B. blumeana* leaf extracts. For N-hexane, when 50 mg/L concentration was compared with 100mg/L, the resulting p-value of 0.191 was obtained; thus, there is no significant difference. However, when it was compared with 200 mg/L, 400 mg/L and 800 mg/L, the obtained p-values were 0.033, 0.000 and 0.000, respectively. This shows that there is a significant difference with the effect, indicating that higher concentrations are more effective in killing the snails. The effect of 100 mg/L n-hexane leaf extracts showed no significant difference on all extracts and their respective concentrations, except at 400 mg/L and 800 mg/l of n-hexane. At 200 mg/L of n-hexane, it was found out that there was no significance with all extracts and concentrations as well, except at 50 mg/l of all solvents used. No significant difference was noted between 400 mg/l n-hexane extracts with 800mg/l of n-hexane. For ethyl acetate extracts, 50mg/L showed significant difference in comparison with 100 mg/L, 200 mg/L, 400 mg/L and 800 mg/L concentrations. A concentration of 100 mg/L of ethyl acetate was found to show no significant difference when compared with 200 up to 800 mg/L of the same extract. The 200 mg/l ethyl acetate leaf extract was found to have no significant difference when compared with 400 and 800 mg/L of the same extract.

Table 4.Multiple Comparison of different concentration of *B. blumeana* leaf extracts

Concentration of *B. blumeana* leaf extract		p-value	Interpretation
50 mg/L NH	100 mg/L NH	0.191	NS
	200 mg/L NH	0.033	S
	400 mg/L NH	0.000	HS
	800 mg/L NH	0.000	HS
100 mg/L NH	50 mg/L NH	0.191	NS
	200 mg/L NH	1.000	NS
	400 mg/L NH	0.009	S
	800 mg/L NH	0.009	S
200 mg/L NH	50 mg/L NH	0.033	S
	100 mg/L NH	0.191	NS
	400 mg/L NH	1.000	NS
	800 mg/L NH	0.062	NS
400 mg/L NH	50 mg/L NH	0.000	HS
	100 mg/L NH	0.009	S
	200 mg/L NH	0.062	NS
	800 mg/L NH	1.000	NS
50 mg/L EA	100 mg/L EA	0.033	S
	200 mg/L EA	0.002	S
	400 mg/L EA	0.001	S
	800 mg/L EA	0.001	S
100 mg/L EA	50 mg/L EA	0.033	S
	200 mg/L EA	1.000	NS
	400 mg/L EA	0.995	NS
	800 mg/L EA	0.995	NS
200 mg/L EA	50 mg/L EA	0.002	S
	100 mg/L EA	1.000	NS
	400 mg/L EA	1.000	NS
	800 mg/L EA	1.000	NS
400 mg/L EA	50 mg/L EA	0.001	S
	100 mg/L EA	0.995	NS
	200 mg/L EA	1.000	NS
	800 mg/L EA	1.000	NS
50 mg/L But vs.	100 mg/L But	0.001	S
	200 mg/L But	0.000	HS
	400 mg/L But	0.000	HS
	800 mg/L But	0.000	HS
100 mg/L But vs.	50 mg/L But	0.001	S
	200 mg/L But	1.000	NS
	400 mg/L But	1.000	NS
	800 mg/L But	0.457	NS
200 mg/L But vs.	50 mg/L But	0.000	HS
	100 mg/L But	1.000	NS
	400 mg/L But	1.000	NS
	800 mg/L But	0.628	NS
400 mg/L But vs.	50 mg/L But	0.000	HS
	100 mg/L But	1.000	NS
	200 mg/L But	1.000	NS
	800 mg/L But	1.000	NS

Legend: Significant at p-value < 0.05 NH - N-hexane EA- Ethyl Acetate But- Butanol NS- Not Significant S- Significant HS-Highly Significant

A concentration of 400 mg/L of ethyl acetate leaf extract when compared with 800 mg/L of ethyl acetate showed no significant difference. For butanol leaf extracts, 50 mg/L showed significant difference with greater concentrations of the same extract, and highly significant difference was found with 200mg/L up to 800 mg/l concentrations.

