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Combination of molluscicides with attractant carbohydrates and amino acids in bait formulation against the snail *Lymnaea acuminata*

P. KUMAR, V.K. SINGH, D.K. SINGH

Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur 273 009 (U.P. India)

Abstract. – *Aim:* **Fascioliasis is an important helminth disease caused by *Fasciola (F.) hepatica* and *F. gigantica* of Asia and Africa. This disease belongs to the plant-borne trematode zoonoses. Human infection has been reported in 51 different countries from 5 continents. One of the possible approaches to control this problem is to interrupt the life cycle of the parasitic trematodes by eliminating the snail.**

Materials and Methods: **Snails attractant pellets (SAP) were prepared from binary combination of carbohydrate + amino acid (20 mM) in 2% agar solution with active molluscicidal component *Ferula asafoetida* (ferulic acid, umbelliferone), *Syzygium aromaticum* (eugenol), *Carum carvi* (limonene). Attraction of snails to different combinations was studied by using clear glass aquaria having diameter of 30 cm. Each aquarium was divided into four concentric zones; zone-3 (central zone), zone-2 and zone-1 (middle zone) and zone-0 (outer zone) had a diameter of 13, 18, 24, and 30 cm, respectively. The behavioral responses of snails to these binary combinations of carbohydrate and amino acid in bait formulation were examined. The fraction of snails that was in contact with the SAP at different times was used as a measure of attraction.**

Results: **Among all the binary combination of carbohydrate+amino acid+molluscicide after 2h of experiment, highest attraction of snail (54.71%) was observed towards the SAP containing starch+histidine+limolene. Limonene+ starch+histidine containing SAP emerged as the strongest bait formulation (96h LC_{50} 0.74%) against *Lymnaea acuminata*.**

Conclusions: **The present study suggested that the molluscicides of plant origin could be used with varying degrees of success in bait formulation.**

Key Words:

Carbohydrate, Amino acids, Bait formulation, Molluscicides, *Lymnaea acuminata*.

Introduction

Human fascioliasis is truly endemic, varying from hypo to hyper-endemic in different parts of the world. In human endemic areas, fascioliasis mainly affects children and female, with fluke infecting even at very early age[1,2]. Human infection has been reported in 51 different countries from 5 continents[3]. This disease is characterized by abdominal pain, hypereosinophilia and acute pancreatitis[4]. It is caused by the digenetic trematode of *Fasciola (F.) hepatica* and *F. gigantica* having two hosts, a final mammalian and a snail intermediate host[5]. Due to higher funding priorities such as SARS, AIDS and malaria and research in immunological approaches to worm control, there is a little current interest in snail control as a means of managing fascioliasis/schistosomiasis. One way to reduce the incidence of fascioliasis is to de-link their 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 for preparing effective bait formulations. Snails, like other gastropod mollusks, use chemical clues to locate food sources[13-19]. The freshwater snails inhabit an environment containing macrophytes algae and bacteria[20]. These aquatic organisms release different type of chemical, such as carbohydrates and amino acids, into the surrounding water[20-24] which acts as attractant for snails. Use of a combination of snail attractant and molluscicides in bait formulation is an effective tool for the pest management. The present study assay the behavioral responses of *Lymnaea (L.) acuminata* to different binary combination of

Corresponding Author: D.K. Singh, Ph.D; dksingh_gpu@yahoo.co.in

carbohydrates + amino acids and identify among them that which of them could preferably be used as a potent attractant for preparing bait along with molluscicides. Active molluscicidal component *Ferula asafoetida* (ferulic acid, umbelliferone), *Syzygium aromaticum* (eugenol), *Carum carvi* (limonene)[10,11] were used inside the preferred snails' attractant pellets (SAP) for the control of snail *L. acuminata.*

Material and Methods

Collection of Snails

Adult *L. acuminata* (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±1°C. 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.

