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Seasonal variation in toxicity of citral against *Fasciola larva*

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ABSTRACT

Objective: To test whether the larvicidal activity of citral against *Fasciola* varies by season.
Methods: Mortality of *Fasciola larva* in different month of year (2011-2012) in *in vitro* and *in vivo* condition were observed at 2 h, 4 h, 6 h and 8 h exposure of citral.
Results: *In vitro* toxicity of citral against redia was highest in between the June to August (8 h LC_{50}: 2.58-2.62 mg/L), whereas against cercaria 8 h LC_{50} was in between 3.44-2.62 mg/L. Highest *in vivo* toxicity against redia was noted in between June to August (8h LC_{50}: 4.20-5.09 mg/L). The lowest toxicity was observed from November to April. The highest temperature, free carbon dioxide, and lowest pH, dissolved oxygen were observed from June to August.
Conclusions: The present study conclusively shows that varying a biotic factor can significantly alter the *in vitro* and *in vivo* toxicity of citral against sporocyst redia and cercaria larva.

1. Introduction

Fasciolosis is one of the most debilitating zoonotic diseases. The World Health Organization has estimated that 2.4 million people are infected with *Fasciola*, and further 180 million are at risk of infection[1]. Two species *Fasciola hepatica* and *Fasciola gigantica* (*F. gigantica*) are found throughout the world with several outbreaks in humans from many countries[2]. The intermediate host of liver fluke *F. gigantica* is a hermaphroditic mollusc *Lymnaea acuminata* (*L. acuminata*) inhabiting freshwater ponds and ditches. Snail *L. acuminata* serves as intermediate host of *Fasciola* species[3]. Incidence of endemic fasciolosis is very common in the eastern region of the state of Uttar Pradesh in India[4-8]. Development of larval digenetic trematodes is complex process involving initial infection of the snail host by the free-swimming miracidium, its sequent transformation to a parasite primary sporocyst stage, followed by asexual reproduction and release of secondary, sporocyst or redia and finally the eventual formation and release of cercaria the next free-swimming s+tage in the life cycle. One of the possible approaches to control or eradicate fasciolosis is to interrupt the life cycle of the parasitic trematodes by eliminating the larva (sporocyst, redia and cercaria) inside the snail body or killing the host snail. These snails are an important component of aquatic ecosystem. Now it is realized that instead of killing the snails, it is better to kill the larvae inside the snail body with the help of certain plant products. Recently, Sunita

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and Singh have reported that the phytotherapy of snail by diverse chemicals found in different plant products have sufficient larvicidal activity[5]. Natural products are eco-friendly and easily biodegradable. It has been noted by us that active molluscicidal component of *Zingiber officinale* (citral) (*Z. officinale*) have sufficient larvicidal effect against *Fasciola* larva in Sunita and Singh's study[5]. The aim of the present study is to explore the possibility that seasonal change in abiotic factors such as temperature, pH, dissolved oxygen and free carbon dioxide in test water can influence *in vitro* and *in vivo* larvicidal activity of citral against different *Fasciola* larva in infected snails in each month of the year 2011-2012.

2. Materials and methods

2.1. Test materials

Citral (3, 7- dimethyl-2, 6-0 octadienal) (Figure 1) was supplied by Sigma USA. Temperature and pH of water were measured by thermometer and digital pH meters, respectively.

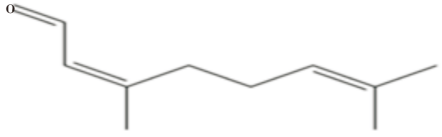

Figure 1. Citral (3, 7- dimethyl-2, 6-0 octadienal).

2.2. Animals

Adult *L. acuminata* [(2.60±0.20) cm in length] were collected locally. Cercaria shedding infected and uninfected snails were separated in two groups. The snails were allowed to acclimatize for 24 h in laboratory condition. Each infected snail was dissected in a glass Petri dish containing 10 mL of dechlorinated water at 22 °C-24 °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. After dissecting sporocyst, redia and cercaria were separated in different Petri dish containing 10 mL of dechlorinated water by the method of Sunita and Singh[5]. These larvae were kept in dechlorinated tap water where they survive up to 48 h in laboratory condition.

