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ENZYME INHIBITION BY HERBAL MOLLUSCICIDES IN THE NERVOUS TISSUE OF THE SNAIL *LYMNAEA ACUMINATA*

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Abstract

The effect of *Annona squamosa*, *Lawsonia inermis* and their combination with other herbal molluscicides were studied on different enzyme activity in the nervous tissue of *Lymnaea acuminata*. Twenty-Four hour *in vivo* exposure to 40% and 80% of 24 h LC_{50} of plant derived molluscicides and their combination with other molluscicides such as *Cedrus deodara*, *Azadirachta indica* oil, *Allium sativum*, *Polianthes tuberosa* bulb and oleoresin of *Zingiber officinale* and acetogenins caused a significant reduction in the activity of acetylcholinesterase, cytochrome oxidase and Na^+/K^+ ATPase in the nervous tissue of *L. acuminata*. Combination of *C. deodara* with *A. squamosa* or *L. inermis* inhibited the acetylcholinesterase activity in the nervous tissue up to 60% and 58.70% of control, respectively. Acetogenin was more effective against Na^+/K^+ ATPase and cytochrome oxidase. Inhibition of these enzymes even at lower concentrations of plant derived molluscicides present in binary mixture with respect to their single treatment inhibit the enzyme activity nearly up to same level.

Keywords: Snails; Plant derived molluscicides; Enzymes

Introduction

It has been observed that seed powder of *Annona squamosa* and *Lawsonia inermis* are potent molluscicides. Combination of other herbal molluscicides with them caused several fold increase in their molluscicidal activity against the aquatic snail *Lymnaea acuminata* and *Indoplanorbis exustus* [1,2]. These snails are the vectors of liver flukes *Fasciola hepatica* and *Fasciola gigantica* which causes endemic fascioliasis in the north eastern part of Uttar Pradesh. One of the solutions to tackle with the problem of fascioliasis is to destroy the carrier snail and thus remove a link in their transmission cycle [3]. Most of the researches have been carried on the use of synthetic molluscicides. However their use at present is restricted due to their persistence in the environment and their high toxicity towards the aquatic flora and fauna. As the use of synthetic molluscicides is impeded by its deleterious effects, there is a demand for environmentally safe natural molluscicides. Herbal molluscicides have gained more and more importance because of being the product of biosynthesis, they are easily biodegradable in the biosphere [3,4]. In the present study, attention has been focused on the effect of seed powder of *A. squamosa* and *L. inermis* and their combinations with other herbal molluscicides on different enzymes in the nervous tissue of *Lymnaea*

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acuminata.

Experimental Section

Annona squamosa and *Lawsonia inermis* seed powder were prepared by the method of Singh and Singh [1,2]. *Azadirachta indica*, *Cedrus deodara* oil were supplied by the Indians Herbs, Saharanpur, *Allium sativum*, *Polianthes tuberosa* bulbs and oleoresin of *Zingiber officinale* were prepared using the method of Singh and Singh [5], Singh *et al.*, [6] and Singh *et al.*, [7]. Acetogenins were extracted from the seeds of *A. squamosa* by the method of Li *et al.*, [8] as modified by Singh and Singh [1].

In vivo Enzyme Assays

Adult *L. acuminata* (2.6 ± 0.30 cm long) were collected locally and used as test animals. Snails were acclimatized to laboratory conditions for 96 h. Snails were maintained and treated with molluscicides according to the method of Singh and Singh [1,2]. Batches of ten snails were kept in glass aquarium, containing 3 liters of dechlorinated water and left exposed to sublethal concentrations, 40% and 20% of 24 h LC_{50} of *Annona squamosa* and *Lawsonia inermis* singly and with binary combination (1:1) of other plant derived molluscicides for 24 h as given in Table 1. These concentrations were based on the LC_{50} values reported from the earlier observation of Singh and Singh [1,2]. Six batches were prepared for each concentration. Control aquaria contained only dechlorinated tap water without treatment. After 24 h, the treated snails were removed from the aquaria and rinsed with water. For *in vivo* enzyme assays, the nervous tissue in snail were quickly dissected out and placed on ice-cubes. Afterwards the nervous tissue was placed on filter paper to remove the adherent water and weighed. Enzyme assays were performed in treated as well as in control groups of test animals.

