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Enzyme Activity in the Nervous Tissue of *Lymnaea Acuminata* Fed to Different Bait Formulations

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Abstract Lymnaeid snails transmit medical and veterinary trematodiases, mainly fascioliasis. Vector specificity of fasciolid parasites defines disease distribution and characteristics. Control of fascioliasis is to de-link the life cycle of fluke, by destroying the Lymnaeid snails. Different Bait formulations were fed to snail *Lymnaea acuminata* in clear glass aquaria having diameter of 30 cm. Snail attractant containing bait formulations were prepared from different binary combination of amino acid (valine, aspartic acid, lysine and alanine 10 mM) in 100 ml of 2% agar solution + sub-lethal (20% and 60% of 24h LC_{50} and 96h LC_{50}) doses of different molluscicides (eugenol, ferulic acid, umbelliferone, and limonene). Snails were fed bait with sub-lethal concentration of different molluscicides inside the snail attractant pellets, which caused a significant inhibition in alkaline phosphatase (ALP) and acetylcholinesterase (AChE) activity in the nervous tissue of the vector snail *L. acuminata*. Combination of different amino acids such as valine + aspartic acid, lysine + valine, lysine + alanine and alanine + valine was used as attractant with active molluscicidal components. Maximum inhibition in ALP (23.57% of control) and AChE (49.48% of control) activity were observed in the nervous tissue of the *L. acuminata* exposed to 60% of 96h LC_{50} of ferulic acid, umbelliferone, respectively in the bait pellets containing valine + aspartic acid as attractant.

Keywords Molluscicides, Amino Acids, Bait Formulation, Ache, Alkaline Phosphatase, *Lymnaea Acuminata*

1. Introduction

Fasciola hepatica and *F. gigantica* are the causative agent of endemic fascioliasis in different parts of the world[1]. Normally fascioliasis is reported in live-stock animals now more occurrence of fascioliasis in human population is noted in different part of the world[2,3]. Human fascioliasis is characterized by hypereosinophilia, abdominal pain and, exceptionally, acute pancreatitis[4,5]. One way to reduce the incidence of fascioliasis is to de-link the life cycle of fluke, by destroying the intermediate hosts[6-12]. Bait formulation of different molluscicides would be an effective tool for selective killing of the snail with minimal adverse effect on the non-target animal and environment. It is therefore important to identify strong attractant compounds and molluscicides for effective bait formulations. Use of a combination of snail attractant and molluscicides in bait formulation is an effective tool for the pest management. The present work is a continuation of our earlier studies[13,14]. The aim of the present study is to evaluate the effect of sub-lethal feeding molluscicides eugenol, ferulic acid, umbelliferone, and limonene[11] in bait formulations with attractant amino acid valine, aspartic acid, lysine and alanine[11] on different enzyme activity (alkaline phosphatase and AChE) in the nervous tissue of the *L. acuminata.* Snail *L. acuminata* is the intermediate host of *Fasciola* species.

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2. Materials and Methods

2.1. Collection of Snails

Adult *L. acuminata* (2.25±0.20 cm in length), intermediate host of liver fluke *F. gigantica,* was used as test animals in the present study. The snails were collected locally from lakes and low lying submerged fields. These snails were acclimatized for 72 hours in dechlorinated tap water at 25±1℃. The pH of the water was 7.1-7.3 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, amino acids (98.5%) (valine, aspartic acid, lysine and alanine; CDH, New Delhi, India), different active molluscicidal component such as eugenol, ferulic acid, umbelliferone and limonene were used in bait formulation. The pure active component ferulic acid (99%) (4 - Hydroxy - 3 methoxycinnamic), umbelliferone (99%) (7- Hydroxy coumarin; 7 – hydroxy - 2H – 1 - benzopyran-2-one), eugenol (99%) (2-Methoxy-4-(2-propenyl) phenol) and limonene (97%) (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 of different amino acid (valine, aspartic acid, lysine and alanine 10mM) and sub lethal (20% and 60% of 24h and 96h LC_{50}) concentration of molluscicides were prepared in 100 ml of 2% agar solution by the method of Madsen,[15]. Concentrations of amino acids were based on the earlier reports of Tiwari and Singh[16,17]. These solutions were spread at a uniform thickness of 5mm. After cooling the bait containing sublethal molluscicides were cut out a corer measuring 5mm in diameter.

2.4. Assay Apparatus and Procedure

The bioassay was performed by the method by Tiwari and Singh[16,17]. 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 (zone3), Middle zone (zone2 and 1) and Outer zone (zone0). A small annular elevation of 9 mm height and 2.4 cm diameter was made in the centre of aquarium (Zone 3). Zone 0 had an area of 254 cm^2 on the periphery of aquarium. The aquaria were then filled with 500 ml of dechlorinated tap water to a height of 8 mm and maintained at 25±1℃. 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 bait containing sublethal concentration of different active molluscicidal components were 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 24h 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. Different enzyme activity viz. alkaline phosphatase (ALP) and acetylcholinesterase (AChE) were measured in feeding as well as control group of snails.