2.3. Toxicity determination

2.3.1. In vivo

In vivo toxicity of citral against larvae of *Fasciola* in infected *L. acuminata* was done by the method of Sunita and Singh[5]. Physical parameters of water such as temperature, pH, dissolve oxygen and free carbon dioxide were measured in each month of the year (2011-2012). Dissolved oxygen and CO_2 were estimated according to methods described by American Public Health Association[9]. After 2 h, 4 h, 6 h and 8 h of treatment, infected snails were dissected. Live and dead sporocyst, redia and cercaria were counted. Mortality percent of larvae at each concentration for 2 h, 4 h, 6 h and 8 h were used for determination of LC_{50}.

2.3.2. In vitro

In vitro toxicity of citral was performed in the Petri dish by the method of Sunita and Singh[5]. Ten sporocyst, redia and cercaria larva of *Fasciola* were separated in different Petri dish containing 10 mL dechlorinated tap water. Treatment of citral was made directly in the Petri dish containing 10 sporocyst/redia/cercaria. Mortality of sporocyst, redia and cercaria were observed after 2 h, 4 h, 6 h and 8 h of treatment. Counting of larvae was done with the help of microscope.

Lethal value (LC_{50}), lower and upper confidence limits (LCL and UCL), slop-values, t-ratio, g value and heterogeneity factors were calculated with the help of POLO computer programme of Robertson *et al*[10]. One-way ANOVA and product moment correlation coefficient were done by the method of Sokal and Rohlf[11].

3. Results

In *in vivo* and *in vitro* larvicidal activity of citral against the sporocyst, redia and cercaria larva of *F. gigantica* was time and concentration dependent in each month of year 2011-2012 (Tables 1 and 2). In *in vitro* treatment, highest toxicity of citral was noted against redia and cercaria larva in months of June, July and August (redia-8 h LC_{50}: 2.58, 3.12, and 2.62 mg/L; cercaria-8 h LC_{50}: 3.44, 0.006 and 2.62 mg/L, respectively) and lowest in between September to February (redia-8 h LC_{50}: 6.41, 6.89, 7.74, 7.43, 13.18 and 11.70 mg/L; cercaria-8 h LC_{50}: 4.79, 5.27, 4.64, 6.66, 10.12 and 7.34 mg/L, respectively) (Table 1). In *in vivo* treatment

Table 1

In vitro alteration in toxicity (LC_{50} mg/mL) of citral against *Fasciola* larva (sporocyst, redia and cercaria) in different months of year 2011-2012.

Exposure time	Larvae	March	April	May	June	July	August	September	October	November	December	January	February
2 h	Sporocyst	–	–	–	–	38.71	28.82	82.75	48.13	50.49	–	–	–
	Redia	43.21	44.23	20.42	11.28	10.81	14.62	44.36	37.90	39.02	45.46	53.20	45.73
	Cercaria	39.99	29.95	24.02	10.08	7.88	7.63	22.31	24.00	26.30	45.58	35.72	40.51
4 h	Sporocyst	–	–	–	–	23.48	21.14	55.18	33.58	35.25	–	–	–
	Redia	19.59	21.02	11.68	6.48	7.42	9.45	24.81	22.74	25.41	27.01	40.39	33.54
	Cercaria	19.81	15.82	11.66	6.82	3.25	2.52	11.96	14.42	15.26	25.84	25.84	24.60
6 h	Sporocyst	–	–	–	–	15.13	13.60	30.59	22.19	23.42	–	–	–
	Redia	10.97	9.09	6.32	2.85	3.37	4.04	11.34	12.52	14.22	14.31	24.23	20.51
	Cercaria	8.73	7.60	6.92	4.10	1.96	2.09	7.13	8.40	8.635	13.17	16.49	13.52
8 h	Sporocyst	–	–	–	–	10.04	8.55	14.06	14.72	15.49	–	–	–
	Redia	4.14	5.16	3.62	2.58	3.12	2.62	6.41	6.89	7.74	7.43	13.18	11.70
	Cercaria	6.08	5.79	4.66	3.44	0.006	2.62	4.79	5.27	4.64	6.66	10.12	7.34

