

Research Article

Feeding of Bait to Snail *Lymnaea acuminata* and Their Effect on Certain Enzyme in the Nervous Tissue

Pradeep Kumar, V. K. Singh, and D. K. Singh

Malacology Laboratory, Department of Zoology, Deen Dayal Upadhyay Gorakhpur University, Gorakhpur 273009, India

Correspondence should be addressed to D. K. Singh, dksingh.gpu@yahoo.co.in

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Fascioliasis, a snail-borne parasitic zoonosis, has been recognized for a long time because of its major veterinary and human impact. Different Bait formulations were fed to the snail *Lymnaea acuminata* in clear glass aquaria having diameter of 30 cm. Snail attractant containing bait formulations was prepared from different binary combination (1:1 ratio) of carbohydrates (glucose, starch 10 mM) and amino acid (methionine, histidine 10 mM) in 100 ml of 2% agar solution + sublethal (20% and 60% of 24 h and 96 h LC₅₀) doses of different molluscicides (eugenol, ferulic acid, umbelliferone, and limonene). Snails fed on bait containing sub-lethal concentration of different molluscicides and the snail attractant, causing a significant inhibition in alkaline phosphatase (ALP) and acetylcholinesterase (AChE) activity in the nervous tissue of the vector snail *L. acuminata*. Maximum inhibition in ALP (20% of control) and AChE (49.49% of control) activity was observed in the nervous tissue of the *L. acuminata* exposed to 60% of 96 h LC₅₀ of eugenol in the bait pellets containing starch + histidine, starch + methionine, respectively.

1. Introduction

Fascioliasis is an important cattle and human disease caused by two major species of *Fasciola hepatica* and *F. gigantica* in different parts of the world [1, 2]. Worldwide, 17 million individuals are infected with *Fasciola*, and more than 90 million people are at risk of fascioliasis [3]. Normally, fascioliasis is reported in livestock animals; now, more occurrence of fascioliasis in human population is noted in different parts of the world [4, 5]. In northern India, *Lymnaea acuminata* is the intermediate host of the *Fasciola* species [1, 6–9]. One way to reduce the incidence of fascioliasis is to delink the life cycle of fluke by destroying the intermediate hosts [6, 10–16]. Bait formulation of different molluscicides would be an effective tool for selective killing of the snail with minimal adverse effect on the nontarget animal and environment. The use of a combination of snail attractant and molluscicides in bait formulation [17] is an effective tool for the snails' control. The aim of the present study is to evaluate the effect of sub-lethal feeding of molluscicides eugenol, ferulic

acid, umbelliferone, and limonene [14] in bait formulations containing attractant carbohydrates (glucose, starch) and amino acids (methionine, histidine) attractant on alkaline phosphatase (ALP) and acetylcholinesterase (AChE) activity in the nervous tissue of the snail *L. acuminata*.

2. Materials and Methods

2.1. Collection of Snails. Adult *L. acuminata* (2.25 ± 0.20 cm in length) were collected locally from lakes and low-lying submerged fields. These snails were acclimatized for 72 hours in dechlorinated tap water at 25 ± 1°C. The pH of the water was 7.2–7.3, and dissolved oxygen, free carbon dioxide, and bicarbonate alkalinity were 6.5–7.2 mg/L, 5.2–6.3 mg/L, and 102.0–105.0 mg/L, respectively.

2.2. Pure Compounds. Agar-agar, carbohydrates (glucose, starch), amino acids (methionine, histidine), and different active molluscicides such as eugenol, ferulic acid, umbelliferone, and limonene were used in bait formulations.

The pure active components, eugenol (2-Methoxy-4-(2-propenyl) phenol), ferulic acid (4-Hydroxy-3-methoxycinnamic), umbelliferone (7-Hydroxy coumarin; 7-hydroxy-2H-1-benzopyran-2-one), and limonene ((R)-4-Isopropenyl-1-methyl-1-cyclohexene), were purchased from Sigma chemical Co., USA.

