Effect of bait formulation of plant derived molluscicides on different enzymes of the vector snail *Indoplanorbis exustus*

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Abstract: The effect of sub-lethal treatments (40% and 80% of 24h LC_{50} in AFP) of bait formulation of different plant derived molluscicides on different enzyme activities in the nervous tissue of snail *Indoplanorbis exustus* was studied. Snails fed with sub-lethal concentration of different molluscicides inside attractant food pellets (AFP) caused a significant inhibition in acetylcholinesterase (AChE), acid/alkaline phosphatase (ACP/ALP), lactic dehydrogenase (LDH) and Na^+K^+ ATPase activities in the nervous tissue of *I. exustus*. Bait formulation technique can be used for the mechanical control of the vector snails. Use of molluscicides in the attractant food pellet (AFP) is one of the effective methods of snail control. Attractant food pellets containing amino acid proline and agar plus different concentrations of the molluscicides *Azadirachta indica* bark powder, *Annona squamosa* seed powder and their bio-active components azadirachtin and acetogenin were tested for molluscicidal activity upto 144h against the snail, *I. exustus*. Active components of both the plant derived molluscicides were highly toxic to *I. exustus* compared with their crude forms.

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1. Introduction

The two digenean trematodes *Fasciola hepatica* Linnaeus and *Fasciola gigantica* Cobbold which cause endemic fascioliasis in cattle population of eastern Uttar Pradesh (Singh and Agarwal, 1981; Agarwal and Singh, 1988). The snail *I. exustus* is the vector of these flukes. One way to reduce the incidence of fascioliasis is to de-link the life cycle of fluke by destroying the intermediate hosts (Godan, 1983; Marstson and Hosttetmann, 1985, 1987; Ndamba, 1995; Singh et. al., 1996, Singh and Singh, 2000). The synthetic pesticides play a crucial role in modern agriculture and healthcare programmes. They have been extensively used to control many agricultural pests and insect vectors that transmit a no. of diseases. However, the indiscriminate use of synthetic pesticides has caused great damage to the ecosystem in several ways such as accumulation through biomagnification to alarming toxic levels in the ecosystem, undefined target spectrum that endangers non pathogenic organisms and higher animals and more importantly development of pest resistant target population leading to the induction of secondary pests (Sachidanandam et al., 1996). The development of a selective and safe molluscicide should always be a realistic goal. It must be effective at low concentrations and exert minimal adverse effect on the other biota sharing the same habitat with snail. Lack of contact between molluscicides and target snail population due to meshy vegetation, dilution in upwelling sewage water are two main causes of the failure of snail control programme. Thomas, et al. (1980) reported that the utilization of attractants, arrestants, phagostimulants and toxic factors in control release formulations or bait formulations designed to remove trematode host snails from the fresh water environment is cost effective and ecologically acceptable. The snails use chemical signals for locating food sources. These signals are released from the dead and living aquatic organisms into the modular system of the snails (Maclnnis et. al., 1974; Sterry et. al., 1985; Thomas, 1982; Thomas et. al., 1989; Kpikpi and Thomas, 1992,; Tiwari and Singh 2004 a, b, Tiwari and Singh, 2007; Tiwari et al., 2008, Tiwari, 2011, Tiwari, 2012, 2013 a, b). Proline is the strongest attractant for *L. acuminata* and *I. exustus* at 20 mM concentration (Tiwari, 2011, 2013a). Bait formulation containing attractant and a molluscicide is an expedient approach in order to lure the target snail population to the molluscicide. The aim of the present study is to observe the effect of sub-lethal treatments of these bait formulations on different enzyme activities in the nervous tissue of *I. exustus*.