Acetylcholinesterase

The activity of the enzyme acetylcholinesterase in the nervous tissue of *L. acuminata* was estimated by Ellman *et al.*, [9] as modified by Singh and Agarwal [10]. The nervous tissue was homogenized (50 mg ml^{-1}) in 0.1 M phosphate buffer (pH 8.0) for 5 min in an ice bath. The suspension was centrifuged at 1000 g for 30 min at 4°C. The clear supernatant was taken as an enzyme source. The enzyme activity was measured in a 10 mm path length cuvette using incubation mixture consisting of 0.1 ml of enzyme source, 2.9 ml of 0.1 M phosphate buffer (pH 8.0), 0.1 ml of chromogenic agent DTNB and 0.2 ml freshly prepared acetyl thiocholine iodide solution in distilled water. The absorbance change in optical density at 412 nm was continuously monitored on spectrophotometer for 3 min at 25°C which is a measure of the amount of yellow product formed due to the hydrolysis of DTNB. Enzyme activity has been expressed as μ mole SH hydrolyzed $min^{-1}mg^{-1}$ protein.

Table 1. Sublethal concentrations (40% and 80% of 24 h LC_{50}) of *A. squamosa* and *L. inermis* seed and their combinations with other plant derived molluscicides used for the determination of *in vivo* effect on different enzyme activities in the nervous tissue of *L. acuminata*

Treatments	LC_{50} (mg/l)	40% of 24 h LC_{50}	80% of 24 h LC_{50}
A. squamosa	377.99	151.19	302.39
A. squamosa + *C. deodara*	3.63	1.45	2.90
A. squamosa + *A. indica*	14.59	5.83	11.67
A. squamosa + *A. sativum*	162.33	64.93	129.86
A. squamosa + *P. tuberosa*	156.97	62.78	125.57
A. squamosa + oleoresin	96.13	38.45	76.90
Acetogenins	42.97	17.18	34.37
L. inermis seed	327.35	130.94	261.88
L. inermis seed + *C. deodara*	6.78	2.71	5.42
L. inermis seed + *A. indica*	7.88	3.15	6.30
L. inermis seed + *A. sativum*	169.83	67.93	135.86
L. inermis seed + *P. tuberosa*	175.75	70.3	140.6
L. inermis seed + oleoresin	211.81	84.72	169.44

Cytochrome Oxidase

Activity of cytochrome oxidase was measured according to Cooperstein and Lazarow [11] as modified by Singh and Agarwal [12]. One hundred milligrams of nervous tissue around the buccal mass were homogenized in 1.0 ml of 1/30 mol/l phosphate buffer, pH 7.4, for 5 min in an ice bath and centrifuged at 10,000 g for 30 min at 4°C. Supernatants were used as an enzyme source. Enzyme activity at 25°C was measured in a 10 mm path length cuvette. Three milliliters of reduced cytochrome C solution (1.7×10^{-5} mol L^{-1}) were taken into the cuvette and 0.2 ml of tissue homogenate was added. The reactants were mixed by inverting the cuvette several times, absorption at 550 nm was monitored for 3 min. A few grains of potassium ferricyanide were added (to oxidise cytochrome C completely) and the extinction was redetermined. Enzyme activity has been expressed as the average enzyme activity min^{-1} mg^{-1} protein.