2.3. Preparation of Bait Formulations. Bait formulations containing binary combination (1:1 ratio) of different carbohydrates (glucose, starch 10 mM), amino acids (methionine, histidine 10 mM), and sublethal (20% and 60% of 24 h and 96 h LC₅₀) concentration of molluscicides (eugenol, ferulic acid, umbelliferone, and limonene) were prepared in 100 mL of 2% agar solution by the method of Madsen, [18]. Concentrations of carbohydrates and amino acids were based on the earlier reports of Tiwari and Singh [19, 20]. These solutions were spread at a uniform thickness of 5 mm. After cooling, the bait containing sub-lethal molluscicides was cut out by a corer measuring 5 mm in diameter.

2.4. Assay Apparatus and Procedure. The bioassay was performed by the method of Tiwari and Singh [19, 20]. The bioassay chamber consists of a clean glass aquarium having a diameter of 30 cm. Each aquarium was divided into four concentric zones with diameters of 13, 18, 24, and 30 cm: central zone (zone 3), middle zone (zone 2 and 1), and outer zone (zone 0). A small annular elevation of 9 mm height and 2.4 cm in diameter was made in the centre of aquarium (zone 3). Zone 0 had an area of 254 cm² on the periphery of the aquarium. The aquaria were then filled with 500 mL of dechlorinated tap water to a height of 8 mm and maintained at 25 ± 1°C. At the start of the assay, ten individually marked snails of uniform size were placed on the circumference of zone 0. The distance between two snails was 66 mm. Simultaneously, one of the prepared baits containing sublethal concentration of different active molluscicidal components was added on the small annular elevation in the center (zone 3). Six sets of experiments have been designed with ten snails, each for all molluscicides used in this study.

2.5. Biochemical Estimations. After 24 h of bait feeding, the snails were washed with water, and the nervous tissue was dissected out from snail brain and used for the measurement of enzyme activities. Alkaline phosphatase (ALP) and acetylcholinesterase (AChE) activity were measured in treated as well as control groups of snails.

In the withdrawal experiment, ALP and AChE activities in the nervous tissue of snail fed on bait formulations were measured in withdrawn snails after 96 h feeding of 60% of 96 h LC₅₀ of bait for the next 72 h in fresh water.

2.6. Alkaline Phosphatase Activity. The alkaline phosphatase activity was measured by the method of Bergmeyer, [21] as modified by Singh and Agarwal, [22]. The nervous tissue was homogenized (2% w/v) in ice-cold 0.9% NaCl and centrifuged at 5000 xg for 20 minutes at 4°C. Standard curves were drawn with p-nitrophenol. 0.1 mL of enzyme

source supernatant was added in 1.0 mL of alkaline buffer substrate solution (prepared by dissolving 375 mg glycine, 10 mg MgCl₂·6H₂O and 165 mg p-nitrophenyl 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 mixed thoroughly and incubated for 30 min. at 37°C. 10 mL of 0.02 N NaOH was added to the incubation mixture. The reaction was stopped by the addition of an excess of NaOH. The alkaline phosphatase activity was measured colorimetrically at 420 nm which is a measure of the yellow colour of nitrophenol produced by the hydrolysis of p-nitrophenyl phosphate buffer. The enzyme activity was expressed in moles substrate hydrolyzed/30 min/mg protein.

2.7. Acetylcholinesterase. Acetylcholinesterase activity was measured by the method of Ellman et al., [23] as modified by Singh and Agarwal [24]. The nervous tissue of *L. acuminata* was homogenized (50 mg/mL) in 0.1 M phosphate buffer (pH 8.0) for 5 minutes in an ice bath and centrifuged at 1000 xg for 30 minutes 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 an 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-nitrobenzoate)) and 0.2 mL of freshly prepared acetylthiocholine iodide. The change in optical density at 412 nm was continuously observed on spectrophotometer for 3 minutes at 25°C. Enzyme activity was expressed as moles SH hydrolyzed/minute/mg protein.

2.8. Statistical Analysis. Each experiment was six times replicated estimation (measurement in six different pool of nervous tissue). The values were expressed as mean ± SE. Student's *t*-test was applied to determine the significant ($P < 0.05$) difference between treated and control animals [25].