Based on the multiple comparison per extract and concentration, it is clearly shown that molluscicidal activity is exhibited starting from 200mg/L of N-hexane, 200 mg/L of ethyl acetate and 100mg/L of butanol leaf extracts up to 800 mg/L concentration, and such effect has statistically no significant difference as compared with the positive control. This is more likely due to the ability of the three solvents to extract greater amount of phytochemicals saponins and flavonoids. The concentrations of 200 mg/L of both n-hexane and ethyl acetate leaf extracts and 100mg/L butanol may have contain optimum concentration and amount of phytochemicals that may induce the mortality of snails.

It is evident that as concentrations of *B. blumeana* leaf extracts increases, the number of dead snails per extract also increases. The findings regarding the effect of concentration of the extract as to the amount of phytochemicals positive for testing was in accordance with the work of Valentino, et al. (2015), where in *B. blumeana* leaf extracts' saponins and flavonoids were only detected in leaf extracts in greater concentrations. The study done by Chaieb and Tayeb (2009) was also in agreement in the present study's findings with molluscicidal activity, where in mortality was only induced in higher concentration of plant extracts. In relation to the organic solvent used for extraction, it may be implied that butanol leaf extracts of *B. blumeana* may have induced the most effective extraction of phytochemicals when compared with n-hexane and ethyl acetate, since at a concentration of 100 mg/L, the extract was able to produce results with statistically no significant difference compared with the positive control. The result was in line with the findings of Musman et al. (2010) wherein they obtained molluscicidal effects that were statistically similar as to their positive control at the same concentration and in their extract.

IV. Biochemical Analysis

The specimens analyzed were the pooled hemolymph of the snails that survived from the 24-hour exposure, and are grouped according to the concentration of each organic extract.

After the bioassay, hemolymph samples were extracted from the snails that were still alive from N-hexane at 50mg/L, 100mg/L, and 200mg/L, for butanol extracts at 50 mg/L, 100mg/l, 200 mg/L and 400 mg/L and all concentrations for ethyl acetate since there were still snails that survived in each concentration of ethyl acetate after the 24 hour exposure.

A. Total Protein

Table 5. Total Protein Concentration

	Concentrations of *B. blumeana* leaf extracts	**Concentration g/L**
K Control group	Distilled water	10
N-hexane	50 mg/L	18
	100 mg/L	23.33
	200 mg/L	34.67
Ethyl Acetate	50 mg/L	18
	100 mg/L	21.33
	200 mg/L	28
	400 mg/L	36
	800 mg/L	48
Butanol	50 mg/L	14.67
	100 mg/L	20.67
	200 mg/L	22
	400 mg/L	27.33

Table 5 shows the total protein concentration of the hemolymph. To determine the total protein concentration, results obtained from the spectrophotometry assays were compared against the control group which comprises snails that were exposed with distilled water. The obtained total protein result of the control group was 10g/L. For snails exposed with n-hexane leaf extract, 50mg/L, 100mg/L and 200mg/L obtained total protein results of 18g/L, 23.33g/L and 34.67g/L respectively, which are higher than the control group. With ethyl acetate, Increasing results were also obtained at 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L and 800 mg/L with total protein concentrations of 18 g/L, 21.33 g/L, 28 g/L, 36 g/L and 48 g/L, respectively. With *B. blumeana* leaf extracts using butanol, the results for 50 mg/L, 100 mg/L, 200 mg/L, and 400 mg/L were 14.67 g/L, 20.67 g/L, 22 g/L and 27.33 g/L, respectively, which again has higher concentrations than the control group. Based on the results, *B. blumeana* leaves induced increasing effects to the total protein of the snails' hemolymph. This is more likely due to the phytochemicals present. As stated in the study of Rawi et al.

(2011), plant phytochemicals such as saponins and flavonoids cause alterations in the stability of cell membranes, particularly within hepatocytes. Such changes within the mollusks may result into leakage of protein content; thus, increasing the total protein concentration in the snails' circulation. This study, however, acquired different results compared to Rawi et al. (2011), since the hemolymph was extracted and tested immediately, and the effects of the extracts were from short exposure time only. Proteins that leaked out of the mollusk's cells might not have yet underwent conversion into amino acids; thus, allowing the proteins to react with the Biuret reagent.

