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Original article

Behavioural responses of *Lymnaea acuminata* against apigenin, morusin and quercetin in bait pellets.

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ABSTRACT

Snail control plays an important role in control of fasciolosis. In order to achieve this objective the method of bait formulation containing an attractant and a molluscicide is an appropriate approach to ensure good levels of contact between the molluscicide and the target snail populations. In the present study bait pellets were prepared by addition of attractants that also act as molluscicide i.e. apigenin, morusin and quercetin (10 mM), derived from *Morus nigra* bark, leaf and fruit in 2% agar solution. These were used against *Lymnaea acuminata*, an intermediate host of the digenean trematode *Fasciola gigantica*. The behavioural response of snails to these attractants (apigenin, morusin and quercetin) was examined. The fraction of snails that were in contact with the bait pellet in zone-3 was used as a measure of attraction process. Apigenin emerged as the strongest attractant (89.1% after 2h) against *L. acuminata* in comparison to morusin (80.2% after 2h) and quercetin (72.5% after 2h) at 5% concentration in bait. The molluscicidal activity of quercetin (96h LC_{50}- 0.59% in bait) was more pronounced than that of morusin (96h LC_{50}- 1.01% in bait) and apigenin (96h LC_{50}- 1.32% in bait).

1. Introduction

Fasciolosis is an important helminth disease caused by two trematodes *Fasciola hepatica* and *Fasciola gigantica*. This disease belongs to the plant-borne trematode zoonoses. In Europe, the Americas and Oceania only *F. hepatica* is a concern, but the distributions of both species overlap in many areas of Africa and Asia (Mas-Coma et al., 2005). Fasciolosis is well-known disease of livestock for decades (Singh, et al., 1996; Mas-Coma, et al., 2009). This disease ranks as a major cause of morbidity and mortality both in man and livestock and contribute to socio-economic problem (Mas-Coma et al., 2005). Human fasciolosis is an emerging disease (Chen and Mott, 1990; Mas-Coma, et al., 1999) that is characterized by abdominal pain and exceptionally, acute pancreatitis (Saba, et al., 2004; Echenique- Elizondo, et al., 2005). Human infection has been reported in 51 different countries from 5 continents (Estaben et al., 1998). Several attempts have been made to control the vector snail population for solving the problem of fasciolosis (Agarwal and Singh, 1988, Singh, et al., 1996, Shukla, et al., 2006, Jaiswal and Singh, 2009, Kumar, et al., 2010; Srivastava, et al., 2013).

Freshwater snails inhabit an environment containing macrophytes, algae and bacteria (Thomas, 1982). These organisms release different type of chemicals, such as carbohydrates and amino acids, into the surrounding water (Thomas, et al., 1989; Kpikpi and Thomas, 1992). Snails use these chemicals to locate their food sources (Kpikpi and Thomas, 1992, Tiwari and Singh, 2004a, b, Kumar and Singh, 2009, 2010, Kumar, et al., 2011, Kumar, et al., 2012). Use of attractants and molluscicide, in a bait formulation, may be an effective tool for control of snails and ultimately fasciolosis. Bait formulation feeding by snails is safe as less amount of molluscicide will be diffuse in the aquatic environment. Only attracted snails feeding on bait will be killed. The present study assays the behavioural responses of *L. acuminata* towards bait containing apigenin or morusin or quercetin from *Morus nigra* bark, leaf and fruit, respectively. These active components act as attractant as well as molluscicide.

2. Materials and methods

2.1. Collection of snails

Adult *L. acuminata* snails (average length 2.25±0.20 cm) collected locally from lakes and low-lying submerged fields in Gorakhpur, were used as the test animal. The snails were acclimatized for 72h in de-chlorinated tap water at 25±1°C.

2.2. Preparation of bait pellets

Purified apigenin, morusin were isolated from bark, leaf of *M. nigra* by the method of (Liu et al., 2008) and (Tati et al., 2009), respectively. Quercetin was purchased from the Sigma Chemical Co. USA. Active components apigenin, morusin and quercetin (10 mM) each at 0.9%, 1%, 3% and 5% were added in 100 ml of 2% agar solution separately and the bait pellets were prepared by the method of Madsen, (1992), as modified by Tiwari and Singh, (2004 a, b). These solutions were spread at a uniform thickness of 5 mm. After cooling pellets, were cut from the solidified agar with help of 5 mm corer. These pellets were used for the attraction and toxicity determination against *L. acuminata*.

2.3. Assay apparatus and procedure

Chemo-attraction studies of the bait pellets prepared using attractants apigenin, morusin and quercetin having the function of molluscicide too against *L. acuminata* were made in a circular glass aquarium having a 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), having a diameter of 13, 18, 24 and 30 cm, respectively. Zone 0, 1, 2 and 3 had an area of 254.35, 197.82, 121.68 and 132.66 cm^2, respectively. A small annular elevation of 9 mm and 1.5 cm diameter was made in the center of the aquarium (zone-3). The aquaria were then filled with 500ml of de-chlorinated tap water to the height of 8 mm and maintained at 25±1°C. At the start of the assay 10 individually marked snails of uniform size were placed on the circumference of zone-0. The distance between two snails was 66 mm. Simultaneously, the attractant bait pellet was placed in the center of zone-3. The location of each snail was noted every 15 min for 2h. The mortality rates of snails were noted after every 24h up to 96h. Pellets containing each of the three test chemicals were tested, in six replicates, with 10 snails each, at the required concentration were used.