3. Results and Discussion

Sub-lethal feeding to 20% and 60% of 24 h and 96 h LC₅₀ of eugenol, ferulic acid, umbelliferone, and limonene in bait formulations caused a significant ($P < 0.05$) inhibition in alkaline phosphatase activity in the nervous tissue of snail *L. acuminata* (Table 1). Maximum inhibition (20% of control) in alkaline phosphatase activity was observed in the nervous tissue of *L. acuminata* fed on 60% of 96 h LC₅₀ of eugenol (Table 1). Significant ($P < 0.05$) recovery in alkaline phosphatase activity was observed in the nervous tissue of *L. acuminata* earlier fed on 60% of 96 h LC₅₀ of eugenol bait (20% of control), when discontinued for the next 72 h (34.66% of control). The sub-lethal feeding on 20% and 60% of 24 h and 96 h LC₅₀ of eugenol, ferulic acid, umbelliferone, and limonene caused a significant inhibition in the AChE activity in the nervous tissue of the snail *L. acuminata* (Table 2). Maximum inhibition (49.49% of control) in the AChE activity was observed in the nervous tissue of the snail fed on 60% of 96 h LC₅₀ of eugenol containing bait (Table 2). There was a significant ($P < 0.05$) recovery in the AChE activity in the nervous tissue of the 72 h withdrawn (56.70%)

TABLE 1: Effect of sublethal exposure (20% and 60% of 24 h and 96 h LC₅₀) of bait formulations containing eugenol, ferulic acid, umbelliferone, and limonene on the activity of alkaline phosphatase (ALP) in the nervous tissue of the snail *L. acuminata*.

Treatment	24 h LC ₅₀		96 h LC ₅₀		Withdrawal
	20%	60%	20%	60%	60% (96 h LC ₅₀)
Control (Agar)		2.75 ± 0.13 (100)			2.25 ± 0.61 (100)
Control (a) Glu. + Meth.		2.82 ± 0.51 (100)			2.75 ± 0.32 (100)
Control (b) Star. + Meth.		2.65 ± 0.32 (100)			2.61 ± 0.16 (100)
Control (c) Glu. + Hist.		2.75 ± 0.92 (100)			2.64 ± 0.15 (100)
Control (d) Star. + Hist.		2.65 ± 0.82 (100)			2.31 ± 0.25 (100)
Glu. + Meth. + Eug.	1.95 ± 0.06* (70.90)	1.32 ± 0.12* (48.00)	1.75 ± 0.23* (63.63)	1.28 ± 0.72* (46.54)	1.99 ± 0.72 ⁺ (88.44)
Glu. + Meth. + Fer.	1.66 ± 0.25* (60.36)	1.25 ± 0.07* (45.45)	1.85 ± 0.36* (67.27)	1.33 ± 0.20* (48.36)	1.75 ± 0.26 ⁺ (77.77)
Glu. + Meth. + Umb.	1.75 ± 0.11* (63.63)	1.55 ± 0.20* (56.36)	1.75 ± 0.13* (63.63)	1.55 ± 0.09* (56.36)	1.86 ± 0.23 ⁺ (82.66)
Glu. + Meth. + lim.	1.25 ± 0.03* (45.45)	1.03 ± 0.26* (37.45)	1.85 ± 0.77* (67.27)	1.66 ± 0.21* (60.36)	1.89 ± 0.73 ⁺ (84.00)
Star. + Meth. + Eug.	1.98 ± 0.72* (72.00)	1.70 ± 0.08* (61.81)	1.65 ± 0.26* (60.00)	1.60 ± 0.13* (58.18)	1.82 ± 0.23 ⁺ (80.88)
Star. + Meth. + Fer.	1.34 ± 0.06* (48.72)	1.22 ± 0.38* (44.36)	1.55 ± 0.26* (56.36)	1.13 ± 0.07* (41.09)	1.38 ± 0.07 ⁺ (61.33)
Star. + Meth. + Umb.	1.68 ± 0.23* (61.09)	1.38 ± 0.16* (50.18)	1.40 ± 0.39* (50.90)	1.28 ± 0.36* (46.54)	1.55 ± 0.38* (68.88)
Star. + Meth. + Lim.	1.35 ± 0.72* (49.09)	1.25 ± 0.07* (45.45)	1.48 ± 0.21* (53.81)	1.28 ± 0.77* (46.54)	1.49 ± 0.24 ⁺ (66.22)
Glu. + hist. + Eug.	1.85 ± 0.11* (67.27)	1.63 ± 0.07* (59.27)	1.53 ± 0.23* (55.63)	1.48 ± 0.20* (53.81)	1.65 ± 0.23 ⁺ (73.33)
Glu. + Hist. + Fer.	1.79 ± 0.38* (65.09)	1.58 ± 0.28* (57.45)	1.51 ± 0.01* (54.90)	1.31 ± 0.19* (47.63)	1.52 ± 0.23 ⁺ (67.55)
Glu. + Hist. + Umb.	1.62 ± 0.25* (58.90)	1.35 ± 0.09* (49.09)	1.68 ± 0.98* (61.09)	1.36 ± 0.22* (49.45)	1.49 ± 0.26 ⁺ (66.22)
Glu. + Hist. + Lim.	1.48 ± 0.63* (53.81)	1.25 ± 0.71* (45.45)	1.60 ± 0.23* (58.18)	1.25 ± 0.85* (45.81)	1.51 ± 0.73 ⁺ (67.11)
Star. + Hist. + Eug.	0.98 ± 0.23* (35.63)	0.75 ± 0.03* (27.27)	0.65 ± 0.75* (23.63)	0.55 ± 0.32* (20.00)	0.78 ± 0.23 ⁺ (34.66)
Star. + Hist. + Fer.	1.23 ± 0.07* (44.72)	1.11 ± 0.98* (40.36)	1.05 ± 0.60* (38.18)	0.99 ± 0.23* (36.00)	1.20 ± 0.03 ⁺ (53.33)
Star. + Hist. + Umb.	1.69 ± 0.08* (61.45)	1.68 ± 0.23* (61.09)	1.51 ± 0.23* (54.90)	1.38 ± 0.75* (50.18)	1.59 ± 0.23 ⁺ (70.66)
Star. + Hist. + Lim.	1.42 ± 0.72* (51.63)	1.24 ± 0.29* (45.09)	1.48 ± 0.05* (53.81)	1.28 ± 0.65* (46.54)	1.55 ± 0.26 ⁺ (69.33)

