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Anti-reproductive Activity of Chlorophyllin on Fresh Water Snail *Lymnaea acuminata*

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ABSTRACT

The effects of Sub-lethal treatments (20 and 60% of 24 h LC_{50}) of chlorophyllin on the reproduction and development of *Lymnaea acuminata* has been studied. There was a significant decrease in the fecundity, hatchability and survival of the young snails. Treatment with 60% of 24 h LC_{50} of chlorophyllin caused minimum fecundity (57 eggs/20 snails, 48 h) in summer (sunlight). After 48 h no egg laying and hatching was noted in snails exposed to 60% of 24 h LC_{50} of chlorophyllin in both winter (sunlight) as well as in summer (sunlight). Development could not proceed beyond the veliger stage due to the death of the embryo. The developmental arrest was noticed in many egg strings leading to the high rate of mortality and there by reduction in hatchability of the eggs. Growth rate of survived snails was also very slow. This present study clearly shows that the efficacy of chlorophyllin gives significant control of reproduction of the snails even at sub-lethal exposure. The use of the natural products would have an added advantage against aquatic snails as it would be non toxic to the non target animals and less hazardous to the environment.

Key words: Fasciolosis, chlorophyllin, reproduction, fecundity, hatchability, survival, snail

INTRODUCTION

Fasciolosis, a disease caused by two species of *Fasciola* viz *F. gigantica* and *F. hepatica* is cosmopolitan in occurrence (Singh *et al*., 2012). Parasitic flat worms found in large number in bile duct of infested cattle sheep and other mammal (Mas-Coma *et al*., 2009). The liver-fluke passes to eggs in water bodies where it develops into miracidium larva, which penetrate the soft body of snail (Lymnaeidae and Planorbidae) and reaches to lymph sinuses. In lymph sinuses it metamorphosed into cercaria larva and come out from snail body and infect primary host the mammal (Agarwal and Singh, 1988; Mas-Coma *et al*., 2009; Singh *et al*., 2012). One single miracidium may produce 4000 infective larvae (metacercaria) (Boray, 2005). There are a large number of snails as well as miracidium in water body, so that there is a good chance of multiplication of *Fasciola* through snail host *L. acuminata*. Aquatic gastropods generally deposit their egg in gelatinous masses that are attached to a hard surface. The reproduction rate of snail under favorable condition is very high and theoretically, one surviving snail can produce one million snails in 60 days (Boray, 2005). Earlier studies have shown that the reproductive capacity of snail significantly altered from one season to other (Ter Maat *et al*., 1983; Wayne, 2001; Jigyasu *et al*., 2010; Srivastava and Singh, 2015). Ovipositions in snails are induced by

neuroendocrine hormone of caudo-dorsal cells (CDCs) in the cerebral ganglion (Ter Maat *et al*., 1982; Singh *et al*., 2008; Srivastava and Singh, 2015). An effective method to reduce the incidence of fasciolosis is to control the population of vector snails, there by breaking the life cycle of flukes, or by reducing the reproductive capacity of snails (Godan, 1983; Agarwal and Singh, 1988; Singh *et al*., 1996, 2012). Several attempts have been made to reduce the incidence of fasciolosis by using synthetic and plant derived molluscicides against snail (Singh and Agarwal, 1983; Upadhyay and Singh, 2011; Singh *et al*., 2012, 2014; Hanif and Singh, 2013; Kumar *et al*., 2013, 2014; Kumar and Singh, 2014). Recently, it has been reported that chlorophyllin is potent molluscicides (Singh and Singh, 2015). It is considered to be more advantageous in snail control program if, molluscicides besides killing snails also reduce the reproductive capacity even at sub-lethal concentration. The aim of present study is to evaluate the effect of sub-lethal exposure of chlorophyllin on the fecundity, hatchability and survival of young snails of *Lymnaea acuminata* in summer and winter season in the year 2014-15.

