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Feeding of Binary Combination of Carbohydrates and Amino Acids with Molluscicidesin Baits and Their Effects on Reproduction of *Lymnaea acuminata*

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Abstract: Snail *Lymnaea acuminata* (Lymnidae) serve as intermediate host for parasitic flat worm of *Fasciola* species. Fascioliosis disease is major cause of morbidity and mortality both in man and lives-stock and contributes to major socioeconomic problem in developing countries. One of the possible approaches to control this problem is to interrupt the life cycle of the parasitic trematodes by reducing the number of snail intermediate host population, which is an essential link in life cycle of *Fasciola*. The effect of sublethal (20% and 60% of 24h LC_{50}) feeding of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in bait containing binary combination (1:1 ratio) of amino acids (methionine, histidine) + carbohydrate (glucose, starch) on the, hatchability and survival of young snails of *Lymnaea acuminata* was studied. It was observed that the different amino acid combinations of bait formulation with molluscicides significantly reduced the reproductive capacity (fecundity, hatchability and survival of young snails) of the snail *Lymnaea acuminata.*Maximum reduction in fecundity was observed in starch + histidine + eugenol (25.80 % of control) fed snails. In withdrawal group there is a significant recovery observed in all feeder group (83.46 % of control). The hatching period in treated group was prolonged from 10 to 18 days from 6 to 10 days in control snails.

Key words: Molluscicides · Bait Formulation · Carbohydrates · Amino Acids · Fecundity · Hatchability · *Lymnaea acuminata*

INTRODUCTION

Fascioliosis is a hepatic disease caused by *Fasciola hepatica* and *F. gigantica* that affect, mainly ruminants and occasionally human, in several countries of America, Africa, Europe, Egypt and Asia [1]. Human fascioliosis in India has been reported in the state of Assam, Bihar, Maharashtra, Uttar Pradesh, Arunchal Pradesh and West Bengal [2-6]. This disease has great expansive powers due to the large colonization capacities of its causal agents and vector species [7]. It is caused by the digenetic trematode*Fasciola hepatica* and *F. gigantica* having two hosts, a final mammalian hosts and a molluscan intermediate snail hosts [8-10]. Snail *Lymnaea acuminata* breeds all the year round and lay eggs on the lower surface of the aquatic plants. Snail control is the best means to control the trematode infections [9, 11, 12]. Direct releases of molluscicides in aquatic environment have played a very significant role in controlling the population of molluscan pest problem [9]. With growing awareness of environmental pollution caused by synthetic molluscicides, now the recent research focus is turned on to plant products as alternatives, as they seemed to cause fewer side effects, more effective and readily available [9, 13-15]. Use of bait formulations containing a snail attractant and molluscicides is very effective in controlling the population of target molluscan pest [16-20]. Recently, we have observed that different preparations of bait formulation of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in binary combination with different amino acids as an attractant are very effective against *L. acuminata* [19, 20]. It has been

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advocated that a molluscicide is very effective if it also kill snail eggs and reduce the reproductive capacity of snail [12, 21]. The aim of present study is to evaluate the effect of sub lethal(20and 60 % of 24h LC_{50}) feeding of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in binary combination (1:1 ratio) of amino acids (methionine and histidine) and carbohydrates (glucose and starch), in bait formulations on the fecundity, hatchability and survival of young snails of *L. acuminata.*