2. Materials and Methods

Agar-agar, proline, different plant derived molluscicides such as *Annona squamosa* seed powder, *Azadirachta indica* bark powder and their bio-active components, acetogenin (extracted from the seeds of *A. squamosa* by the method of Li et al., (1990) as

modified by Singh and Singh, (2001), and azadirachtin (supplied by T-Stains and Co. Ltd., India) were used in bait formulation. Adult *I. exustus* (2.25±0.20 cm in length) were collected locally from lakes and low lying submerged fields in Gorakhpur. The snails were acclimatized for 72 hours in dechlorinated tap water at 25±10 C. The pH of the water was 7.1-7.3 and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were set to 6.5-7.2 mg/l, 5.2-6.3 mg/l and 102.0-105.0 mg/l, respectively.

2.1 Chemicals Used

Acetylthiocholine iodide (ATChI); 5,5-dithiobis-2-nitrobenzoate (DTNB); Ouabain (1β, 3β, 5β, 11α, 14, 19-hexahydroxycard-20[22] enolide 3-[6 deoxy α-L-mannopyranoside]; β, nicotinamide adeninedinucleotide (p NADH); sodium pyruvate; all these chemicals were supplied by Sigma Chemicals Co. USA. The snails were fed with sub-lethal concentrations of 40% and 80% of 24h LC_{50} of different molluscicides in AFP for 24h (Table-1).

2.2 Biochemical estimations

Snails were treated with prepared different attractant food pellets of molluscicides according to the method of Madsen (1992) as modified by Tiwari and Singh (2004 a, b; 2007; Tiwari et al., 2008). Snails were exposed to sublethal concentrations of 40% and 80% of 24h LC_{50} of different molluscicides in pellets for 24 h (Table-1). Six batches were prepared for each concentration. Control aquarium contained pellets without molluscicicdes.

Table-0 Sublethal concentrations of different plant molluscicides incorporated inside the bait formulation used in biochemical assay against the snail *I. exustus*.

Molluscicides	24h LC_{50} (% in AFP)	40% of 24h LC_{50} (% in AFP)	80% of 24h LC_{50} (%in AFP)
Annona squamosa seed powder	1.57	0.62	1.25
Azadirachta indica bark powder	1.35	0.54	1.08
Acetogenin	1.19	0.47	0.95
Azadirachtin	1.25	0.5	1.0

Abbreviation: AFP-attractant food pellets

2.3 Enzyme assays *in vivo*

2.3.1Acetylcholinesterase

Acetylcholinestease activity in the nervous tissue of *I. exustus* was measured according to the method of Ellman et al. (1961) as modified by Singh and Agarwal (1983). The nervous tissue was homogenized (50mg/ml) in 0.1 M phosphate buffer (pH 8.0) for 5 minutes in an ice bath and centrifuged at 1000xg for 30 minutes at 40 C. The clear supernatant was taken as 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 (5,5-dithiobis-2 nitro-benzoate) and 0.2 ml freshly prepared acetylthiocholine. The absorbance change in optical density at 412 nm was continuously observed on spectrophotometer for 3 minutes at 25^0 C.

2.3.2 Phosphatase Activity

The activities of phosphatases were measured by the method of Bergmeyer (1967) as modified by Singh and Agarwal (1989). The nervous tissue was homogenized (2%w/v) in ice cold 0.9% NaCl and centrifuged at 5000xg for 20 minutes at 40 ^{0}C. The supernatant was taken and used as enzyme source. Standard curves were drawn with p-nitrophenol.

2.3.3 Alkaline phosphatase

For the assay of alkaline phosphatase 0.1 ml of enzyme source supernatant was added in 1.0 ml alkaline buffer substrate solution (prepared by dissolving 375 mg glycine, 10 mg $MgCl_2$.6H2O and 165 mg p-nitrophenol phosphate sodium salt in 42 ml of 0.1 N NaOH and mixture was made up to 100 ml with double distilled water). The mixture was mix thoroughly and incubated for 30 minutes at 37 ^{0}C. In the incubation mixture, 10 ml 0f 0.02 N NaOH was added. The reaction was stopped by the addition of an excess of NaOH solution. The activity of alkaline phosphatase was measured colorimetrically at 420 nm which is a measure of yellow colour of nitrophenol produced by the hydrolysis of p-nitrophenyl phosphate buffer. The enzyme activity was expressed in µ moles substrate hydrolyzed/30min/mg protein.