The current results were in agreement with the study conducted by Gohary, Laila, and Genena (2011), wherein the total protein increase was comparable as to the long term effects of niclosamide. Aside from the phytochemicals' effects, increasing total protein measurements may also be attributed to other factors, such as the length of exposure time of the snails with the extract, pH and temperature of the extract, and the health of the snails used.

In this study, dehydration may also contribute to the increased results, since the snails' lack any hydration source during the exposure, as the water was replaced by the leaf organic extracts. Also, in accordance with the study of Gohary et al. (2011), the increase in total protein could be due to a variety of pathologic conditions aside from hepatotoxicity induced by the extract. Such conditions include effects against the respiratory system of the animals, muscular damage, and leakage of enzymes and other proteins from organ sources other than the mollusks' liver.

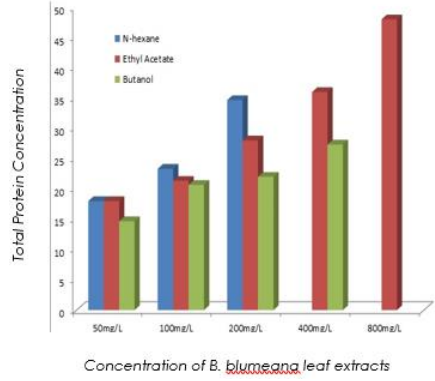


Figure 5. Effect of *B. blumeana* leaf extracts to the total protein of snails

Table 6. Comparison of Total Protein Concentration against the Control Group

	Concentration of *B. blumeana* leaf extract		p-value	Interpretation
n-hexane	50 mg/L		0.100	NS
	100 mg/L		0.130	NS
	200 mg/L		0.060	NS
Ethyl Acetate	50 mg/L		0.020	S
	100 mg/ L		0.050	S
	200 mg/L		0.060	NS
	400 mg/L		0.000	HS
	800 mg/L	0.000	HS	
Butanol	50 mg/L		0.020	S
	100 mg/L		0.100	NS
	200 mg/L		0.030	S
	400 mg/L		0.030	S

Legend: Significant at p-value < 0.05 HS- Highly Significant S- Significant NS- Not Significant

Table 6 shows the comparison of total protein concentration against the control group. Using n-hexane, concentrations at 50 mg/L, 100 mg/L, and 200 mg/L were greater than 0.05; thus, all the obtained total protein results were similar to the control group. With ethyl acetate, at 200 mg/L concentration, the p-value obtained was 0.060, which has similarity with the control group; however, at 50 mg/L and 100 mg/L, both have p-values less than 0.05. This shows significant difference against the control group. On the other hand, at 400 mg/L and 800 mg/L concentrations, both p-values were 0.000, which is highly different with the control group. With butanol, at 100 mg/L concentration, a p-value of 0.100 was obtained, which is similar with the control group; however, at concentrations of 50 mg/L, 200 mg/L and 400 mg/L, p-values were 0.020, 0.030, and 0.030, respectively, which are all interpreted to have significant difference against the control group. It is evident that in terms of total protein concentration, n-hexane at varying concentrations can produce similar total protein results with distilled water; thus, concentrations at 50 mg/L to 200 mg/L is not hepatotoxic, which is contrary to its killing effect against the snails. On the other hand, using ethyl acetate, high concentrations at 400mg/L and 800mg/L, induce hepatotoxic activity. It was found out that at lower concentrations of 50mg/L was enough to cause hepatotoxic effect. With butanol, concentration at 200mg/L and 400mg/L induced hepatotoxic effect. It was also found out that a concentration of 50mg/L mal already induce hepatotoxic activity

against the snails. It is also noticeable that varying effects of different organic solvents and concentrations were observed.

This is may be due to various factors such as pH and temperature of the exposed extract solution, the health of the snail exposed to such concentration, in which some snails may exhibit other total protein values that were slightly different with the control group's 10g/L concentration. This variation is in accordance with the observation of Gohary et al. (2011) regarding the factors that may affect induced slight changes of biochemical parameters.