Na^+/K^+ ATPase

Activity of the enzyme Na^+/K^+ ATPase was measured by the method of Svobodo Mossinger [13] as modified by Singh and Singh [14]. Fifty mg nervous tissue was homogenized in 1.0 ml of 0.32 M chilled sucrose solution for 5 min and centrifuged at 800 g for 10 min at 4°C.The supernatant obtained was used as an enzyme source. Mg^{++}–ATPase and Mg^{++}, Na^+/K^+ activated ATPase activities were simultaneously assayed. The difference in the enzyme activity between these two was considered as the Na^+/K^+ ATPase activity. The incubation medium for total ATPase contained 0.2 ml of supernatant, 0.25 ml Tris HCl buffer (50 mM, pH 7.5), 0.25 ml NaCl (100 mM), 0.25 ml KCl (20 mM) and 0.25 ml of $MgCl_2$ (4 mM). The incubation medium for Mg^{++}–ATPase was similar to the prior one except that it contained 120 mM NaCl, 2×10^{-4} M Ouabain to inhibit the ATPase and no KCl. Both the mixtures were pre-incubated for 10 min, at 37°C before the addition of the substrate to start the enzyme reaction. The reaction mixture was incubated with the substrate for 15 min at 37°C.The reaction was stopped by adding 0.5 ml of 10% perchloroacetic acid (PCA) and kept in ice cold water for 5 min. The inorganic phosphate (Pi) liberated by the method of Fiske and Subbarow [15]. One milliliter of the reaction mixture (containing lipid layer) was pipetted out and 0.4 ml of 10% TCA was added to the reaction mixture and heated. After heating, 0.4 ml of 2.5% ammonium molybdate solution and 0.2 ml of amino napthosulphonic acid (ANSA reagent) were added and the reaction mixture were heated at 80°C for 15 min. The reaction mixture was cooled at room temperature and diluted with 4.0 ml of distilled water and was kept for 5 min. The absorbance was read at 640 nm against blank. The blank consisted of 1.0 ml distilled water, 0.4 ml of TCA, 0.4 ml ammonium molybdate solution, 0.2 ml of ANSA reagent and 4.0 ml of distilled water, but no tissue homogenate. The unit of the enzyme activity was expressed as µ mole Pi liberated mg^{-1} protein h^{-1}.

In vitro Enzyme Assays

In vitro experiments were performed by dissolving the acetogenins in ether and an appropriate volume containing *i.e.* 7, 9, 12, 15 µg was added to 10 mm path length cuvette. Ether was then allowed to evaporate and the molluscicides were pre-incubated with the enzyme source for 15 min at 25°C. The enzyme activity of acetylcholinesterase (AChE), Na^+/K^+ ATPase and cytochrome-oxidase were determined using the same assay procedure as mentioned before (*in vivo* experiments). Control cuvette contained only ether.

Estimation of Protein

Protein estimation was made according to Lowry et al [16] using bovine serum albumin as a standard.

Results have been expressed as mean ± SE of six replicates. Students 't' test were applied between the control and the tested groups to locate the significant variations ($P < 0.05$) [17].

Results and Discussion

In vivo Inhibition of Enzymes

In vivo 24h exposure to 40% and 80% of 24 h LC_{50} of *A. squamosa* and *L. inermis* seed powder alone and their binary combinations with *C. deodara* oil, *A. indica* oil, *A. sativum*, *P. tuberosa* powder, oleoresin of *Z. officinale* and acetogenins extracted from *A. squamosa* seed caused a significant dose dependent decrease in the AChE, Na^+/K^+ ATPase and cytochrome oxidase activity in the nervous tissue of *L. acuminata*.

Twenty-Four hour treatment with 40% and 80% of 24 h LC_{50} of *A. squamosa* seed caused a significant inhibition in the AChE activity (77.50 and 62.50% of control) in the nervous tissue of *L. acuminata*. Treatment with 80% of 24 h LC_{50} of *A. squamosa* + *C. deodara* oil combination caused a greater inhibition of AChE (60% of control) in comparison to *A. squamosa* alone (62.50% of control) and other combinations (Table 2).