2.3.4 Acid phosphatase

Acid phosphatase activity was determined by adding 0.2 ml of enzyme source containing supernatant, 1.0 ml of pre-incubated (10 min) acid buffer substrate solution (prepared by dissolving 0.41 gm of citric acid, 1.125 gm sodium citrate and 165 mg of disodium salt of p-nitrophenyl phosphate to 100 ml of double distilled water), the mixture was mixed thoroughly and incubated for 30 minutes at 370 C. Then 4.0 ml of 0.1 N NaOH was added to the incubation mixture. The yellow colour developed due to the formation of p-nitrophenol. The activity of acid phosphatase was measured at 420 nm. Enzyme activity is expressed as µ moles substrate hydrolyzed/30min/mg protein.

2.3.5 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.

2.3.6 Lactic dehydrogenase

The activity of LDH was measured according to Anon [12] as modified by Singh and Agarwal [13]. The tissue was homogenized (50 mg/ml) in 0.1 M phosphate buffer (pH-7.5) for 5 min and centrifuged (10000 g×30 min) at –4°C. To 0.01 ml of an enzyme source (supernatant), 0.5 ml of pyruvate substrate (10 mg NADH in 10 ml of 0.75 mM/l pyruvate buffer, pH-7.5) was added and kept for incubation for 45 min at 37°C. To this 0.5 ml of 2,4- dinitrophenyl hydrazine solution (0.2 g 2,4-dinitrophenyl hydrazine in 8.5 ml of concentrated HCl and volume made to 1 liter) was added and the mixture was left standing for 20 min at room temperature. Finally 5.0 ml of 0.4 N NaOH was added to the mixture and left for 30 min at room temperature. LDH activity was measured by monitoring the decrease in absorbance at 540 nm. Values were converted into LDH units and expressed as pyruvate reduced/min/mg protein.

2.3.7 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].

3. Results

In vivo 24h sublethal exposure of 40% and 80% of 24h LC_{50} of plant derived molluscicides in AFP caused a significant dose dependent decrease in the AChE, ACP/ALP, LDH and Na+K+ATPase activity in the nervous tissue of snail *I. exustus*. The AChE activity in the nervous tissue of L. acuminate was 0.087 -µ moles –SH hydrolyzed/min/mg protein in control group of animals. Feeding of 40% and 80% of 24h LC_{50} *Annona squamosa* seed powder containing AFP caused significant ($P<0.05$) inhibition in the AChE activety (96.25% of the control) in the nervous tissue of *I. exustus* (Table 1). Snails fed with 80% of 24h LC_{50} of acetogenin and azadirachtin in AFP for 24h caused significant reduction in AChE activity (72.5% of control) was observed in nervous tissue of snail *I. exustus*.

The alkaline phosphatase (ALP) activity in the nervous tissue of the snails was 1.08-µ moles/30 min/mg protein in control group of animals. There was a significant inhibition in the alkaline phosphatase activity in the nervous tissue of *I. exustus* fed with AFP containing sublethal concentrations (40% and 80% of 24h LC_{50}) in different plant derived molluscicides. Maximum inhibition was observed

when the snails were fed with AFP containing 80% of 24h LC_{50} of *Azadirachta indica* bark powder (63.86% of control). There was no significant inhibition in ALP activity in the nervous tissue of snails fed with AFP containing 40% of 24h LC_{50} of all the plant derived molluscicides, except AFP containing azadirachtin (Table 3).

The acid phosphatase (ACP) activity in the nervous tissue of the vector snail *I. exustus* was observed 2.05-µ moles/30 min/mg protein in control group of animals. There was a significant inhibition in the acid phosphatase activity in the nervous tissue of *I. exustus* after the exposure of 40% and 80% of 24h LC_{50} of the plant derived molluscicides containing AFP. Maximum inhibition (43.9% of control) in ACP activity was observed in the nervous tissue of *I. exustus* fed with 80% of 24h LC_{50} of azadirachtin (Table 4).