Pure Compounds

Agar-agar, amino acids, different active component (Molluscicides) such as eugenol, ferulic acid, umbelliferone and limonene were used in bait formulation. The pure active component ferulic acid (4-Hydroxy-3 methoxycinnamic), umbelliferone (7-Hydroxy coumarin; 7-hydroxy-2H-1-benzopyran-2-one), eugenol (2-methoxy-4-(2-propenyl) phenol) and limonene ((R)-4-isopropenyl-1-methyl-1-cyclohexene); were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Preparation of Snail-Attractant Pellets (SAP)

Attractant food pellets (AFP) were prepared according to the method of Madsen[25] as modified by Tiwari and Singh[16,17]. Briefly, 0.02 g of amino acid (20 mM) was added to 2% agar solution. After boiling, each of the selective active component (molluscicides) was added to the solution in different concentrations (Table I), the mixture was stirred constantly for 30 minutes and spread to a uniform thickness (5 mm). After cooling, the pellets were cut out from the layer with a corer (5 mm diameter).

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; 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 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 bait of different active component (molluscicides) was added on the small annular elevation in the center (zone 3). The location of each snail was noted after every 15 min for two hours. Six sets of experiments have been designed with ten snails each for all molluscicides used in this study.

Statistical Analysis

The mortality data were observed after every 24h up to 96h. Lethal values (LC_{50}), lower and upper confidence limits (LCL and UCL), slope values, t-ratio, "g" value and heterogeneity factor were calculated using POLO computer programme[26]. Two-way ANOVA and product moment correlation coefficient was applied between the different data obtained in Table I[27].

Results

Table I gives the distribution of *L. acuminata* in the zone-3 around the snail attractant pellets (SAP) of different binary combination of carbohydrate, amino acid with various active molluscicidal components after 1 and 2h from the start of the experiment. Placement of SAP in center (zone 3) affected the behavior of the snails. The effect of binary combination of carbohydrate, amino acid with various active molluscicidal components in SAP on the proportion of snail in zone 3 was analyzed by one-way ANOVA. After 1h lowest attraction (26.33%) of the snails, in zone-3 was observed, when they were fed with bait containing starch + histidine and 0.7% umbelliferone (Table I). 0.7% eugenol, ferulic acid, umbelliferone, limonene in bait containing glucose + histidine (39.36%, 37.33%, 37.09%, and

Table I. Mean number of snail *L. acuminata* in zone three in contact with the attractant food pellets (AFP) that contain different molluscicides after one and two hours from beginning of experiment.