Each experiment was replicated six times. Six batches of 15 snails were exposed different concentrations of the above molluscicidal treatments. Mortality of redia was recorded every 2 h. Concentrations given are the final concentration (W/V) in the glass aquarium water. t-ratio was more than 1.96. The heterogeneity factor was less than 1.0. The g-values were less than 0.5. Significant negative regression ($P<0.05$) was observed between exposure time and LC_{50} of treatments. TS-testing significance of the regression coefficient. March- sporocyst/redia/cercaria (0, -7.55^{++}, -6.39^{++}), April (0, -9.92^{++}, -9.34^{++}), May (0, -8.28^{++}, -8.25^{++}), June (0, -2.27^{++}, -0.25^{+}), July (-0.07^{+}, -0.57^{++}, -2.42^{++}), August (-9.10^{+}, -1.39^{+}, 0.74^{+}), September (-47.38^{+}, -3.82^{++}, -1.46^{++}), October (-17.90^{+}, -4.04^{++}, -9.30^{++}), November (-17.81^{+}, -5.14^{+}, -6.63^{+}), December (0, -9.64^{+}, -7.34^{+}), January (0, -20.36^{+}, -4.70^{+}), February (0, -31.89^{+}, -8.62^{++}): linear regression between x and y, $^{++}$: non-linear regression.

Table 2

In vivo alteration in toxicity (LC_{50} mg/mL) of citral against *Fasciola* larva and different abiotic parameters in different months of year 2011-2012.

Exposures LC_{50} mg/mL	Larvae	March	April	May	June	July	August	September	October	November	December	January	February
2 h	Sporocyst	–	–	–	–	25.63*	69.89*	90.18*	55.52*	60.44*	–	–	–
	Redia	59.61*	71.03*	44.98*	21.51*	21.19*	19.74*	44.36*	39.84*	61.96*	66.56*	52.20*	62.14*
	Cercaria	49.03*	59.80*	41.94*	12.66*	11.86*	10.63*	25.57*	31.10*	58.25*	63.84*	48.32*	54.31*
4 h	Sporocyst	–	–	–	–	23.00*	54.51*	67.45*	37.47*	39.91*	–	–	–
	Redia	39.28*	59.37*	35.38*	13.44*	12.37*	9.89*	24.81*	17.38*	36.52*	41.20*	39.43*	47.31*
	Cercaria	24.33*	34.96*	20.57*	7.29*	7.90*	6.22*	17.50*	20.68*	37.10*	41.35*	45.60*	37.28*
6 h	Sporocyst	–	–	–	–	9.23*	27.84*	42.14*	22.94*	23.45*	–	–	–
	Redia	26.29*	41.00*	14.64*	8.72*	7.63*	7.14*	10.06*	10.06*	19.42*	23.42*	30.34*	30.69*
	Cercaria	14.63*	22.43*	18.50*	6.74*	6.97*	5.47*	10.35*	13.70*	21.32*	24.50*	40.32*	21.26*
8 h	Sporocyst	–	–	–	–	1.23*	23.72*	25.40*	14.62*	14.04*	–	–	–
	Redia	13.21*	21.33*	9.68*	5.09*	5.00*	4.20*	7.97*	7.97*	10.52*	13.21*	14.34*	20.12*
	Cercaria	8.65	12.77*	7.26*	5.34*	4.84*	3.27*	7.55*	9.82*	12.34*	14.35*	37.56*	13.33*
Physical parameters	Temperature	20.16±0.30*	22.00±0.25*	22.33±0.33*	34.30±0.33*	27.60±0.21*	24.83±0.30*	24.66±0.42*	22.16±0.30*	10.33±0.33*	10.30±0.33*	11.50±0.34*	19.50±0.22*
	pH	8.16±0.16*	7.56±0.23*	7.46±0.11*	6.04±0.83*	7.10±3.06*	7.09±4.76*	8.01±0.02*	8.08±0.01*	8.08±4.20*	8.75±0.76*	8.95±0.03*	8.92±0.01*
	DO	4.25±0.02*	3.01±5.15*	2.50±5.76*	1.80±5.76*	1.01±5.15*	1.47±0.01*	2.29±4.27*	3.00±6.13*	4.97±0.01*	5.04±0.01*	6.02±0.02*	5.04±0.01*
	CO_2	14.21±0.45*	24.91±0.33*	24.96±0.18*	24.80±0.34*	29.90±0.33*	25.11±0.23*	20.15±0.17*	20.51±0.36*	19.86±0.17*	14.98±0.21*	14.98±0.04*	14.78±0.26*