Table 2. *In vivo* effect of 24 h exposure to 40% and 80% of 24 h LC_{50} of *A. squamosa* and its different combinations with plant derived molluscicides on acetylcholinesterase and ATPase activity in the nervous tissue of *L. acuminata*

Treatment	AChE-moles SH hydrolysed $min^{-1}mg^{-1}$protein		ATPase-µmole Pi liberated mg^{-1}protein h^{-1}	
	40% of 24 h (LC_{50} mg/l)	80% of 24 h (LC_{50} mg/l)	40% of 24 h (LC_{50} mg/l)	80% of 24 h (LC_{50} mg/l)
Control	0.080 ± 0.002 (100)	0.080 ± 0.002 (100)	4.06 ± 0.052 (100)	4.06 ± 0.052 (100)
A. squamosa seed	0.062 ± 0.004* (77.50)	0.050 ± 0.002* (62.50)	2.78 ± 0.067* (68.47)	2.22 ± 0.035* (54.67)
A. squamosa seed + *C. deodara*	0.058 ± 0.004* (72.50)	0.048 ± 0.003* (60.0)	2.44± 0.044*† (60.09)	2.50 ± 0.035*† (61.57)
A. squamosa seed + *A. indica*	0.066 ± 0.002* (82.50)	0.059 ± 0.003* (73.75)	2.56 ± 0.044*† (63.05)	2.21 ± 0.123* (54.43)
A. squamosa seed + *A. sativum*	0.071 ± 0.003* (88.75)	0.067 ± 0.001*† (83.75)	2.82 ± 0.167* (69.45)	2.42 ± 0.033*† (59.60)
A. squamosa seed + *P. tuberosa*	0.067 ± 0.003* (83.75)	0.061 ± 0.002*† (76.25)	2.88 ± 0.098* (70.90)	2.60 ± 0.044*† (64.03)
A. squamosa seed + Oleoresin	0.060 ± 0.002* (75.00)	0.056 ± 0.003* (70.00)	2.70 ± 0.060* (66.50)	2.49 ± 0.059*† (61.33)
Acetogenins	0.061 ± 0.002* (76.25)	0.058 ± 0.003* (72.50)	2.71 ± 0.063* (66.74)	1.41 ± 0.66* (34.72)

Values are mean ± SE of six replicates. Concentrations (w/v) have been expressed as final concentrations in the aquarium water. Values in parentheses indicate percent enzyme activity with control taken as 100%.
* Significant ($P < 0.05$) when student t-test was used for locating differences between experimental and control groups of animals.
† Significant ($P < 0.05$) when student t-test was used for locating differences between *A. squamosa* seed and its different combinations with plant derived molluscicides.

Table 3. *In vivo* effect of 24 h exposure to 40% and 80% of 24 h LC_{50} of *L. inermis* and its different combinations with plant derived molluscicides on acetylcholinesterase and ATPase activity in the nervous tissue of *L. acuminata*.

Treatment	AChE-moles SH hydrolysed $min^{-1}mg^{-1}$protein		ATPase-µmole Pi liberated mg^{-1}protein h^{-1}	
	40% of 24 h (LC_{50} mg/l)	80% of 24 h (LC_{50} mg/l)	40% of 24 h (LC_{50} mg/l)	80% of 24 h (LC_{50} mg/l)
Control	0.080 ± 0.002 (100)	0.080 ± 0.002 (100)	4.06 ± 0.052 (100)	4.06 ± 0.052 (100)
L. inermis seed	0.058 ± 0.003* (72.50)	0.051 ± 0.003* (63.75)	2.57 ±0.015* (63.30)	2.24 ± 0.061* (55.17)
L. inermis seed + *C. deodara*	0.056 ± 0.004* (70.0)	0.047± 0.002* (58.78)	2.80 ± 0.039*† (68.96)	2.49 ± 0.033*† (61.33)
L. inermis seed + *A. indica*	0.060 ± 0.002* (75.00)	0.052 ± 0.002* (65.00)	2.92 ± 0.010*† (71.92)	2.66 ± 0.038*† (65.51)
L. inermis seed + *A. sativum*	0.066 ± 0.004* (82.50)	0.059 ± 0.001* (73.75)	3.10 ± 0.010*† (76.35)	2.86 ± 0.086*† (70.44)
L. inermis seed + *P. tuberosa*	0.068 ± 0.002*† (85.00)	0.059 ± 0.003* (73.75)	3.00 ± 0.016*† (73.89)	2.54 ± 0.067*† (62.56)
L. inermis seed + Oleoresin	0.070 ± 0.004* (87.50)	0.62 ± 0.003*† (77.50)	2.96 ± 0.016*† (72.90)	1.59 ± 0.069*† (39.16)