The lactic dehdrogenase (LDH) activity in the nervous tissue of the vector snails *I. exustus* was 333.59 µ moles/30 min/mg protein in control group of animals. Maximum reduction in LDH activity (64.13% of control) was observed in the nervous tissue of *I. exustus* fed with 80% of 24h LC_{50} of acetogenin in AFP (Table 5). Feeding of 40% of 24h the nervous tissue of *I. exustus*.

The Na^+K^+ ATPase activity in the nervous tissue of the vector snail *I. exustus* was 1.05 Pi liberated µ moles/30 min/mg protein in the control group of animals. Feeding of 40% and 80% of plant derived molluscicides inside AFP caused significant reduction in the activity of Na^+K^+ATPase in the nervous tissue of *I. exustus*. Maximum inhibition (20.95% of control) was observed in nervous tissue of *I. exustus* fed with 80% of 24h LC_{50} of *A. squamosa* seed powder containing AFP (Table 6).

Table-1 Effect of *in vivo* 24h exposure to 40% and 80% of 24h LC_{50} in AFP of different molluscicides on acetylcholinesterase activity in the nervous tissue of *I. exustus*.

Treatments	**AChE-µ moles-SH hydrolyzed/min/mg protein**	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	0.080 ± 0.002 (100)	0.080 ± 0.002 (100)
A. squamosa seed powder	0.077 ± 0.004* (96.25)	0.050 ± 0.002* (62.50)
A. indica bark powder	0.058 ± 0.004* (72.50)	0.048 ± 0.003* (60.0)
Acetogenin	0.061 ± 0.002* (76.25)	0.058 ± 0.003* (72.50)
Azadirachtin	0.064±0.001* (80)	0.058±0.003* (72.5)

Values are mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between experimental and control group of animals.

Table-2 Effect of *in vivo* 24h exposure to 40% and 80% of 24h LC_{50} in AFP of different molluscicides on alkaline phosphatase activity in the nervous tissue of *I. exustus*.

Treatments	**ALP-µ moles/30min/mg protein**	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	1.08±0.06 (100)	1.08±0.06 (100)
A. squamosa seed powder	0.98±0.07 (90.74)	0.77±0.06 (71.29)*
A. indica bark powder	1.02±0.06 (94.44)	0.69±0.02 (63.86)*
Acetogenin	1.04±0.06 (96.29)	0.75±0.05 (69.44)*
Azadirachtin	0.86±0.06 (79.62)	0.79±0.07 (73.14)*

Values are mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between experimental and control group of animals.

Table-3 Effect of *in vivo* 24h exposure to 40% and 80% of 24h LC_{50} in AFP of different molluscicides on acid phosphatase activity in the nervous tissue of *I. exustus*.

Treatments	**ACP- µ moles/30min/mg protein**	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	2.05±0.05 (100)	2.05±0.05 (100)
A. squamosa seed powder	1.18±0.02 (57.56)*	0.99±0.02 (48.29)*
A. indica bark powder	1.53±0.02 (74.63)*	1.4±0.04 (68.29)*
Acetogenin	1.13±0.02 (55.12)*	0.93±0.02 (45.36)*
Azadirachtin	1.04±0.03 (50.73)*	0.90±0.02 (43.9)*

Values are mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between experimental and control group

Table-4 Effect of *in vivo* 24h exposure to 40% and 80% of 24h LC_{50} in AFP of different molluscicides on lactic dehydrogenase activity in the nervous tissue of *I. exustus*.