In withdrawal experiment ALP and AChE activity in the nervous tissue of snail were measured in withdrawan snails after 96h feeding of 60% of 96h LC_{50} of bait for next 72h to fresh water.

2.6. Alkaline Phosphatase Activity

The alkaline phosphatase activity was measured by the method of Bergmeyer[18] as modified by Singh and Agarwal[19]. The nervous tissue was homogenized (2% w/v) in ice cold 0.9% NaCl and centrifuged at 5000xg for 20 minutes at 4℃. 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 $MgCl_2$. $6H_2O$ and 165 mg p- nitrophenyl phosphate sodium salt in 42 ml of 0.1N NaOH and mixture was made upto 100 ml with double distilled water). The mixture was mix thoroughly and incubated for 30 min. at 37℃. 10 ml of 0.02N NaOH was added to the incubation mixture. The reaction was stopped by addition of an excess of NaOH. The alkaline phospahatase activity 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/30 min/mg protein.

2.7. Acetylcholinesterase

Acetylcholinesterase activity was measured by the method of Ellman et al.,[20] as modified by Singh and Agarwal[21]. 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 1000xg for 30 minutes at 4℃. 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 (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℃. Enzyme activity was expressed as μ moles SH hydrolyzed/minute/mg protein.

2.8. Statistical Analysis

Each result was six times replicate 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[22].

3. Results

Sub-lethal feeding to 20% and 60% of 24h and 96h LC_{50} of ferulic acid, umbelliferone, eugenol 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 (23.57% of control) in alkaline phosphatase activity was observed in the nervous tissue of *L. acuminata* fed to 60% of 96h LC_{50} of ferulic acid (Table-1). Significant ($P<0.05$) recovery in alkaline phosphatase activity was observed in the nervous tissue of *L. acuminata* earlier fed to 60% of 96h LC_{50} of ferulic acid bait (23.57% of control), when discontinued for the next 72h (38.43% of control). The sub-lethal feeding to 20% and 60% of 24h LC_{50} and 96h LC_{50} 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 (49.48% of control) in the AChE activity was observed in the nervous tissue of the snail fed to 60% of 96 LC_{50} of umbelliferone containing bait (Table-2).

There was a significant (P<0.05) recovery in the AChE activity in the nervous tissue of 72h withdrawn (57.77 %) snails with respect to snails fed to 60% of 96h LC_{50} of umbelliferone bait.

Table.1. Effect of sublethal exposure (20% and 60% of 24h LC_{50} and 96h LC_{50}) of bait formulation with active molluscicidal component (ferulic acid, umbelliferone, eugenol and limonene) on the level of alkaline phosphatase (ALP) activity in the nervous tissue of *L. acuminata*