Molluscicides	Time (hrs)	Concentration of molluscicides			
		0.7%	1.0%	2.0%	3.0%
Glu + His + Eug	1	1.99 ± 0.52 (33.16)$^{+}$	2.24 ± 0.43 (32.00)	2.49 ± 0.83 (31.12)	1.74 ± 0.28 (28.00)
	2	4.33 ± 0.36 (39.36)$^{+}$	4.08 ± 0.37 (40.80)	3.58 ± 0.34 (35.80)	2.41 ± 0.76 (40.16)
Glu + His + Feru	1	3.01 ± 0.28 (34.22)$^{+}$	3.49 ± 0.61 (34.90)	3.49 ± 0.16 (34.94)	2.33 ± 0.65 (33.28)
	2	2.24 ± 0.64 (37.33)$^{+}$	2.24 ± 0.72 (37.35)	2.65 ± 0.62 (38.00)	2.83 ± 0.50 (40.42)
Glu + His + Umb	1	2.53 ± 0.52 (33.28)$^{+}$	1.83 ± 0.28 (26.14)	2.08 ± 0.67 (29.71)	3.00 ± 0.81 (33.33)
	2	4.08 ± 0.49 (37.09)$^{+}$	4.16 ± 0.31 (41.60)	3.74 ± 0.64 (37.40)	4.16 ± 0.21 (37.81)
Glu + His + Lim	1	2.24 ± 0.43 (32.00)$^{+}$	3.08 ± 0.63 (34.22)	2.41 ± 0.21 (34.42)	2.24 ± 0.55 (35.21)
	2	3.83 ± 0.16 (38.30)$^{+}$	3.08 ± 0.63 (39.50)	4.41 ± 0.45 (36.75)	3.84 ± 0.23 (38.22)
Sta + His + Eug	1	2.25 ± 0.47 (37.50)$^{+}$	2.24 ± 0.79 (32.00)	1.66 ± 0.30 (27.66)	1.41 ± 0.37 (28.20)
	2	3.33 ± 0.23 (41.62)$^{+}$	3.08 ± 0.75 (38.50)	3.41 ± 0.34 (37.88)	2.33 ± 0.49 (46.60)
Sta+ His + Feru	1	3.08 ± 0.37 (34.22)$^{+}$	1.74 ± 0.28 (29.00)	1.83 ± 0.42 (30.50)	3.99 ± 0.59 (36.23)
	2	2.33 ± 0.62 (38.83)$^{+}$	3.58 ± 0.25 (39.74)	4.24 ± 0.68 (42.40)	3.14 ± 0.67 (45.14)
Sta+ His + Umb	1	1.58 ± 0.43 (26.33)$^{+}$	3.33 ± 0.82 (33.30)	2.33 ± 0.70 (33.28)	4.24 ± 0.76 (38.54)
	2	3.83 ± 0.44 (38.30)$^{+}$	3.66 ± 0.69 (44.33)	4.99 ± 0.59 (41.58)	2.91 ± 0.82 (48.50)
Sta + His + Lim	1	1.81 ± 0.32 (30.16)$^{+}$	2.75 ± 0.54 (39.28)	2.16 ± 0.68 (30.58)	3.41 ± 0.90 (34.10)
	2	3.41 ± 0.24 (31.00)$^{+}$	4.57 ± 0.82 (41.54)	3.58 ± 0.57 (38.16)	3.82 ± 0.90 (54.71)
Control (Agar)	1	0.82 ± 0.08 (18.02)	1.07 ± 0.12 (19.13)	1.33 ± 0.04 (19.88)	1.30 ± 0.20 (20.01)
	2	1.41 ± 0.31 (25.11)	1.11 ± 0.03 (26.01)	2.03 ± 0.42 (25.99)	2.33 ± 0.13 (25.98)
Control (Glu + His)	1	3.28 ± 0.21 (52.33)	4.28 ± 0.72 (53.92)	4.80 ± 0.03 (56.33)	4.02 ± 0.67 (58.33)
	2	4.30 ± 0.12 (53.89)	4.82 ± 0.77 (55.90)	4.21 ± 0.39 (60.32)	4.20 ± 0.55 (62.99)
Control (Sta + His)	1	3.82 ± 0.11 (51.49)	4.32 ± 0.81 (55.10)	4.88 ± 0.59 (57.20)	4.21 ± 0.33 (59.21)
	2	4.29 ± 0.72 (54.72)	4.92 ± 0.87 (56.21)	4.11 ± 0.29 (59.00)	4.25 ± 0.38 (62.81

Values in parentheses are percentages of snails in zone 3 (in contact with attractant food pellet) with snail in zone 1 and 2. Statistically significant (p<0.05) when two way ANOVA was applied in between different molluscicides ($^{+}$) and their different concentrations (*). *Abbreviations:* Glu-glucose, His-histidine, Sta-starch, Eug-eugenol, Umb-umbelliferone, Feru-ferulic acid, Lim-limonene.

38.30%, respectively) and starch + histidine (41.62%, 38.83%, 38.30%, and 31.00%, respectively) caused more attraction after 2h (Table I). However, attraction of snails in bait containing molluscicide + attractant was lower than control pellet containing agar + attractant (glucose + histidine/ starch + histidine).

Molluscicidal activity of different SAP containing active component against *L. acuminata* was time and dose dependent (Tables II and III). There was a significant (p<0.05) negative correlation between exposure period and LC_{50} of different molluscicides. The active component umbelliferone (24h LC_{50}- 1.77%) and limonene (24h LC_{50}- 1.87%) were more toxic than eugenol and ferulic acid (Tables II and III). The 96h LC_{50} of different SAP containing umbelliferone (96h LC_{50}- 0.93%), limonene (96h LC_{50}- 0.74%) was higher than of eugenol and ferulic acid 0.93%, 0.74%, respectively (Tables II and III).

The slope values given in Tables II and III were steep. Separate estimate of LC_{50} based on each of the six replicates was found to be within 95% confidence limits. The t-ratio was greater than 1.96 and the heterogeneity less than 1.0. The g value was less than 0.5 at all probability levels (90, 95 and 99) (Tables II and III).

Discussion

Present study clearly demonstrates that the snail *L. acuminata* showed a significant behavioral response towards the different binary combinations of carbohydrates + amino acids with molluscicides. Earlier, it has been observed that gastropods detect the amino acids/carbohydrates as indicator of their food[12,16-19]. Among all the binary combinations of carbohydrate + amino acid

Table II. Bait formulation of different molluscicides and their toxicity against *L. acuminata* at different time exposure.