Treatments	**LDH- µ moles/30 min/mg protein**	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	333.59±2.3 (100)	333.59±2.3 (100)
A. squamosa seed powder	266.38±0.83 (79.85)*	259.26±0.88 (77.71)*
A. indica bark powder	330.59±2.3 (99.1)	265.09±0.88 (79.46)*
Acetogenin	277.32±4.5 (83.13)*	213.96±1.3 (64.13)*
Azadirachtin	306.4±1.04 (91.84)*	269.87±0.88 (80.89)*

Values are mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between experimental and control group of animals.

Table-5 Effect of *in vivo* 24h exposure to 40% and 80% of 24h LC_{50} in AFP of different molluscicides on Na^+/K^+ stimulated ATPase activity in the nervous tissue of *I. exustus*.

Treatments	**Na^+/K^+ stimulated ATPase- moles/30min/mg protein**	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	1.05±0.003 (100)	1.05±0.003 (100)
A. squamosa seed powder	0.56±0.006 (53.33)*	0.22±0.01 (20.95)*
A. indica bark powder	1.01±0.01 (96.16)*	0.74±0.001 (70.47)*
Acetogenin	1.0±0.01 (95.23)*	0.94±0.001 (89.52)*
Azadirachtin	0.9±0.008 (85.71)*	0.46±0.02 (43.8)*

Values are mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between experimental and control group of animals.

1. Discussion

It is clear from the result section that snails fed with sublethal concentration i.e. 40% and 80% of 24h LC_{50} of different molluscicides inside AFP caused a significant inhibition in acetylcholinesterase (AChE), acid/alkaline phosphatase (ACP/ALP), lactic dehydrogenase (LDH) and Na^+K^+ATPase activity in the nervous tissue of *I. exustus*. Inhibition of AChE causes accumulation of ACh at the synapses, so that the post synaptic membrane is in a permanent stimulation which results in paralysis, ataxia, general lack of co-ordination in the neuromuscular system and eventual death. Alkaline phosphatase (ALP), which plays a critical role in protein synthesis, shell formation and other secretory activities its inhibition may result reduction in protein level in gastropods. Acid phosphatase (ACP), a lysosomal enzyme which play an important role in catabolism, pathological necrosis, autolysis and phagocytosis was also inhibited by these plant derived molluscicides inside AFP. There was a significant reduction in the activity of Na^+K^+ATPase when the snails fed with AFP of different plant derived molluscicides. Na^+K^+ATPase an essential enzyme of neurotransmitter process that maintain ion gradient, electric potential of membrane and osmotic balance. The persistence of sodium channel activation stimulates sodium influx, which alters the activity of Na^+K^+ATPase to pump out sodium and evoke neurotransmitter release. The disturbance in action potential during the exposure of AFP might be critical for snails. Inhibition of LDH activity by all the plant derived molluscicides incorporated inside AFP indicates that they act on anaerobic metabolism in the snail body. Effect of all these plant derived molluscicides taken in the present study when dissolved in aquarium water directly also caused significant alteration in different enzyme activities in the nervous tissue of vector snail *L. acuminata*.

The treatment of *Azadirachta indica* oil and azadirachtin caused significant inhibition of AChE, ACP/ALP, LDH and Na^+K^+ATPase activities in the nervous tissue of snail *L. acuminata*. *Annona squamosa* seed powder and acetogenin caused a significant inhibition of AChE and Na^+K^+ATPase activities in the nervous tissue of the snail *L. acuminate*. It is clear from the present study that the plant derived molluscicides which are used earlier in aquarium water directly against harmful snails *I. exustus*, when used in AFP (which is consumed by the snail) is effective selectively in similar manner in killing them. It can also be concluded that the delivery of the molluscicide might be improved by the development of bait formulation containing both an attractant and a molluscicide which would be ingested by the snails. An ideal molluscicide would release no poison into the environment and would contain a diffusion or slow-release attractant or chemical stimulant that attracted the snail to its surface or induced the snail to ingest a particle.

The use of bait formulation would allow target snails to be removed selectively with minimal adverse effect on the environment. The harmlessness of the plant derived molluscicides inside the bait formulation to non target organisms and environment makes these suitable for integrated vector management.

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