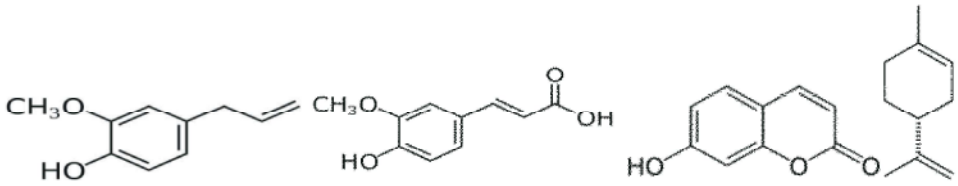
MATERIALS AND METHODS

Test Animal: Adult snails *Lymnaea acuminata* (2.60 ± 0.30 cm in length) were collected locally from lakes; ponds and low lying submerged fields and were used as test animals. The snails were acclimatized for 72h in laboratory condition. The pH of the water was 7.1-7.3 and dissolved O_2, free CO_2 and bicarbonate alkalinity were 6.5-7.2 mg/l, 5.2-6.3 mg/l and 102.0-105.0 mg/l, respectively. Twenty experimental snails were kept in glass aquaria containing 3L of dechlorinated tap water at 22to 24°C. *L.acuminata* laid their eggs on the lower surface of leaves of the aquatic plants in the form of elongated gelatinous capsules containing 5-180 eggs.

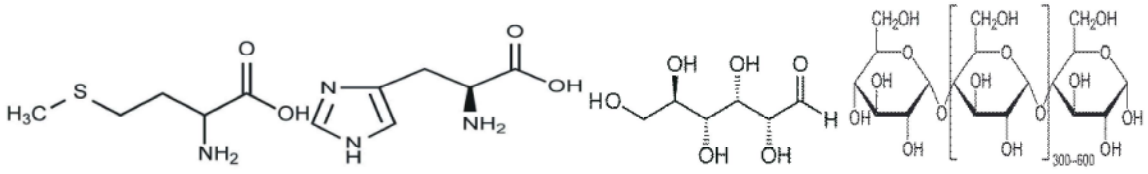
Pure Compounds: Agar-agar, amino acid (methionine and histidine), carbohydrates (glucose and starch) and different active molluscicidelcomponents eugenol, ferulic acid, umbelliferone and limonene were used in bait formulations. The pure active components eugenol (2-Methoxy-4-(2-propenyl) phenol), ferulic 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).

Bait Formulation with Molluscicides: Bait formulations containing binary combination (1:1 ratio) of amino acid (methionine and histidine10mM) and carbohydrates (glucose and starch 10mM) and sub-lethal (20% and 60% of 24h and 96h LC_{50}) molluscicides(eugenol, ferulic acid, umbelliferone and limonene) were prepared in 100 ml of 2% agar solution by the method of Madsen, [22]. Concentrations of amino acids were based on the earlier reports of Tiwari and Singh, [23, 24]. Sublethal concentrations of molluscicides in bait were based on the earlier study of Kumar *et al.* [20]. These solutions were spread at a uniform thickness of 5mm. After cooling the bait containing sublethal molluscicides were cut out a corer measuring 5 mm in diameter. Six replicates were prepared for each concentration. Control snails were fed with bait without molluscicide.

Assay Apparatus and Procedure: The bioassay was performed by the method by Tiwari and Singh, [23, 24]. The bioassay chambers consist of a clean glass aquarium having a diameter of 30 cm. Each aquarium was divided into four concentric zones; Zone 3 (Central zone), 2, 1 (Middle zone) and zone 0 (Outer zone) had diameters of 13, 18, 24 and 30 cm, respectively. 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 3L of dechlorinated tap water to a height of



(Eugenol)(Ferulic acid)(Umbelliferone)(Limonene)



(Methionine)(Histidine) (Glucose) (Starch)

8 mm and maintained at 25±1° C. At the start of the assay twenty snails of uniform size were placed on the circumference of zone 0. Simultaneously, one of the prepared bait of different active component (molluscicides) was added on the small annular elevation in the center (Zone 3). Six sets of experiments have been designed with twenty snails in each replicate.

Treatments: Snail fed to bait formulation containing sublethalmolluscicide (eugenol, ferulic acid, umbelliferone and limonene) and binary combination (1:1 ratio) of amino acid (methionine and histidine) and carbohydrates (glucose and starch) as attractant on snail reproduction was studied by the method of Kumar *et al.* [20]. Groups of 20 snails in 3L water were fed to sublethal concentrations (20% and 60% of 24h LC_{50}) of different combinations of molluscicides with amino acids + carbohydrates.