Each value is mean ± SE of six replicates. Values in parentheses are percent change with control taken as 100%.

Concentration (w/v) has been expressed as final concentration in aquarium water. *Significant ($P < 0.05$) when t -test was applied in between treated and control groups and ⁺in between 60% of 96 h LC₅₀ and withdrawal group. Glu.: glucose; Star.: starch; Meth.: methionine; Hist.: histidine; Eug.: eugenol; Fer.: ferulic acid; Umb.: umbelliferone; Lim.: limonene.

snails with respect to snails fed on 60% of 96 h LC₅₀ of eugenol bait.

The result of the present study indicates that the sub-lethal feeding on 20% and 60% of 24 h and 96 h LC₅₀ of the active components, eugenol, ferulic acid, umbelliferone, and limonene, with attractant carbohydrates and amino acids was more effective in killing the *L. acuminata*. Earlier, it has been reported that direct release of eugenol, ferulic acid, umbelliferone, and limonene in aquarium water has significant molluscicidal activity against *L. acuminata* [6, 12]. Kumar et al. [15] have demonstrated that when these active molluscicidal components in bait formulations were fed to snails, they also act as potent molluscicides. Kumar et al. [26] have reported that the combination (1:1) of amino acids such as valine + aspartic acid, lysine + valine, lysine + alanine, and alanine + valine with active molluscicides, eugenol, ferulic acid, umbelliferone, and limonene, in bait formulations caused maximum inhibition in ALP (23.57% of control) and AChE (49.48% of control) in nervous tissue of *L. acuminata* exposed to 60% of 96 h LC₅₀ of ferulic acid and umbelliferone, respectively. In the present study, the mode of entry of molluscicides into the snail body is through the digestive system. In an earlier study, it was through the body surface, when molluscicides were released directly in water. Although the entry of molluscicide inside the body is different, both methods are equally effective in killing the