MATERIALS AND METHODS

Preparation of chlorophyllin from extract of spinach: Chlorophyllin was prepared by the method of Wohllebe *et al*. (2011) as modified by Singh and Singh (2015). Chlorophyll was extracted from spinach with the help of macerated leaves and kept for 2 h in 100% ethanol at 55°C. To avoid transformation of chlorophyll into pheophytin by the acidic content of the cell vacuoles 1.0 mg $CaCO_3$ per gram leaves were added as a buffer. The extract was subsequently filtered and equal volume of petroleum benzene was added. After shaking the mixture the chlorophyll moved into the lipophillic benzene phase. The two phases were separated in separatory funnel and about 1 mL methanolic KOH was added to 50 mL of the benzene phase. The chlorophyll came into contact with the methanolic KOH and was transformed into water soluble chlorophyllin (this process occurs due to the breakage of the ester bond between the chlorophyllin and the phytol tail by saponification).

Fecundity experiment: Sexually mature *L. acuminata* of average size (2.25±0.30 cm in length) were collected locally from ponds, pools and lakes of Gorakhpur district India. These snails were kept in glass aquarium containing dechlorinated tap water for 72 h to go acclimatization. The snail *L. acuminata* lays eggs in the form of elongated gelatinous capsules (egg masses or egg strings) on the lower surface of leaves of aquatic vegetation. These egg masses may have 2-4 rows of eggs with number of egg ranging from 5 up to 200 or sometimes even more. Groups of 20 snails kept in glass jars containing 3 L of dechlorinated tap water and *Nelumbo nucifera* leaves (egg laying surface) were exposed separately to sublethal concentration (20 and 60% of 24 h LC_{50}) of chlorophyllin. The experiment was conducted in summer season (March to June) and winter season (November to February) in the year of 2014-15. The total number of egg masses and eggs laid by the groups of snails were counted every 24-96 h.

As it is difficult to identify the mother snails for a particular spawn, capsule containing eggs from each treated groups were separated carefully with the help of scalpel from the lower surface of lotus leaves in covered petridishes containing the same concentration as given to adult snails. The development of embryos was observed under microscope up to their hatching and the duration of each stage and hatching period was noted. As the eggs were transparent and surrounded by mucus string, changes taking place up to the time of hatching could be observed directly. The egg capsules besides conveniently permitting observations of growing embryos also act as a diffusion

barrier and prevent passage of large molecules. Hatchability was studied with the eggs laid after 24 h exposure. Dead embryos lacks embryonic movement and become opaque were removed to avoid any contamination. Immediately after hatching the miniature snails were transferred to fresh water. The snail's survival was observed up to 72 h.

Statistical analysis: A student t-test was applied to determine the significant ($p<0.05$) differences between treated and control animals. Product moment correlation coefficient was applied between exposure time and fecundity survival of snails by the method of Sokal and Rohlf (1995).

RESULTS

In control groups each snails laid egg per day and each 20 snails laid 200-251 eggs per day. There was a significant ($p<0.05$) reduction in the fecundity of snail *L. acuminata* on the treatment of 20 and 60% of 24 h LC_{50} of chlorophyllin. No egg laying after 48 h, it was observed in snails to 60% of 24 h LC_{50} of chlorophyllin in winter as well as in summer (Table 1). In the present result 2-3 embryo were observed in some eggs. The data on the morphological observation i.e., fecundity, different developmental periods, hatchability and survivability of young snails in *L. acuminata.* A decrease in the number of egg masses and its size was observed with the increase in exposure time and the number of eggs per egg masses was also reduced. Effect of these treatments could also be seen on the duration of different stages of development hatching time and hatchability of the snails. The duration of the larval stages in treated groups was increased in comparison to control i.e., the embryos in treated groups experienced delay in development compared to the controls without treatment.

In the controls morula stage lasted for 1 day, trochophore 1-2 days, veliger 2 days and hippo 2-3 days while in treated snails morula stage extended up to 3 days, trochophore 3-4 days, veliger 5-6 days and hippo 5-7 days. The hatching time was prolonged in the treated groups (16-19) days with respect to the control group 7-9 days. Eggs exposed to 60% of chlorophyllin failed to hatch due to the death of the embryo after the veliger stage.