The total number of eggs laid by bait fed and control snails were counted after every 24h for 96h. Since it is difficult to detect the mother snails for particular spawn, capsules containing eggs from each feeder group were incubated at 30°C in covered petridishes. The development of embryos at regular intervals was observed under binocular microscope until hatching. Percent hatching was studied only with eggs laid after the 24h feeding period. Dead embryo was removed to avoid any contamination. Survival of young snails was observed up to 72h. Snails were transferred to fresh water after a 96h feeding period to observe the effect of the above bait formulations after withdrawal.

Statistical Analysis: Each experiment was replicated at least 6 times. Values were expressed as Mean±SE. Students't' test was applied to determine the significant ($P<0.05$) difference between bait feeder and control animals. Product moment correlation coefficient was applied in between exposure time and different values of fecundity/ survival of hatched snails [25].

RESULTS

In control groups of 20 snails laid 180-184 eggs/day. There was a significant ($p<0.05$) reduction in the fecundity of snail *L. acuminata* feeding to 20 and 60 % of LC_{50}/24h of ferulic acid, umbelliferone, eugenol and limonene withbinary combination (1:1 ratio) of amino acids (methionine, histidine) and carbohydrates (glucose, starch) as a snail attractants in bait formulations (Table 1).

Table1: Effect of sublethal (20% and 60% of 24h LC_{50}) molluscicide (eugenol, ferulic acid, umbelliferone and limonene) in bait containing binary combinations (1:1 ratio) of amino acid (methionine and histidine) and carbohydrates (glucose and starch) on the fecundity of the snail *L. acuminata*.

Treatment	Fecundity after 24h (eggs/20 snail)	Fecundity after 48h (eggs/20 snail)	Fecundity after 72h (eggs/20 snail)	Fecundity after 96h (eggs/20 snail)	Withdrawal after 96h exposure Fecundity after 72h (eggs/20 snail)
Control (Agar)	176.82±0.91	177.26±0.90	175.72±0.45	173.45±0.62	174.22±0.95
Control (a) Glu+Meth	154.62±0.73	156.23±0.73	154.29±0.15	157.31±0.26	153.62±0.36
Control (b) Star+Meth	158.61±0.63	156.12±0.42	160.42±0.92	155.73±0.11	156.42±0.45
Control © Glu+Hist	162.75±0.42	165.23±0.85	166.72±0.93	169.48±0.62	168.45±0.62
Control (d) Star+Hist	165.20±0.94	163.73±0.23	164.70±0.31	166.42±0.18	165.30±0.24
Glu+Meth+Eug	55.73±0.32*+	45.68±0.82	38.92±0.88	-	146.78±0.36
	35.60±0.71*+	25.92±0.73	-	-	128.92±0.96
Glu+Meth+Fer	54.86±0.91*+	48.72±0.45	36.76±0.48	-	139.95±0.63
	27.65±0.42*+	26.69±0.31	26.00±0.39	24.85±0.78	127.62±0.81
Glu+Meth+Umb	51.73±0.23*+	42.73±0.66	40.73±0.42	35.36±0.92	138.96±0.24
	28.93±0.48*+	27.64±0.31	26.40±0.85	24.07±0.68	127.58±0.38
Glu+Meth+lim	60.58±0.90*+	50.13±0.98	48.31±0.72	39.48±0.73	142.63±0.68
	31.45±0.91*+	28.62±0.73	27.96±0.63	25.64±0.56	127.85±0.85
Star+Meth+Eug	51.75±0.54*+	48.70±0.33	-	-	150.51±0.93
	28.29±0.81*+	-	-	-	129.42±0.73
Star+Meth+Fer	51.96±0.42*+	49.75±0.86	47.58±0.25	-	148.58±0.96
	29.75±0.23*+	28.11±0.60	27.94±0.63	25.59±0.36	127.15±0.37
Star+Meth+Umb	50.76±0.11*+	48.82±0.79	46.82±0.40	44.96±0.63	145.73±0.92
	27.62±0.16*+	26.77±0.11	25.82±0.49	24.48±0.96	125.76±0.88