B. Alanine Aminotransferase

Alanine aminotransferase activity determination was utilized as part of the hepatic panel, being more specific compared to aspartate aminotransferase. Measurement of the activity of this analyte enabled a molluscicidal test assay to investigate and confirm the hepatotoxicity if the mode of action of the animal's mortality is caused by phytochemicals against the liver. The assays done in the present study were in accordance with biochemistry assays performed by the studies conducted by Rawi et al. (2011) and Gohary et al. (2011) which employed such analytes as well.

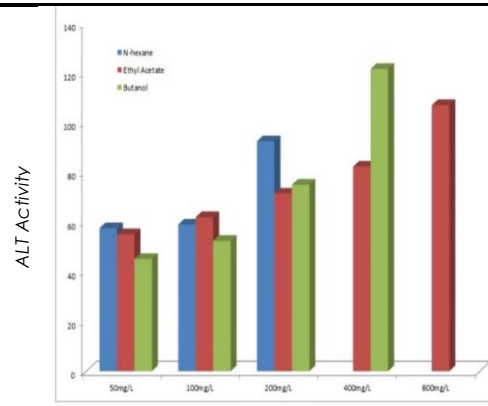
Table 7 shows the effect of *B. blumeana* leaf extracts to alanine aminotransferase activity. Results obtained were interpreted based on the control group, which was acquired by testing the alanine aminotransferase activity of the normal control group composed of the snails that were exposed in distilled water. ALT result of the control group was 61.15 U/I, and was also near the values of the control group (both 62.00 U/l) obtained by Rawi et al. (2011) and Musman et al. (2013). For snails exposed with n-hexane leaf extracts, at 50mg/l concentration and 100 mg/L, ALT activity of the snails were slightly lower than the control group with 57.51 U/I and 58.96 U/I, respectively. For the 200 mg/L concentration, 92.46 U/I was obtained. Ethyl acetate leaf extracts at 50mg/L induced ALT activity result of 55.06 U/I, while at 100 mg/L, 200 mg/L, 400 mg/L and 800 mg/L, ALT results were 61.15 U/I, 71.44 U/I, 82.27 U/I and 107.01 U/I, respectively. Butanol leaf extracts obtained the least ALT activity result in 50mg/l with 45.04 U/I, while exhibiting increasing activities of 52.14 U/I, 74.98 U/I, and 121.58 U/I at 100 mg/L, 200 mg/L and 400 mg/L, respectively.

Based on the results, *B. blumeana* leaf extracts were able to induce increasing ALT activity when snails were exposed to higher concentrations of the extracts. The increasing results of

ALT activity may be attributed to the hepatotoxic effect of the *B. blumeana* leaves brought about by the synergistic hepatotoxic activity of saponins and flavonoids against the hepatic cells of the snails. Increased destruction of hepatocytes not only released the proteins within the cells, but also the hepatic enzymes, one of which is ALT. This finding is supported by the biochemical analyses of the liver enzymes done by Rawi et al. (2011). The previous study along with the work of Gohary et al. (2011), also support the findings on the decreased or normal levels of ALT on lower concentrations, in which the authors have proven that considerable damage on other organs of the animal must occur first, and in relation to the current investigation, such destruction may have not yet occurred within the low extract concentrations in the span of 24 hours exposure.

Table 7.Alanine Aminotransferase Activity

Concentration of *B. blumeana* leaf extracts		Concentration U/l
Control Group	Distilled water	61.15
N-hexane	50mg/L	57.51
	100mg/L	58.96
	200mg/L	92.46
Ethyl Acetate	50mg/L	55.06
	100mg/L	61.95
	200mg/L	71.44
	400mg/L	82.27
	800mg/L	107.01
Butanol	50mg/L	45.14
	100mg/L	52.41
	200mg/L	74.98
	400mg/L	121.58



Concentration of B. blumeana leaf extracts

In correlation with the total protein results, changes on the transaminase activities particularly on its elevation in the present study may also be attributed to increased protein and amino acid leakage on other organs of the mollusk's body. As reported by Rawi et al. (2011), such effects may induce liver parenchymal cell destruction, leading to the release of ALT enzymes. Based on the results of this study on biochemical parameters, total protein and ALT enzyme levels were different on the lower concentration groups as this may be a result of other proteins aside from the hepatic system to be present in higher levels with the plant phytochemicals causing damage on other physiologic systems of the mollusks. Such proteins may have been detected by the total protein assay; thus, producing abnormally higher results than ALT enzyme levels. The increasing levels of both parameters in greater extract concentrations as well as the mortality of the mollusks may have been the effect of greater hepatic involvement as stated by Gohary et al. (2011).