Each experiment was replicated six times. Temperature, pH, dissolve oxygen and free carbon dioxide were measured at intervals of 2 h, 4 h, 6 h and 8 h. Product movement correlation coefficient in between the LC_{50} and different parameters indicate significant ($P<0.05$) (*) positive correlation. Six batches of 15 snails were exposed different concentrations of the above molluscicidal treatments. Mortality of redia was recorded every 2h. Concentrations given are the final concentration (W/V) in the glass aquarium water. t-ratio was more than 1.96. The heterogeneity factor was less than 1.0. The g-values were less than 0.5. Significant negative regression ($P<0.05$) was observed between exposure time and LC_{50} of treatments. TS-testing significance of the regression coefficient. March- sporocyst/redia/cercaria (0, -1.21^{++}, -11.06), April (0, -6.20_{+}, -9.00^{++}), May (0, -4.79^{+}, -5.15^{++}), June (0, -14.69^{++}, -7.16^{++}), July (-5.93^{+}, -11.58^{+}, -7.17^{++}), August (-13.83^{+}, 0.21^{+}, -9.73^{++}), September (-10.72^{+}, -1.23^{++}, -7.36^{++}), October (-8.54^{+}, -2.68^{+}, -10.08^{+}), November (-8.79^{+}, -6.92^{+}, -9.71^{+}), December (0, -7.98^{+}, -9.39^{+}), January (0, -13.55^{+}, -2.72^{+}), February (0, -16.35^{+}, -6.15^{++}): linear regression between x and y, $^{++}$: non- linear regression.

of citral against *F. gigantica* larva inside the snail was also highest in June, July and August (redia 8 h LC_{50}: 5.09, 5.00 and 4.20 mg/L; cercaria 8 h LC_{50}: 5.34, 4.84 and 3.27 mg/L, respectively). The lowest toxicities were observed in between the November to April (Table 2).

The slope values were steep and separate estimation of LC_{50} based on each six replicate were found to be within the 95% confidence limit of LC_{50}. The t-ratio was greater than 1.96 and the heterogeneity factor is less than 1.0. The g value was less than 0.5 at all probability levels (90, 95 and 99 respectively) (Tables 1 and 2).