Table1: Continued

Treatment	Fecundity after 24h (eggs/20 snail)	Fecundity after 48h (eggs/20 snail)	Fecundity after 72h (eggs/20 snail)	Fecundity after 96h (eggs/20 snail)	Withdrawal after 96h exposure Fecundity after 72h (eggs/20 snail)
Star+Meth +Lim	58.48±0.62*+	56.96±0.32	53.61±0.73	50.98±0.81	152.99±0.31
	36.72±0.58*+	34.75±0.42	32.96±0.43	30.82±0.70	131.85±0.48
Glu+hist+Eug	50.72±0.43*+	47.46±0.96	-	-	148.65±0.96
	28.92±0.23*+	-	-	-	129.00±0.72
Glu+Hist+Fer	51.63±0.24*+	50.76±0.32	-	-	151.60±0.63
	26.78±0.42*+	25.18±0.73	24.82±0.73	23.92±0.48	125.96±0.72
Glu+Hist+Umb	48.45±0.92*+	47.66±0.28	46.92±0.31	45.31±0.55	146.82±0.62
	27.68±0.31*+	26.69±0.73	25.96±0.72	23.31±0.98	125.92±0.86
Glu+Hist+Lim	58.76±0.43*+	55.72±0.48	51.72±0.76	48.73±0.81	150.62±0.73
	27.68±0.85*+	26.55±0.62	24.63±0.28	23.96±0.90	125.85±0.63
Star+Hist+Eug	45.62±0.42*+	44.63±0.25	-	-	145.42±0.92
	36.11±0.92*+	-	-	-	137.07±0.62
Star+Hist+Fer	46.70±0.15*+	44.82±0.68	-	-	145.72±0.72
	29.64±0.38*+	28.75±0.76	27.98±0.93	26.60±0.18	128.12±0.83
Star+Hist+Umb					
	48.96±0.73*+	47.06±0.48	45.66±0.48	44.92±0.63	146.73±0.55
	25.68±0.44*+	23.70±0.39	20.78±0.36	19.63±0.70	122.96±0.82
Star+Hist+Lim	63.75±0.81*+	60.82±0.76	55.92±0.76	51.73±0.82	147.63±0.96
	48.42±0.95*+	46.79±0.38	44.76±0.72	42.98±0.66	145.38±0.73

Each value is mean±SE of six replicates. Each replicates represents the egg laid by the group of 20 snails. (*) significant ($P<0.05$) when student "t" test was applied to treated and control groups. (+) product moment correlation coefficient showed that there was significant ($P<0.05$) negative correlation in between the exposure period and fecundity of snail *L. acuminata*. (-) No fecundity was observed. Abbreviation-Gul=glucose, Star=starch, Meth=methionine, His=histidine, Eug=euhenol, Feb=ferulic acid, Umb=umbelliferone, Lim=limonene

Table2: Effect of sublethal (20% and 60% of 24h LC_{50}) molluscicide (eugenol, ferulic acid, umbelliferone and limonene) in bait containing binary combinations (1:1 ratio) of amino acid (methionine and histidine) and carbohydrates (glucose and starch) on the hatchability and survival of the snail L.acuminata eggs obtained after 24h in table 1