2.4. Data analysis

Proportion of snails was arcsine transformed for each replicate in zone-3. Comparisons for each test chemical at different concentrations were made using one-way ANOVA (Sokal and Rohlf, 1973) lethal values (LC_{50}), lower and upper confidence limits (LCL and UCL), slope values, t-ratio, g-values and heterogeneity factor were calculated using POLO computer programme of Russell *et al*., (1977).

3. Results

Table 1 gives the distribution of *L*. *acuminata* in the 3rd zone around the bait containing purified component of *M*. *nigra* apigenin, morusin and quercetin. All these components act as attractant and molluscicide. Placement of bait in center (zone-3) affected the behaviour of the snails. The effect of attractants in bait on the proportion of snail in zone-3 was analyzed by one-way ANOVA. Four concentrations (0.9%, 1%, 3% and 5%) were taken for each attractant. Number of attracted snails was noted after 1 and 2h from the beginning of experiment. Apigenin showed the highest attraction at every concentration after 1 and 2h in comparison to morusin and quercetin (Table 1). At 0.9% concentration after 1 and 2h apigenin had attracted 36.9% and 38.2%, while morusin 32.8% and 35.5% and quercetin 27.9% and 28.7%, respectively. Bait containing 5% of the apigenin had attraction 63.7% and 89.1% of snails after 1 and 2h, respectively. Morusin attracted 63.2%, 80.2% and quercetin 62.5%, 72.5% after 1h and 2h exposure respectively (Table1).

The molluscicidal activity of morusin used in bait formulation at 24h (24h LC_{50}- 8.48% in bait) was more pronounced than that of apigenin (24h LC_{50}- 9.66% in bait) and quercetin (24h LC_{50}- 10.15% in bait). At 96h exposure toxicity of quercetin was more pronounced (96h LC_{50}- 0.59% in bait) than morusin (96h LC_{50}- 1.01% in bait) and apigenin (96h LC_{50}- 1.32% in bait) (Table 2). The attractants used in the bait pellet caused significant variation ($P < 0.05$) in number of snails reaching in zone-3. After 1h (for apigenin $F_{(4, 25)} = 25.06$, for morusin $F_{(4, 25)} = 22.37$ and for quercetin $F_{(4, 25)} = 20.25$) and for 2h (for apigenin $F_{(4, 25)} = 28.97$, for morusin $F_{(4, 25)} = 25.15$ and for quercetin $F_{(4, 25)} = 21.06$), respectively. Significant negative regression ($P < 0.05$) was observed between exposure time and LC_{50} of treatments in case of toxicity determination.

The slope values were steep and separate estimation of LC_{50} based on each of the six replicates was found to be within 95% confidence limits (Table 2). The t-ratio was higher than 1.96 and heterogeneity factor was less than 1.0. The g-value was less than 0.5 at all the probability levels i.e. 90, 95, 99 (Table 2).

Table 1
Mean number of snail *L*. *acuminata* in zone-3 with the bait pellets of Apigenin, Morusin and Quercetin after 1 and 2h from the beginning of experiment.

Attractants	Time (h)	Concentrations			
		0.9%	1%	3%	5%
Apigenin	1h	1.70±0.35(36.9)	2.01±0.27(43.6)	2.78±0.33(52.7)	3.58±0.40(63.7)
	2h	1.76±0.34(38.2)	2.60±0.29(50.7)	3.99±0.55(69.9)	5.60±0.36(89.1)
Morusin	1h	1.51±0.22(32.8)	1.78±0.47(38.7)	2.36±0.30(47.9)	3.55±0.22(63.2)
	2h	1.66±0.20(35.5)	2.11±0.36(44.7)	3.63±0.22(64.5)	4.82±0.20(80.2)
Quercetin	1h	1.24±0.43(27.9)	1.74±0.35(37.6)	2.13±0.33(45.1)	3.50±0.22(62.5)
	2h	1.28±0.45(28.7)	1.95±0.30(42.9)	3.43±0.54(61.4)	4.20±0.44(72.5)
Control	1h	0.92±0.19(23.9)	0.92±0.19(23.9)	0.92±0.19(23.9)	0.92±0.19(23.9)
	2h	0.87±0.16(22.7)	0.87±0.16(22.7)	0.87±0.16(22.7)	0.87±0.16(22.7)

Values in parentheses indicate the percentages of snails successfully locating bait pellets i.e. snails in zone-3 compared with that failed in their location. Statistically significant ($P < 0.05$) when one-way ANOVA was applied in between different attractants having different concentrations.

Table 2
Toxicity of different bait formulations of molluscicides against the snail *L. acuminata* at different time exposure.