4. Discussion

The toxicity of citral against sporocyst, redia and cercaria in *in vitro* and *in vivo* condition significantly altered with respect to change in abiotic factors in different months of the year 2011-2012. Temperature, pH, dissolved oxygen and free carbon dioxide are important

factors, which alter the toxicity of citral during each month of the year 2011–2012. When the water temperature is higher in the summer months (June to August), the toxicity of citral is highest. Contrarily, in the winter season the temperature of water is low and the toxicity of citral is less, as evident by a higher LC_{50} value in both *in vitro* and *in vivo* treatments. Possibly citral become more soluble at high temperature, free carbon dioxides, causing more larval (sporocyst, redia and cercaria) mortality inside the body of treated snails. However, dissolved oxygen is also one of the factors that alter the toxicity of citral. In winter, water holds more oxygen Waterwatch Australia[12], and as a result, less mortality of larvae occurs during this period. The anthelmintic activity of ethanol extracts of rhizomes of *Z. officinale* against human *Ascaris lumbricoldes* is appreciable noted by Raj[13,14]. Goto *et al*. reported the lethal effect of *Z. officinale* on *Anisakis* larvae in *in vitro* treatments[15]. The anti filarial effect of *Z. officinale* against *Dirofilaria immitis* has been reported by Datta and Sukula[16]. Adewunmi *et al*. and Singh *et al*. have reported the molluscicidal activity of *Z. officinale*[17,18]. They have also reported that the active molluscicidal component in *Z. officinale* is citral. It has been speculated that the mechanism of action of ginger may be both central and peripheral *i.e*. ant cholinergic and antihistaminic[19]. Citral used as larvicidal to kill *Fasciola* larva in snail body[5]. At higher temperature, the increasing rate of snail metabolism release more CO_2 which effect the pH of water[20,21]. This was evident from the elevated concentration of CO_2 which decreases the pH of water during summer season. Trematode parasite is highly sensitive to abiotic factors. However, increasing problems of development of resistance in helminthes Greek and Dorny against anthelmintic have led to the proposal of screening medicinal plants for their anthelmintic activity[22]. A number of medicinal plants have been used to treat parasitic infection in man and animals[23]. Present study clearly demonstrates that different larval stages in the snail's body as well as outside of snail body can be killed without killing the snails. Earlier it has been reported that citral (24 h LC_{50}: 68.95 mg/L) is the active molluscicides against *L. acuminata*[18]. 8 h LC_{50} of citral used against sporocyst, redia and cercaria larva is many time lower than used to kill the *L. acuminata*.

The concentration used to kill sporocyst, redia and cercaria in not toxic to snail, even in 24 h exposure period. So that use of citral in killing the sporocyst, redia and cercaria of *F. gigantica* within or outside snail body directly, without killing the host snail is important. Snails are crucial component of aquatic ecosystem. The present study is a direct phytotherapy of snails to control fascioliosis. In *in vivo* and *in vitro*, killing of sporocyst, redia and cercaria of *F. gigantica* is beneficial as it kills directly target larva of *F. gigantica*. Generally, citral inhibits activity of acetylcholinesterase, acid/alkaline phosphates and ATPase in the nervous tissue of *L. acuminata*[24,25]. Earlier it has been reported the functioning of acetylcholinesterase and cytochrome oxides system in cercaria for efficient release of energy[26,27]. To elucidate the mechanism of the larvicidal activity by citral, their action on acetylcholinesterase/cytochrome oxidase is required in further study.

The steep slope value indicates that a small increase in the concentration of larvicidal caused higher larval mortality in different months of year 2011–2012 in respect of physical parameter. A t-ratio value greater than 1.96 indicates that the regression is significant. Heterogeneity factor value less than 1.0 denote that in the replicate test of random sample the concentration responds limits and thus the model fits the data adequately. The index of significance of the potency estimation g indicates that the value of mean is within the limit at all probability level (90, 95 and 99, respectively) since it is less than 0.5.

It can be concluded from the present study that sublethal treatment of citral significantly killed the sporocyst, redia and cercaria larva of *F. gigantica* inside the body of vector snail *L. acuminata*. Phytotherapy of infected snails by citral is one of the new approaches to control the fascioliosis. The high temperature, pH, free carbon dioxides and low dissolved oxygen were observed in the month of the June– August. The variant abiotic factor can significantly alter the *in vitro* and *in vivo* toxicity of citral against *Fasciola* larvae.

Conflict of interest statement

We declare that we have no conflict of interest.

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