Table 8.Comparison of Alanine Aminotransferase against the Control Group

	Concentration of *B. blumeana* leaf extracts	p-value	Interpretation
N-hexane	50 mg/L	0.130	NS
	100 mg/L	0.817	NS
	200 mg/L	0.004	S
Ethyl Acetate	50 mg/L	0.379	NS
	100 mg/L	0.854	NS
	200 mg/L	0.148	NS
	400 mg/L	0.035	S
	800 mg/L	0.020	S
Butanol	50 mg/L	0.069	NS
	100 mg/L	0.195	NS
	200 mg/L	0.119	NS
	400 mg/L	0.002	S

Legend: Significant at p-value < 0.05 HS- Highly Significant S- Significant NS- Not Significant

Table 8 shows the comparison of alanine aminotransferase against the control group. Using n-hexane, concentrations at 50 mg/L and 100 mg/L were greater than the 0.05 level of significance; thus, both extracts show ALT activities that were similar with distilled water. On the other hand, at 200 mg/L concentration, the p-value obtained was 0.004, confirming the higher than normal ALT result obtained at higher concentration group.

With ethyl acetate, concentrations at 50mg/L, 100mg/L and 200 mg/L obtained p-values of 0.379, 0.854, and 0.148, respectively. This shows no significant difference against the control group. With higher concentrations at 400 mg/L and 800 mg/L, the resulting p-values were 0.035 and 0.020; which reveals higher ALT levels than the control group. Using butanol, 50 mg/L, 100 mg/L, and 200 mg/L obtained p-values of 0.069, 0.195 and 0.119, respectively; thus purification of extract must be performed. On the other hand, higher concentration at 400mg/L showed a p-value of 0.002, which indicates significant difference against distilled water.

Based on the statistical analysis for ALT activity, it is noticeable that the greater the concentration of the extract, the more different the ALT activity of the snails against the control group It may be implied that as more phytochemicals from *B. blumeana* extracts are exposed to the snails, the greater the damage that the liver of the snails acquire; thus, increasing the amount of ALT being released. In relation to the solvents used, it may also be implied that n-hexane, ethyl acetate and butanol were able to extract saponins and flavonoids that were able to elicit hepatotoxic effect to the liver, since such extracts were known to increase the ALT activity as the extract concentration increases. These findings were in agreement with the findings obtained by Rawi et al. (2011) and Gohary et al. (2011), where in ALT activities were not significantly affected by phytochemicals at lower concentrations, but were found to be increasing as the concentration of the extract was increased.

CONCLUSIONS

From the present study, it was concluded that *B. blumeana* leaves have exhibited molluscicidal effect starting at 400mg/L of n-hexane, 200mg/L of ethyl acetate and 100mg/L butanol, and were being considered to have the same efficacy in comparison with the synthetic and commonly used niclosamide. Using the three organic solvents, *B. blumeana* leaf extract was ound to contain phytochemicals, particulaly saponins and flavonoids that may have contributed to the mortality of the snails. Moreover, molluscicidal property of *B. blumeana* leaf was supported by increase concentrations of Total protein and alanine aminotransferase at higher concentrations of plant extracts.

RECOMMENDATIONS

In the future, molluscicidal activity tests may give emphasis on purified saponin and the effect of saponins. In determining short and long term effects of the extracts, tissue homogenate analyses instead of hemolymph assays may be done. Further studies may also be done on snails infected with actual parasitic infections. And lastly, studies for effects of the leaf extracts against other systems of the mollusk aside from the liver.

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