Exposure period	Molluscicides	LC_{50} % AFP	LCL	UCL	Slope value	t-ratio	g-value	Heterogeneity
24h	Glu + His + Eug	3.81	2.54	12.00	1.29 ± 0.35	3.63	0.29	0.20
	Glu + His + Feru	2.31	1.76	3.83	1.46 ± 0.34	4.25	0.21	0.15
	Glu + His + Umb	2.45	1.94	3.63	1.81 ± 0.35	5.09	0.14	0.19
	Glu + His + Lim	2.96	2.18	5.74	1.49 ± 0.35	4.29	0.21	0.19
48h	Glu + His + Eug	3.03	2.09	8.38	1.19 ± 0.34	3.49	0.31	0.24
	Glu + His + Feru	1.81	1.35	2.82	1.29 ± 0.33	3.84	0.26	0.26
	Glu + His + Umb	1.82	1.44	2.53	1.60 ± 0.34	4.67	0.17	0.23
	Glu + His + Lim	2.10	1.69	2.93	1.79 ± 0.34	5.15	0.14	0.19
72h	Glu + His + Eug	1.83	1.44	2.60	1.54 ± 0.34	4.53	0.18	0.28
	Glu + His + Feru	1.31	0.98	1.69	1.55 ± 0.34	4.56	0.18	0.39
	Glu + His + Umb	1.36	1.05	1.73	1.67 ± 0.34	4.88	0.16	0.25
	Glu + His + Lim	1.60	1.26	2.10	1.63 ± 0.34	4.80	0.16	0.31
96h	Glu + His + Eug	1.43	1.15	1.81	1.81 ± 0.34	5.25	0.13	0.53
	Glu + His + Feru	0.97	0.57	1.29	1.32 ± 0.34	3.89	0.25	0.45
	Glu + His + Umb	0.98	0.52	1.33	1.20 ± 0.33	3.55	0.30	0.32
	Glu + His + Lim	1.28	0.98	1.61	1.68 ± 0.34	4.94	0.15	0.43

Abbreviations: Glu-glucose, His-histidine, Eug-eugenol, Umb-umbelliferone, Lim-limonene, LCL – lower confidence limits, UCL – upper confidence limits. Six batches of ten snails were exposed different concentration of the above molluscicides inside the attractant food pellets (AFP). Mortality was determined after every 24h. Significant negative regression (p<0.05) was observed between exposure time and LC_{50} of treatments. Ts - testing significant of the regression coefficient – Glu + His + Eug – 8.07+; Glu + His + Feru – 16.31+; Glu+ His +Umb – 12.16+; Glu + His + Lim – 13.56++. +: linear regression between x and y; ++: non – linear regression between log x and log y.

Table III. Bait formulation of different molluscicides and their toxicity against *L. acuminata* at different time exposure.

Exposure period	Molluscicides	LC_{50} % AFP	LCL	UCL	Slope value	t-ratio	g-value	Heterogeneity
24h	Sta + His + Eug	2.51	1.85	4.91	1.32 ± 0.34	3.85	0.25	0.17
	Sta + His + Feru	2.52	1.73	3.61	1.49 ± 0.34	4.34	0.20	0.13
	Sta + His + Umb	1.77	1.44	2.30	1.86 ± 0.34	5.38	0.13	0.27
	Sta + His + Lim	1.87	1.44	2.81	1.42 ± 0.34	4.18	0.20	0.25
48h	Sta + His + Eug	1.77	1.39	2.46	1.56 ± 0.34	4.58	0.18	0.16
	Sta + His + Feru	1.71	1.31	2.47	1.40 ± 0.33	4.16	0.22	0.29
	Sta + His + Umb	1.50	1.16	2.00	1.53 ± 0.33	4.52	0.18	0.45
	Sta + His + Lim	1.40	0.93	2.06	1.13 ± 0.33	3.40	0.33	0.34
72h	Sta + His + Eug	1.24	0.94	1.56	1.66 ± 0.34	4.87	0.16	0.24
	Sta + His + Feru	1.38	1.04	1.80	1.52 ± 0.33	4.49	0.19	0.39
	Sta + His + Umb	1.16	0.85	1.46	1.61 ± 0.34	4.73	0.17	0.46
	Sta + His + Lim	1.10	0.74	1.44	1.40 ± 0.33	4.15	0.22	0.47
96h	Sta + His + Eug	1.03	0.74	1.29	1.71 ± 0.34	4.95	0.15	0.31
	Sta + His + Feru	1.17	0.77	1.57	1.30 ± 0.33	3.85	0.25	0.50
	Sta + His + Umb	0.93	0.50	1.26	1.24 ± 0.33	3.69	0.28	0.60
	Sta +His + Lim	0.74	0.29	1.06	1.17 ± 0.34	3.43	0.32	0.53