† Significant ($P < 0.05$) when student t- test was used for locating differences between *L. inermis* seed and its different combinations with plant derived molluscicides.
For other explanations see Table 2.

Table 4. *In vivo* effect of 24 h exposure to 40% and 80% of 24 h LC_{50} of *A. squamosa*, *L. inermis* and its different combinations with plant derived molluscicides on Cytochrome oxidase activity ($min^{-1}mg^{-1}$protein) in the nervous tissue of *L. acuminata*

Treatment	40% of 24 h (LC_{50} mg^{-1})	80% of 24 h (LC_{50} mg^{-1})	Treatment	40% of 24 h (LC_{50} mg^{-1})	80% of 24 h (LC_{50} mg^{-1})
Control	18.32 ± 0.27 (100)	18.32 ± 0.27 (100)	Control	18.32 ± 0.27 (100)	18.32 ± 0.27 (100)
A. squamosa seed	13.27 ± 0.42* (72.43)	11.21 ± 0.32* (61.18)	*L. inermis* seed	14.36 ± 0.26* (78.38)	12.26 ± 0.34* (66.92)
A. squamosa seed + *C. deodara*	11.33± 0.35*† (61.84)	9.47 ± 0.56*† (51.69)	*L. inermis* seed + *C. deodara*	11.75 ± 0.36*† (64.13)	9.89 ± 0.54*† (53.98)
A. squamosa seed + *A. indica*	12.05 ± 0.29* (65.77)	10.46 ± 0.36*† (57.09)	*L. inermis* seed + *A. indica*	12.98 ± 0.45*† (70.85)	12.06 ± 0.42* (65.82)
A. squamosa seed + *A. sativum*	15.51 ± 0.36*† (84.66)	14.31 ± 0.24* (78.11)	*L. inermis* seed + *A. sativum*	14.79 ± 0.33* (80.73)	13.38 ± 0.39* (73.03)
A. squamosa seed + *P. tuberose*	15.10 ± 0.16*† (82.42)	13.34 ± 0.35*† (72.81)	*L. inermis* seed + *P. tuberosa*	15.26 ± 0.24* (83.29)	13.25 ± 0.44* (75.32)
A. squamosa seed + Oleoresin	12.75 ± 0.47* (69.59)	11.67 ± 0.56* (63.70)	*L. inermis* seed + Oleoresin	14.94 ± 0.38* (81.55)	13.78 ± 0.44*† (75.21)
Acetogenins	10.32 ± 0.36*† (56.33)	7.36 ± 0.34*† (40.17)			

† Significant ($P < 0.05$) when student t-test was used for locating differences between *A. squamosa* or *L. inermis* seed and its different combinations with plant derived molluscicides.
For other explanations see Table 2.