After hatching the treated miniature snails, shell was very thin and tentacles were smaller in comparison to the control groups. Also their movement was slow than the control snails. Survival of these snails was observed for about 72 h and the survivability progressively decreased with time.

Table 1: Effect of sublethal concentration (20 and 60% of 24 h LC_{50}) of chlorophyllin kept in sunlight on the no. of spawns and fecundity of snail *Lymnaea acuminata* in winter and summer season

Molluscicides/ season and treatment (mg L^{-1})	No. of spawns (h)				Fecundity (No. of eggs of 20 snails) (h)			
	24	48	72	96	24	48	72	96
Winter chlorophyllin (sunlight)								
Control	+6.66±0.20*	6.16±0.16*	5.33±0.20*	4.50±0.22*	+251.33±0.49*	150.66±0.33*	101.00±0.81*	56.66±2.77*
20% of 24 h LC_{50} (19.11 mg L^{-1})	+6.33±0.20*	5.50±0.22*	4.50±0.22*	3.16±0.30*	+179.50±0.98*	78.33±1.76*	70.66±0.49*	52.33±1.04*
60% of 24 h LC_{50} (57.33 mg L^{-1})	+5.16±0.16*	4.91±0.21*	0	0	+72.00±1.02*	59.50±0.98*	0	0
Summer chlorophyllin (sunlight)								
Control	+6.16±0.16*	5.66±0.20*	5.16±0.16*	4.00±0.20*	+201.50±0.75*	140.16±2.48*	82.66±1.91*	28.33±3.33*
20% of 24 h LC_{50} (10.55 mg L^{-1})	+5.33±5.20*	5.16±0.24*	0	0	+123.33±1.10*	70.00±1.14*	0	0
60% of 24 h LC_{50} (31.36 mg L^{-1})	+5.12±0.26*	4.00±0.25*	0	0	+62.00±0.88*	57.00±0.67*	0	0

Each value is Mean±SE of six replicates, each replicate represent the No. of spawn/egg laid by a group of 20 snail, 0: No spawns and egg laying by a group of 20 snail was observed, *$p<0.05$ significant when student t-test was applied to treated and control groups, (+) Product moment correlation coefficient showed that there was significant negative correlation between LC_{50} of treatment and no. of spawns and fecundity of *L. acuminata*

Table 2: Effect of sublethal concentration (20 and 60% of 24 h LC_{50}) of chlorophyllin kept in sunlight on the fecundity, hatchability and survival of snail *Lymnaea acuminata* in winter and summer season

Molluscicides/ season and treatment (mg L^{-1})	Fecundity after 24 h (No. of eggs/20 snails)	Hatchability (hatching periods in days) (%)	Survival (%)		
			24 h	48 h	72 h
Winter chlorophyllin (sunlight)					
Control	+251.33±0.49*	100.00 (7-9)	100.00	100.00	100
20% of 24 h LC_{50} (19.11 mg L^{-1})	+179.50±0.98*	+92.00±1.14* (13-15)	+87.31±0.82*	69.18±2.97*	52.87±1.56*
60% of 24h LC_{50} (57.33 mg L^{-1})	+72.00±1.02*	+84.51±0.95* (16-19)	+80.77±2.04*	58.96±2.37*	0
Summer chlorophyllin (sunlight)					
Control	+201.50±0.75*	100.00 (7-9)	100.00	100.00	100
20% of 24 h LC_{50} (10.55 mg L^{-1})	+123.33±1.10*	+88.20±2.87* (15-16)	+78.90±1.63*	50.97±3.83*	0
60% of 24 h LC_{50} (31.36 mg L^{-1})	+62.00±0.88*	+83.12±3.31* (16-19)	+71.48±1.90*	47.09±1.93*	0

Each value is Mean±SE of six replicates, each replicate represent the egg laid by a group of 20 snail, 0: No hatchability and survivability was observed, *p<0.05 significant when student t-test was applied to treated and control groups, (+) Product moment correlation coefficient showed that there was significant negative correlation between LC_{50} of treatment and fecundity, hatchability and survival of *L. acuminata*

There was a significant (p<0.05) negative correlation between exposure period and survival of the young snails hatched from the eggs laid by treated snails. Maximum decrease in survivability was noticed in the snails exposed to 60% of 24 h LC_{50} of sunlight chlorophyllin in summer (Table 2). The growth rate was also very slow.