Abbreviations: Sta - starch, His – histidine, Eug - eugenol, Umb - umbelliferone, Lim - limonene, LCL – lower confidence limits, UCL - upper confidence limits. Six batches of ten snails were exposed different concentration of the above molluscicides inside the attractant food pellets (AFP). Mortality was determined after every 24h. Significant negative regression (p<0.05) was observed between exposure time and LC_{50} of treatments. Ts - testing significant of the regression coefficient – Sta +His + Eug – 11.63++; Sta + His +Feru – 128.52++; Sta + His + Umb – 20.12+; Sta + His +Lim – 15.49+. +: linear regression between x and y; ++: non – linear regression between log x and log y.

and molluscicides after 2h, starch + histidine + limolene shows highest (54.71%) prepared attracted SAP towards the snail *L. acuminata.* In nature starch is the major carbohydrate stored in aquatic plants and maltose is released by some epiphytic algae[23,25]. Present study clearly demonstrates that the binary combinations of different carbohydrate + amino acids + molluscicides are rapidly recognized by the *L. acuminata*. Behavioral responses of the snail *Biomphalaria alexandrina* and *Lymnaea acuminata* against different carbohydrates and amino acids in snail-attractant pellets has been reported earlier[12,16-19,28]. Starch is recognized more rapidly by the chemoreceptor present in the snails[18]. It may be possible that differences in behavioral responses between *L. acuminata* and other snails may be due to differences in the feeding behavior and metabolism of different species or it may be due to variation in receptors that detect the attractants. Significant variation in the number of snails in zone-3 attracted by different carbohydrate + amino acid in SAP clearly demonstrates that snails are capable of differentiating type of carbohydrate in the SAP. Snails like other gastropods, are able to detect their food sources by using chemical sense of carbohydrate and amino acid as sign for the presence of their food[16-18]. Gastropod molluscs are attracted to some of the chemical diffusion out from dead and living aquatic organisms in to the modular system of snails[20,22-24,29].

In present study SAP containing starch + histidine +3% limolene shows maximum attraction (54.71%) towards the snail *L. acuminata* after 2h. Significant variation in the number of snails in zone 3 following the addition of the different binary combinations clearly demonstrate that snails are capable of differentiating the different types of combinations in the SAP.

The active molluscicidal component *Ferula asafoetida* (ferulic acid, umbelliferone), *Syzygium aromaticum* (eugenol), *Carum carvi* (limonene)[10,11] caused a time and concentration dependent toxicity against *L. acuminata* after 2h. Snails were less attracted towards the molluscicide containing bait formulation with respect to their control containing only attractant. It shows molluscicides in bait have some repellent action. Active component eugenol, ferulic acid, umbelliferone and limonene are very effective molluscicides when release directly in aquatic environment[10,11]. The bait formulation in the present study is very effective in the snail control programme as they use less amount of molluscicide than their direct release in water. Among all the SAP containing molluscicides umbelliferone and limonene is more effective in killing the snails.

The steep slope value indicates that a small increase in the concentration of different molluscicides caused higher snail mortality. A t-ratio value greater than 1.96 indicates that the regression is significant. Heterogeneity factor values less than 1.0 denote that in the replicate tests of random sample the concentration response curve would fall within the 95% confidence limits and thus the model fits the data adequately. The index of significance of the potency estimation g indicates that the value of the mean is within the limit at all probability level (90, 95 and 99) since it is less than 0.5.

The present study reveals that molluscicides of plant origin could be used with varying degrees of success in bait formulation. This concept is a new approach and technique for the control of harmful snails.

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