Treatment	Hatchability percentage (hatching period)	Percent survival after 24h	Percent survival after 48h	Percent survival after 72h
Control (Agar)	100 (6-7)	100	100	100
Control (a)				
Glu+Meth	100 (6-7)	100	100	100
Control (b)				
Star+Meth	100 (6-7)	100	100	100
Control (c)				
Glu+Hist	100 (7-8)	100	100	100
Control (d)				
Star+Hist	100 (7-10)	100	100	100
Glu+Meth+Eug	61.38±0.25 (11-14)	-	-	-
	25.66±0.95 (11-15)	-	-	-
Glu+Meth+Fer	39.12±0.45 (10-11)	36.92±0.76*+	34.85±0.18	30.99±0.72
	19.85±0.76 (11-13)	19.62±0.32*+	18.62±0.23	18.15±0.63
Glu+Meth+Umb	45.18±0.36 (12-13)	41.38±0.62*+	38.72±0.31	33.62±0.96
	28.42±0.92 (12-14)	25.18±0.19*+	22.82±0.63	20.55±0.36
Glu+Meth+lim	58.75±0.32 (13-14)	45.96±0.28*+	36.69±0.21	30.86±0.23
	41.96±0.80 (13-14)	38.21±0.32*+	30.72±0.62	-
Star+Meth+Eug	28.11±0.32 (14-16)	-	-	-
	15.62±0.13 (14-17)	-	-	-
Star+Meth+Fer	32.72±0.23 (12-14)	32.12±0.23*+	28.75±0.46	21.65±0.85
	20.36±0.23 (13-15)	18.56±0.92*+	18.10±0.18	16.33±0.11
Star+Meth+Umb	45.72±0.16 (12-13)	40.62±0.38*+	38.62±0.23	31.89±0.66
	24.76±0.96 (13-15)	23.99±0.23*+	22.16±0.73	20.65±0.28

Table 2: Continued

Treatment	Hatchability percentage (hatching period)	Percent survival after 24h	Percent survival after 48h	Percent survival after 72h
Star+Meth +Lim	56.12±0.76 (12-14)	51.96±0.28*+	48.66±0.75	41.23±0.29
	23.75±0.36 (13-15)	23.11±0.23*+	20.75±0.62	19.18±0.36
Glu+hist+Eug	48.72±0.76 (14-16)	38.62±0.76*+	33.62±0.96	30.75±0.23
	21.60±0.23 (14-17)	20.61±0.35*+	-	-
Glu+Hist+Fer	38.58±0.66 (13-14)	35.25±0.75*+	-	-
	33.62±0.85 (13-15)	32.76±0.18*+	30.18±0.23	28.66±0.32
Glu+Hist+Umb	48.75±0.28 (14-15)	45.62±0.92*+	41.63±0.28	37.25±0.23
	40.26±0.16 (15-17)	37.62±0.33*+	31.62±0.18	30.56±0.23
Glu+Hist+Lim	58.23±0.26 (12-13)	57.32±0.28*+	56.11±0.96	42.76±0.33
	28.19±0.11 (12-15)	26.30±0.16*+	22.75±0.36	20.86±0.12
Star+Hist+Eug	33.98±0.76 (14-16)	-	-	-
	22.85±0.16 (15-18)	-	-	-
Star+Hist+Fer	39.26±0.66 (13-15)	36.76±0.56*+	33.82±0.76	28.67±0.81
	26.76±0.31 (14-15)	24.96±0.36*+	22.70±0.18	20.36±0.22
Star+Hist+Umb				
	43.13±0.62 (12-14)	40.85±0.76*+	38.26±0.96	35.80±0.16
	21.11±0.26 (13-16)	19.65±0.32*+	19.11±0.32	18.65±0.36
Star+Hist+Lim	55.26±0.66 (14-15)	48.36±0.96*+	42.26±0.71	39.26±0.62
	33.18±0.71 (15-16)	30.26±0.26*+	28.65±0.96	-

Each value is mean±SE of six replicates. Each replicates represent the egg laid by the group of 20 snails. (*) significant ($P<0.05$) when student "t" test was applied to treated and control groups. (+) product moment correlation coefficient showed that there was significant ($P<0.05$) negative correlation in between survival period and survival of the snail *L. acuminata*. (-)No fecundity was observed. Abbreviation- Gul=glucose, Star=starch, Meth=methionine, His=histidine, Eug=euhenol, Feb=ferulic acid, Umb=umbelliferone, Lim=limonene

No egg lying after 48h feeding, was observed in snails feed to 60% of 24h LC_{50} of bait formulation in binary combination (1:1 ratio) of starch + methionine, starch + histidinewith eugenol. The hatching period was prolonged in treated group (10-18days) with respect to control group (6-10 days) (Table 2). Withdrawal of snail after 96h feeding of baits for 72h in fresh water caused a significant ($P<0.05$) recovery in the fecundity of snails with respect to their corresponding treatment (Table 1).