Treatment	24h LC_{50}		96h LC_{50}		Withdrawal
	20%	60%	20%	60%	60%
Control (Agar)	2.69 ±0.31 (100)				2.13±0.68 (100)
Control (a) Vali+Aspa	2.63±0.82 (100)				2.55±0.61 (100)
Control (b) Lysi+Vali	2.75±0.19 (100)				2.66±0.38 (100)
Control (c) Lysi+Ala	2.55±0.18 (100)				2.86±0.76 (100)
Control (d) Ala+Vali	2.45±0.32 (100)				2.11±0.18 (100)
Vali+Aspa+Eug	1.86±0.09* (70.72)	1.32±0.15* (50.19)	1.55±0.08* (58.93)	1.12±0.04* (42.58)	1.81±0.03+ (70.98)
Vali+Aspa+Fer	1.11±0.02* (42.20)	0.96±0.09* (36.50)	0.86±0.11* (32.69)	0.62±0.21* (23.57)	0.98±0.32+ (38.43)
Vali+Aspa+Umb	0.89±0.06* (33.84)	0.76±0.10* (28.89)	0.85±0.06 * (32.31)	0.80±0.06* (30.41)	0.88±0.05+ (34.50)
Vali+Aspa+lim	1.62±0.07* (61.59)	1.10±0.08* (41.82)	0.98±0.16 * (37.21)	0.80±0.32* (30.41)	0.99±0.13+ (38.82)
Lysi+Vali+Eug	1.75±0.19* (63.63)	0.96±0.06* (34.20)	0.86±0.12* (31.27)	0.76±0.19* (27.63)	0.98±0.10+ (36.84)
Lysi+Vali+Fer	1.04±0.03* (37.81)	0.96±0.12* (34.90)	0.98±0.62* (35.63)	0.82±0.06* (29.81)	0.99±0.03+ (37.21)
Lysi+Vali+Umb	1.30±0.40* (47.27)	0.97±0.04* (35.27)	0.96±0.03* (34.90)	0.82±0.08* (29.81)	0.98±0.32+ (36.84)
Lysi+Vali+Lim	1.11±0.22* (40.36)	0.98±0.62* (35.63)	0.90±0.04* (32.72)	0.80±0.12* (29.09)	0.92±0.62+ (34.58)
Lysi+Ala+Eug	1.89±0.04* (46.27)	0.97±0.06* (38.03)	0.95±0.07* (37.25)	0.85±0.09* (33.33)	0.98±0.10+ (34.26)
Lysi+Ala+Fer	1.05±0.06* (41.17)	0.99±0.08* (38.82)	0.95±0.09* (37.25)	0.89±0.03* (34.90)	0.98±0.10+ (34.26)
Lysi+Ala+Umb	0.88±0.06* (34.50)	0.82±0.03* (32.15)	0.80±0.17* (31.37)	0.76±0.33* (25.60)	0.81±0.12+ (28.32)
Lysi+Ala+Lim	1.60±0.03* (62.74)	0.92±0.06* (36.07)	0.91±0.08* (35.68)	0.88±0.13* (34.50)	0.92±0.02+ (32.16)
Ala+Vali+Eug	1.76±0.07* (71.83)	0.99±0.08* (40.40)	0.96±0.04* (39.18)	0.91±0.03* (37.14)	0.98±0.04+ (46.44)
Ala+Vali+Fer	1.05±0.06* (42.85)	0.98±0.02* (40.00)	1.02±0.06* (41.63)	0.99±0.08* (40.40)	1.10±0.19+ (52.13)
Ala+Vali+Umb	0.92±0.04* (37.55)	0.98±0.06* (36.32)	0.93±0.02* (37.95)	0.87±0.08* (35.51)	0.94±0.13+ (44.54)
Ala+Vali+Lim	1.58±0.09* (64.48)	0.97±0.02* (39.59)	0.98±0.19* (40.00)	0.88±0.06* (35.91)	0.98±0.07+ (46.44)

Each value is mean ±SE of six replicates.
Value is parentheses are per cent 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 group and (+) in between 60% of 96h LC_{50} and withdrawal group.
Vali=valine, Aspa=aspartic acid, Lysi=lysine, Ala=alanine, Eug=euhenol, Feb=ferulic acid, Umb=umbelliferone, Lim=limonene

Table.2. Effect of sublethal exposure (20% and 60% of 24h LC_{50} and 96h LC_{50}) of bait formulation with active molluscicidal component (ferulic acid, umbelliferone, eugenol and limonene) on the level of acetylcholinesterase (AchE) activity in the nervous tissue of *L. acuminata*

Treatment	24h LC_{50}		96h LC_{50}		Withdrawal
	20%	60%	20%	60%	60%
Control (Agar)	0.098 ±0.006 (100)				0.096±0.002 (100)
Control (a) Vali+Aspa	0.097±0.005 (100)				0.090±0.003 (100)
Control (b) Lysi+Vali	0.095±0.004 (100)				0.094±0.007 (100)
Control (c) Lysi+Ala	0.097±0.003 (100)				0.096±0.005 (100)
Control (d) Ala+Vali	0.098±0.004 (100)				0.095±0.003 (100)
Vali+Aspa+Eug	0.072±0.003* (74.22)	0.068±0.006* (70.10)	0.067±0.005* (69.07)	0.062±0.008* (63.91)	0.078±0.002+ (86.66)
Vali+Aspa+Fer	0.059±0.005* (60.82)	0.055±0.002* (56.70)	0.056±0.003* (57.73)	0.051±0.007* (52.57)	0.057±0.006+ (63.33)
Vali+Aspa+Umb	0.060±0.004* (61.85)	0.052±0.009* (53.60)	0.051±0.006* (52.52)	0.048±0.002* (49.48)	0.052±0.007+ (57.77)
Vali+Aspa+lim	0.072±0.006* (74.20)	0.068±0.003* (70.10)	0.066±0.008* (68.04)	0.060±0.004* (61.85)	0.065±0.005+ (72.22)
Lysi+Vali+Eug	0.070±0.006* (73.68)	0.068±0.005* (71.57)	0.065±0.003* (68.42)	0.060±0.003* (63.15)	0.064±0.002+ (68.08)
Lysi+Vali+Fer	0.060±0.002* (63.15)	0.056±0.009* (58.94)	0.054±0.007* (56.84)	0.050±0.004* (52.63)	0.053±0.006+ (56.38)
Lysi+Vali+Umb	0.059±0.006* (62.10)	0.056±0.004* (58.94)	0.055±0.006* (57.89)	0.05±0.007* (53.68)	0.055±0.008+ (58.51)
Lysi+Vali+Lim	0.070±0.003* (73.68)	0.066±0.006* (69.47)	0.063±0.003* (66.31)	0.059±0.002* (62.10)	0.061±0.004+ (64.89)
Lysi+Ala+Eug	0.071±0.009* (73.19)	0.067±0.003* (69.07)	0.070±0.005* (72.16)	0.066±0.008* (68.04)	0.069±0.006+ (71.87)
Lysi+Ala+Fer	0.060±0.003* (61.85)	0.058±0.008* (58.79)	0.056±0.007* (57.73)	0.052±0.006* (53.60)	0.055±0.003+ (57.29)
Lysi+Ala+Umb	0.062±0.009* (63.91)	0.058±0.006* (59.79)	0.056±0.003* (57.73)	0.050±0.009* (51.54)	0.054±0.002+ (56.25)
Lysi+Ala+Lim	0.071±0.004* (73.19)	0.068±0.005* (70.10)	0.066±0.007* (68.04)	0.060±0.004* (61.85)	0.067±0.008+ (69.79)
Ala+Vali+Eug	0.069±0.006* (70.40)	0.065±0.003* (66.32)	0.061±0.005* (62.24)	0.058±0.003* (59.18)	0.062±0.009+ (65.26)
Ala+Vali+Fer	0.060±0.003* (61.22)	0.058±0.008* (59.18)	0.053±0.006* (54.08)	0.050±0.006* (51.02)	0.054±0.007+ (56.84)
Ala+Vali+Umb	0.062±0.007* (63.26)	0.058±0.003* (59.18)	0.055±0.003* (56.12)	0.051±0.004* (52.04)	0.056±0.003+ (58.94)
Ala+Vali+Lim	0.071±0.005* (72.44)	0.068±0.004* (69.38)	0.065±0.006* (66.32)	0.061±0.007* (62.24)	0.064±0.005+ (67.36)