Exposure period	Molluscicides	LC_{50} % in BP	Limits LCL-UCL	Slope Value	t-ratio	g-value	Heterogeneity
24h	Apigenin	9.66	5.51-42.72	1.20±.30	3.99	0.24	0.29
	Morusin	8.48	5.13-28.85	1.26±.30	4.23	0.22	0.26
	Quercetin	10.15	5.35-75.53	1.02±.29	3.54	0.31	0.13
48h	Apigenin	4.71	3.18-11.04	1.14±.27	4.20	0.22	0.19
	Morusin	5.79	3.45-28.05	0.91±.27	3.38	0.34	0.19
	Quercetin	3.24	2.19-7.19	0.96±.26	3.65	0.29	0.14
72h	Apigenin	2.27	1.65-3.38	1.19±.26	4.51	0.19	0.13
	Morusin	1.87	1.35-2.58	1.28±.27	4.80	0.17	0.26
	Quercetin	0.90	0.36-1.34	1.03±.27	3.79	0.27	0.19
96h	Apigenin	1.32	0.90-1.74	1.38±.27	5.05	0.15	0.33
	Morusin	1.01	0.69-1.29	1.69±.29	5.78	0.12	0.44
	Quercetin	0.59	0.24-0.89	1.33±.30	4.42	0.20	0.20

Six sets of experiments were carried out with ten snails in each for every molluscicide used in this study at required concentration. Mortality was determined at every 24h upto 96h. Abbreviation: BP= bait pellet; LCL= lower confidence limit; UCL= upper confidence limit. Significant negative regression ($p < 0.05$) was observed between exposure time and LC_{50} of treatments. T_s= testing significance of the regression coefficient of Apigenin (7.40^{+}), Morusin (8.12^{+}) and Quercetin (5.26^{+}). (+) Linear regression between x and y. (++) Non-linear regression between log x and log y.

4. Discussion

The present study clearly demonstrates that the *L. acuminata* snails were attracted by the baits. There was a significant variation in behavioural response towards the bait containing apigenin or morusin or quercetin. Bait is something that seduces or has the quality to seduce, it is something used to lure animals so that they can be trapped or killed. *Morus nigra* L. (Moraceae) belongs to the genus *Morus* which is widely distributed in Asia, Europe, North and South America and Africa. Mulberry (genus Morus) is an economically important plant used for sericulture, as a feed for the domesticated silkworm, *Bombyx mori* (Awasthi et al., 2004), and has a long history of medicinal use in Chinese medicine as a herbal medicine called "Sang Bai-Pi" (Nomura, 1988). Moisture, ash, protein, lipids, crude fibers, pectin, carbohydrates and computed calorific values of *M. nigra* were proved to be good nutritional source and balanced diet (Ikhtiar et al., 2008). The bark is purgative and is used to expel tape worms (Chopra et al., 1986). The decoction of the leaves possesses blood purifying properties, reduces fever and is diuretic (Kumar and Gupta, 1996). The fruits are one of the constituent of Unani medicine named "Tut-i-aswad" which is used against cancer (Ahmad et al., 1985). Earlier it has been reported (Hanif and Singh, 2012) that direct release of different organic extract and active component (apigenin, morusin and quercetin) of plant *Morus nigra* shows molluscicidal activity against the snail *L. acuminata.*

Apigenin has been reported in the bark of *M. nigra* (Wang et al., 2007). Apigenin is a natural product belonging to the flavones class that is the aglycone of the several naturally-occurring glycosides. It is a yellow crystalline solid. Apigenin may contribute to the chemopreventive action of vegetables and fruits (Ferreira et al., 2006). Morusin has been reported in the leaves of *M. nigra* (Mazimba et al., 2011). Quercetin has been reported in the fruit of *M. nigra* (Ercisli and Orhan, 2007). It is clear from the result section that use of apigenin, morusin and quercetin up to 5% in bait attract more snails (62.5 to 89.1%) with respect to control bait (22.7-23.9%). Apigenin attract more snails. In toxicity study it was noticed that except 24h quercetin was more toxic than apigenin and morusin. Although apigenin attract more snails yet its molluscicidal activity is less than morusin and quercetin. Earlier Singh et al., (1997) has already reported that quercetin is a potent (96h LC_{50}- 65.91 mg/l) molluscicide when directly released in water against *L. acuminata*. According to him quercetin is not toxic at 24h treatment, whereas in present study in form of bait treatment it is toxic in 24h observation. However, present study clearly demonstrates that its use in bait formulation is also very effective (96h LC_{50}- 0.59%) than direct release (96h LC_{50}- 65.91 mg/l) in killing the snail *L. acuminata*.

The steep slope values indicate that a small increase in the concentration of different molluscicides caused high 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 samples the concentration response curves 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 limits at all probability levels (90, 95 and 99) since it is less than 0.5.

In conclusion, it can be stated that molluscicides of plant origin could be used with varying degrees of success in bait formulation. This concept is a new approach for the control of harmful snails. The bait formulation concept would allow snails to be removed selectively with minimal adverse effect on the environment and other biota.

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