Lymnaea acuminata fed with 20 % or 60 % of 24h LC_{50} of glucose + methionine, starch + methionine and starch + histidine with eugenol 20% or 60% of 24h LC_{50}caused no survival of snail after 24h hatching (Table 2). There was a significant ($P<0.05$) negative correlation between the feeding time and survival of young snails hatched from eggs laid by snail feeder to 20%, 60% of 24h LC_{50} of different preparation of bait formulation (Table 2).

DISCUSSION

The result of the present study indicates thatsublethal feeding (20% and 60% of 24h LC_{50}) of the binary combination (1:1 ratio) of amino acids (methionine, histidine) and carbohydrate (glucose, starch) with molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in bait formulations, significantly reduced the reproductive capacity of snail *L. acuminata*. Bait formulation with 60% of 24h LC_{50} of glucose + methionine, starch + methionine and starch + histidinewith active molluscicidal component eugenol in binary combination (1:1 ratio) reduced the fecundity of *L. acuminata* within 24h.

The plant derived active component eugenol (*Syzygiumaromaticum*),ferulic acid/umbelliferone (*Ferula asafoetida*) and limonene (*Carumcarvi*) have dose dependent influence on the fecundity of snails [12], when directly given in aquarium water. A number of plant products have been effectively used for control of snail reproduction [26-28]. Although, the binary combinations (1:1 ratio) of amino acids with bait formulation of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) are more effective, it reduce the free amino acids, proteins, DNA and RNA level in the ovotestis of *L. acuminata* [19]. Kumar *et al.* [20] have reported the bait containing 60 % of 96h LC_{50} of eugenol with starch + histidine, starch + methionine; respectively inhibit alkaline phosphatase (ALP) (20 % of control) and acetylcholinesterase (AChE) (49.49 % of control) activity in the nervous tissue of *L. acuminata*. Binary 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*feeder to 60 % of 96h LC_{50} of ferulic acid and umbelliferone, respectively [20]. In the present study the mode of entry of molluscicides into the snail body through the digestive system and affect the caudodarsal cells (CDSs), reducing the release of the ovulation hormone that resulted a decrease in the fecundity of bait fed snails. Caudodarsal cell is responsible for the fecundity of snail *L. acuminata* [21, 29, 30]. The inhibition in ALP and AChE activity may be due to the direct interference of these active molluscicidal with enzymes. Kumar *et al*.[19] 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 [31], shell formation [32] and other secretary activity [33] and its inhibition may result in the reduction of protein level [30] in gastropods. It plays an important role in the transport of metabolites across the membrane [34].

The reduced hatchability of *L. acuminata* fed to the different baits may be due to interference of molluscicides with the embryonic growth and development of the snails. In bait fed snails, young larvae were weak, unable to break the egg capsule and died owing to starvation. Young snails hatched from the treated egg masses showed delay in attaining maturity in comparison with the control groups. Most of these eggs were attached to the wall of the aquarium and were apathetic toward feeding. In general, the egg shells were thinner and the hatchlings had shorter tentacles and slower movement and were smaller in size as compared with control group. Mortality and low reproduction in the bait fed snails suggest the active molluscicidal components in bait formulation was able to control the population of snail *L. acuminata* by inhibiting development at any stage of growth.

Transfer of mother snails to fresh water for the next 72h after 96h feeding all the feeder snails leads to a significant recovery in the fecundity of snails. Withdrawal experiments also showed that the feeding of bait formulation were reversible as the levels of free amino acids, proteins, DNA and RNA [19] activity were restored within 7 days. Thus, reversibility of the effects would be an added advantage in their use against aquatic target snails as they would cause only short-lived effects. This concept is new technique and approach to the use of bait formulation with sublethalmolluscicides will be helpful in control of harmful snails, without using more active molluscicide in the aquatic environment and attracting target snails.

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