snails. Snails fed with a sub-lethal dose, that is, 20% and 60% of 24 h and 96 h LC₅₀ of different molluscicides, caused a significant inhibition in ALP and AChE activity in the nervous tissue of snail *L. acuminata*. The inhibition in ALP and AChE activities may be due to the direct interference of these active molluscicidal with enzyme. Kumar et al., [27] reported that there was a depletion of amino acid and reduction of protein and nucleic acid level in the ovotestis of *L. acuminata* when these active molluscicides were fed to snails in bait formulations. Alkaline phosphatase plays a critical role in protein synthesis [28], shell formation [29], and other secretory activities [30] and its inhibition may result in the reduction of protein level [22, 31] in gastropods. It plays an important role in the transport of metabolites across the membrane [32]. The AChE inhibition results in the accumulation of acetylcholine at the nerves synapses, so that the post synaptic membrane is in a state of permanent stimulation producing paralysis, ataxia, general lack of coordination in neuromuscular system, and eventual death [26, 33–38]. Animal behavior is a neurotropically regulated phenomenon which is mediated by neurotransmitter substances such as ACh [39]. The enzyme AChE is found in the synaptic regions and mediates the transmission of impulses by breaking acetylcholine into acetic acid and choline [40]. The acetylcholine at neural and neuromotor regions upon accumulation causes hyperexcitability [41]

TABLE 2: Effect of sublethal exposure (20% and 60% of 24 h and 96 h LC₅₀) of bait formulations containing eugenol, ferulic acid, umbelliferone, and limonene on the activity of acetylcholinesterase (AChE) in the nervous tissue of the snail *L. acuminata*.

Treatment	24h LC ₅₀		96 h LC ₅₀		Withdrawal 60% (96 h LC ₅₀)
	20%	60%	20%	60%	
Control (Agar)			0.099 ± 0.002 (100)		0.097 ± 0.007 (100)
Control (a) Glu. + Meth.			0.098 ± 0.003 (100)		0.097 ± 0.005 (100)
Control (b) Star. + Meth.			0.097 ± 0.002 (100)		0.098 ± 0.006 (100)
Control (c) Glu. + Hist.			0.096 ± 0.005 (100)		0.095 ± 0.001 (100)
Control (d) Star. + Hist.			0.098 ± 0.004 (100)		0.097 ± 0.006 (100)
Glu. + Meth. + Eug.	0.075 ± 0.001* (75.75)	0.063 ± 0.002* (63.63)	0.05 ± 0.001* (58.58)	0.051 ± 0.003* (51.51)	0.067 ± 0.006* (69.07)
Glu. + Meth. + Fer.	0.065 ± 0.003* (65.65)	0.051 ± 0.002* (51.51)	0.086 ± 0.003* (86.86)	0.065 ± 0.004* (65.65)	0.078 ± 0.003* (98.73)
Glu. + Meth. + Umb.	0.073 ± 0.001* (73.73)	0.068 ± 0.008* (68.68)	0.070 ± 0.003* (70.70)	0.055 ± 0.001* (55.55)	0.063 ± 0.001* (64.94)
Glu. + Meth. + lim.	0.078 ± 0.002* (78.78)	0.069 ± 0.001* (69.69)	0.067 ± 0.002* (67.67)	0.060 ± 0.003* (60.60)	0.075 ± 0.002* (77.31)
Star. + Meth. + Eug.	0.055 ± 0.006* (55.55)	0.050 ± 0.003* (50.50)	0.058 ± 0.005* (58.58)	0.049 ± 0.006* (49.49)	0.055 ± 0.004* (56.70)
Star. + Meth. + Fer.	0.062 ± 0.004* (62.62)	0.060 ± 0.008* (60.60)	0.058 ± 0.001* (58.58)	0.054 ± 0.003* (54.54)	0.059 ± 0.003* (60.82)
Star. + Meth. + Umb.	0.072 ± 0.005* (71.71)	0.065 ± 0.001* (65.65)	0.062 ± 0.002* (62.62)	0.057 ± 0.005* (57.57)	0.062 ± 0.003* (63.91)
Star. + Meth. + Lim.	0.072 ± 0.008* (72.72)	0.063 ± 0.004* (63.63)	0.068 ± 0.003* (68.68)	0.058 ± 0.002* (58.58)	0.062 ± 0.001* (63.91)
Glu. + hist. + Eug.	0.057 ± 0.001* (57.57)	0.054 ± 0.002* (54.54)	0.055 ± 0.007* (55.55)	0.050 ± 0.007* (50.50)	0.058 ± 0.006* (59.79)
Glu. + Hist. + Fer.	0.082 ± 0.003* (82.82)	0.075 ± 0.006* (75.75)	0.069 ± 0.003* (69.69)	0.061 ± 0.003* (61.61)	0.069 ± 0.003* (71.13)
Glu. + Hist. + Umb.	0.070 ± 0.008* (70.70)	0.068 ± 0.001* (68.68)	0.069 ± 0.004* (69.69)	0.066 ± 0.002* (66.66)	0.070 ± 0.006* (72.16)
Glu. + Hist. + Lim.	0.069 ± 0.003* (69.69)	0.066 ± 0.003* (66.66)	0.065 ± 0.007* (65.65)	0.060 ± 0.003* (60.60)	0.006 ± 0.003* (68.04)
Star. + Hist. + Eug.	0.057 ± 0.006* (57.57)	0.055 ± 0.004* (55.55)	0.055 ± 0.003* (55.55)	0.051 ± 0.005* (51.51)	0.057 ± 0.008* (58.76)
Star. + Hist. + Fer.	0.060 ± 0.003* (60.60)	0.059 ± 0.003* (59.59)	0.058 ± 0.002* (58.58)	0.055 ± 0.001* (55.55)	0.058 ± 0.002* (59.79)
Star. + Hist. + Umb.	0.078 ± 0.008* (78.78)	0.077 ± 0.006* (77.77)	0.075 ± 0.001* (75.75)	0.070 ± 0.003* (70.70)	0.073 ± 0.006* (75.25)
Star. + Hist. + Lim.	0.074 ± 0.003* (74.74)	0.071 ± 0.002* (71.71)	0.072 ± 0.002* (72.72)	0.068 ± 0.004* (68.68)	0.073 ± 0.003* (75.25)