Maximum inhibition of AChE activity (58.78% of control) was observed when the snails were exposed to 80% of 24 h LC_{50} of *L. inermis* + *C. deodara* oil followed by *L. inermis* seed alone (63.75% of control), *L. inermis* + *A. indica* oil (65.00% of control), both *L. inermis* + *A. sativum* or *P. tuberosa* (73. 75% of control) and *L. inermis* + oleoresin of *Z. officinale* (77.50% of control) (Table 2). There was no significant difference in AChE activity in the nervous tissue of snails treated with either *A. squamosa* or *L. inermis* seed when compared with binary combinations of Tables 2 and 3.

Treatment with 80% of 24 h LC_{50} of *A. squamosa* seed (54.67% of control) and their binary combinations caused a significant reduction in the ATPase activity. Maximum reduction in ATPase activity (34.72% of control) was observed in the nervous tissue of *L. acuminata* exposed to 80% of 24 h LC_{50} of acetogenins (Table 3).

Exposure to 40% and 80% of 24 h LC_{50} of *L. inermis* seed and its binary combinations with other plant derived molluscicides caused significant inhibition in the Na^+/K^+ ATPase activity in the nervous tissue of *L. acuminata*. Maximum reduction (39.16% of control) in the Na^+/K^+ ATPase activity was observed in snails exposed to 80% of the 24 h LC_{50} of *L. inermis* seed + oleoresin of *Z. officinale* (Table 3). Binary combination *A. squamosa* + *C. deodara* or *A. indica* and *L. inermis* + oleoresin caused significantly more inhibition in Na^+/K^+ ATPase activity in the nervous tissue than *A. squamosa* and *L. inermis* seed treated snails (Tables 2 and 3)

Twenty-Four hour treatment with 40% and 80% of 24 h LC_{50} of *A. squamosa* seed powder and their binary combinations caused a significant inhibition in the cytochrome oxidase activity in the nervous tissue of *L. acuminata*. Maximum reduction in cytochrome oxidase in the nervous tissue was observed in the snails exposed to 80% of 24 h LC_{50} of acetogenins (40.17% of control) (Table 4).

Twenty-Four hour treatment with 80% of 24 h LC_{50} of *L. inermis* seed + *C. deodara* oil caused a maximum inhibition in the cytochrome oxidase activity (53.98% of control) followed by *L. inermis* seed + *A. indica* oil (65.82% of control), *L. inermis* (66.92% of control), *L. inermis* seed + oleoresin (72.32% of control), *L. inermis* seed + *A. sativum* (73.03% of control) and *L. inermis* seed + *P. tuberosa* (75.21% of control) (Table 3).

Binary combination of *A. squamosa* + *C. deodara*, *L. inermis* + *C. deodara* and acetogenins caused a significant higher inhibition in cytochrome oxidase activity in the nervous tissue of *L acuminata* with respect to single treatment of *A. squamosa* and *L. inermis* seed.

Table 5. *In vitro* exposure of acetogenins on acetylcholinesterase (AChE), sodium potassium ATPase and cytochrome oxidase activity in the nervous tissue of *L. acuminata*

Enzymes	Enzyme activity				
	Control	7.0 µg	9.0 µg	12.0 µg	15.0 µg
AChE	0.083 ± 0.002 (100)	0.070 ± 0.003* (84.33)	0.068 ± 0.002* (81.92)	0.065 ± 0.004* (78.31)	0.062 ± 0.002* (74.69)
ATPase	4.20 ± 0.09 (100)	2.89 ± 0.15* (68.80)	2.68 ± 0.41* (63.80)	2.16 ± 0.29* (51.42)	1.98 ± 0.03* (47.14)
Cytochrome-oxidase	19.34 ± 0.23 (100)	14.26 ± 0.28* (73.73)	12.50 ± 0.36* (64.63)	10.14 ± 0.33* (52.43)	8.42 ± 0.44* (43.53)

Concentrations have been given as the final concentration of acetogenins in the incubation mixture.
For other explanations see Table 2.