Each value is mean ±SE of six replicates.
Value is parentheses are per cent 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 group and (+) in between 60% of 96h LC_{50} and withdrawal group. Vali=valine, Aspa=aspartic acid, Lysi=lysine, Ala=alanine, Eug=euhenol, Feb=ferulic acid, Umb=umbelliferone, Lim=limonene

4. Discussion

It is evident from the result section that active components of *Syzygium aromaticum* (eugenol), *Ferula asafoetida* (ferulic acid, umbelliferone), and *Carum carvi* (limonene) in bait formulations were 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 have significant molluscicidal activity again *L. acuminata*[8, 23]. Kumar and Singh[11, 12] have demonstrated that when these active molluscicidal components in bait formulations were fed to snails, it also acts as potent molluscicides. In present study mode of entry of molluscicides into the snail body is through the digestive system as it was used in bait. In an earlier study it was through the body surface when molluscicides were released directly in water[8]. 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 i.e. 20% and 60% of 24h and 96h LC_{50} of different molluscicides inside snail attractant pellets, caused a significant inhibition in ALP and AChE activity in the nervous tissue of snail *L. acuminata.* The inhibition in ALP and AChE activity may be due to the direct interference of these active molluscicidal with enzyme. Kumar et al.,[24] 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 molluscicidel were fed to snails in bait formulations. Alkaline phosphatase plays a critical role in protein synthesis[25], shell formation[26] and other secretary activities[27] and its inhibition may result reduction in protein level[19, 28] in gastropods. It plays an important role in transport of metabolites across the membrane[29]. The AChE inhibition results in accumulation of acetylcholine at the nerves synapses, so that the post synaptic membrane is in a state of permanent stimulation producing paralysis, ataxia, and general lack of coordination in neuromuscular system and eventual death[30]. Animal behavior is a neurotropically regulated phenomenon which is mediated by neurotransmitter substances such as ACh[31]. The enzyme AChE is found in the synaptic regions and mediates transmission of impulses by breaking acetylcholine into acetic acid and choline[32]. The acetylcholine at neural and neuromotor regions upon accumulation causes hyper-excitability[33] which in turn might also influence behavior pattern of animals. The present study shows that ferulic acids, umbelliferone, eugenol and limonene that are incorporated in the bait caused significant time and dose dependent inhibition in the activity of enzyme ALP and AChE in the snails *L. acuminata*. Although earlier it has been reported that ferulic acid, umbelliferone, eugenol and limonene inhibited the ALP and AChE activity in the nervous tissue of snails *L. acuminata* when used directly in aquarium water[23], 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.,[23]. Instead of it there is also difference in concentration of molluscicide in direct treatment ferulic acid/ umbelliferone/limonene and eugenol (0.63, 0.66, 0.84 and 0.67 mg/l, respectively) and bait formulations (0.43, 0.45, 0.64 and 0.45 mg/l, respectively). The present study reveals that bait formulations are more appropriate in killing snails, with respect to direct release of molluscicides in aquatic environment. It is more economical and safe.

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