Each value is mean ± SE of six replicates. Values in parentheses are percent change with control taken as 100%.

Concentration (w/v) has been expressed as final concentration in aquarium water. *Significant ($P < 0.05$) when *t*-test was applied in between treated and control groups and ⁺in between 60% of 96 h LC₅₀ and withdrawal group. Glu.: glucose; Star.: starch; Meth.: methionine; Hist.: histidine; Eug.: eugenol; Fer.: ferulic acid; Umb.: umbelliferone; Lim.: limonene.

which in turn might also influence behavior pattern of animals. The present study shows that eugenol, ferulic acids, umbelliferone, and limonene that are incorporated in the bait caused significant time- and dose-dependent inhibition in the activities of enzyme, ALP and AChE, in the snails *L. acuminata*. Nagababu et al. [42] reported that eugenol significantly inhibited the rise in SGOT activity and cell necrosis without protecting the endoplasmic reticulum damage as assessed by its failure to prevent a decrease in cytochrome p450 and G-6-phosphatase activities. The inhibitory mechanism implies that eugenol does not inactivate the enzyme directly but may interfere with fatty acid radical intermediate due to its hydroxyl radical scavenging ability and thus plays a role in inhibiting the propagation of lipid peroxidation [43]. Eugenol pretreatment prevents DNA strand break and improves the antioxidant status in thioacetamide-treated rats [44]. Although earlier it has been reported that ferulic acid, umbelliferone, eugenol, and limonene inhibited the ALP and AChE activities in the nervous tissue of snails *L. acuminata* when used directly in aquarium water [12], yet it has been observed in the present study that inhibition of ALP activity in ferulic acid/limonene/eugenol bait fed snails were 1.09/1.26/1.60 times higher than earlier reports of Kumar et al. [12]. This concept is a new technique and approach for the effective control of harmful snails, without using more active molluscicide directly in the water and attracting specifically the particular target snail.

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