In vitro Inhibition of Enzymes

In vitro pre-incubation of different concentrations (µg) of acetogenins caused a significant dose dependant decrease in the AChE, ATPase and cytochrome oxidase activities in the nervous tissue of the snail *L. acuminata* (Table 5). Treatment of 15 µg of acetogenins caused maximum reduction (43.53% of control) in cytochrome oxidase activity followed by Na^+/K^+ ATPase activity (47.14% of control) and AChE activity (74.69% of control) in the nervous tissue of *L. acuminata*.

It is apparent from the above result, that *in vivo* treatments of *A. squamosa* or *L. inermis* with different combinations of *C. deodara*, *A. indica* oil, *A. sativum* and *P. tuberosa* bulb powders, oleoresin of *Z. officinale* and extracted acetogenins caused a significant inhibition of acetylcholinesterase (AChE). The enzyme acetylcholinesterase controls the hydrolysis of acetylcholine, generated at nerve junctions, into choline. In the absence of effective acetylcholinesterase, the liberated acetylcholine accumulates and prevents the smooth transmission of impulse across the synaptic gap at nerve junctions. This causes the loss of muscular coordination, convulsions and ultimately death [10,18].

A significant decrease in the cytochrome oxidase activity was observed when the snails were treated with sublethal concentration of *A. squamosa*, *L. inermis* and their combination with other plant derived molluscicides and extracted acetogenins. Singh *et al.*, [19] and Singh and Singh [14] have reported that 24 h *in vivo* treatment with 40% and 80% of the 24 h LC_{50} of the plant derived molluscicides *Azadirachta indica* oil, *Allium sativum* powder and oleoresin of *Zingiber officinale* rhizome and their active molluscicidal components azadirachtin, allicin and [6]- gingerol caused a significant inhibition of acetylcholinesterase, Na^+/K^+ ATPase and cytochrome oxidase activity in the nervous tissue of *L. acuminata*. Combination of these plant derived molluscicides with *A. squamosa* and *L. inermis* seed caused a significant inhibition of AChE, Na^+/K^+ ATPase and cytochrome oxidase activity in the nervous tissue of *L. acuminata*. Singh and Singh [1,2] reported that binary combinations taken in the present study represent the joint action of the both components. LC_{50} of binary combination (1:1) of *A. squamosa* or *L. inermis* with other plant derived molluscicides treatment against *L. acuminata* is very low in comparison to their single treatments [1,2]. At the same time if we consider the concentration of each component in binary combination, it is several times lower than single treatment and still it is inhibiting these enzymes significantly with respect to control. Changes in enzyme activity in nervous tissue of *L. acuminata* by binary combinations is nearly equal as most of the times there is no significant variation with their single treatments or significantly higher than single treatments of *A. squamosa* and *L. inermis.* However, certain combinations such as *A. squamosa* or *L. inermis* with *C. deodara* caused significant inhibition of Na^+/K^+ ATPase and cytochrome oxidase in nervous tissue of snail with respect to single treatment.

The acetogenins are very potent inhibitors of NADH: ubiquinone oxidoreductase, which is an essential enzyme in the complex I of the electron transport system (ETS) in the mitochondria which leads to oxidative phosphorylation [20,21]. When insects were exposed to the acetogenins there was a slow decrease in their mobility and increasing letharginess before the onset of death. These symptoms were attributed to the lowered ATP levels caused by respiratory inhibitors [20]. Banu *et al.*, [22] observed that extracts of *L. inermis* inhibit the mitochondrial malate dehydrogenase and malic enzyme of a filarial worm *Setaria digitata*. *In vitro* treatment with acetogenins in the present study clearly demonstrates that acetogenins extracted from *A.*

squamosa seed inhibit the AChE, ATPase and cytochrome oxidase in the nervous tissue of the snail directly. It can be concluded from the present study that combinations can reduce the doses of plant derived molluscicides in binary mixtures, so that areas of treated water where the plant molluscicides are sublethal may exert a lethal effects by inhibiting these enzymes up to same level as inhibited by single treatment.

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