DISCUSSION

It is evident that sublethal exposure of chlorophyllin significantly reduced the fecundity of *Lymnaea acuminata*. The result from this study shows that sunlight exposure and intensity alter the fecundity, hatchability and survival of snails. In the summer season (March to June) the temperature of the water is high (32-40°C) and intensity 1200 W m^{-2}. When the temperature of the water increased above 35°C i.e., in the month of June, there was a marked decrease in the fecundity. An earlier study has shown that the decrease in the temperature from 20-8°C stopped the oviposition of the snail *Lymnaea stagnalis* because of a reduction in the activities of neurosecretory cells (CDCs) (Wayne, 2001; Srivastava *et al*., 2013; Srivastava and Singh, 2015). Chlorophyllin is more effective in sunlight as it is photodynamic product which produces ROS which cause oxidative stress to different target cell such as lipid, protein and DNA (Kohen and Nyska, 2002). In summer season the intensity of sunlight (1200 W m^{-2}) is much more than winter (900 W m^{-2}) and temperature is also noted very high in comparison to winter (>35°C). So the fecundity observed in 60% of 24 h LC_{50} of chlorophyllin in summer sunlight is minimum (57 eggs/20 snails). Changes in abiotic factors (salinity, dissolved oxygen, temperature etc.) as a consequences of change in seasons may therefore influence normal metabolic activities of organisms and the induction of oxidation stress as a consequence of increased generation of Reactive Oxygen Species (ROS) (Van der Oost *et al*., 2003; Ognjanovic *et al*., 2008; Malanga *et al*., 2009). The ROS can be highly toxic to aquatic organisms including molluscs after resulting in oxidation of lipid in membranes, protein and nucleic acids, polysaccharides and inhibition of vital enzymes (Mates, 2000; De Almeida *et al*., 2007; Tripathi *et al*., 2010).

Reduced hatchability of *L. acuminata* exposed to different plant molluscicides is due to interference with embryonic growth and development of snails. In the treated group, egg masses swelled and turned somewhat viscous. The color of the egg capsules in the control group was dark cream but changed to white in the treated samples (Bhide *et al*., 1998). The maximum decrease in the hatchability is observed in 60% of 24 h LC_{50} (83.12% of control) of chlorophyllin in summer with exposure of sunlight. Time dependent reduction in the survival of newly hatched snails, even after transfer to fresh water, indicates that chemical received either from mother snail or in the eggs is

lethal to young newly hatched snails. It is reported that the thin and fragile shell of newly hatched snails in the treated group is due to decalcification as observed in cobaltous sulfate-treated *Planorbis exustus* and thiourea treated *L. acuminata* (Sherbert and Lakshmi, 1964). Erzinger *et al*. (2011) was demonstrated that mosquito larva and cercaria larva can be killed by photosensitizer chlorophyllin in light. Recently, Singh and Singh (2015), observed the larvicidal activity of chlorophyllin with different wavelengths of visible light against *Fasciola gigantica*. The minimum survivability is observed in 60% of 24 h LC_{50} (47.09% of control) of chlorophyllin in summer season (sunlight). In summer water temperature is higher and dissolve oxygen concentration is low, which pose higher mortality of snails. Due to their water solubility, it is accumulated in the intestine of exposed mosquito larvae which immediately cause cell death. Mortality and low reproduction in the treated snails suggest that the plant molluscicide chlorophyllin were able to control the population of snail *L. acuminata* by altered the reproductive capacity and inhibiting development and growth of young ones.

It has been observed that photodynamic molluscicide chlorophyllin used in the control of the snail *L. acuminata* are very effective in the summer (sunlight). These treatments are not only effective in killing the snails but also possess a capability of making them sterile. Besides this it also kills the eggs, causes death of the embryo during developmental stages thereby inhibiting its hatching and increase the mortality of the hatched miniature snails.

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