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ORIGINAL ARTICLE

ANTHELMINTIC ACTIVITY OF CHLOROPHYLLIN AGAINST DIFFERENT LARVAL STAGES OF *Fasciola gigantica*

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SUMMARY

Fasciolosis is a food borne zoonosis, caused by the digenetic trematode *Fasciola*. Freshwater lymnaeid snails are the intermediate host of the trematodes. Chlorophyllin, a semi-synthetic derivative of chlorophyll and its formulations obtained from freeze dried cow urine (FCU) had their toxicity tested against redia and cercaria larvae of *F. gigantica*. The larvicidal activity of chlorophyllin and its formulations were found to depend on both, time and concentration used against the larvae. Toxicity of chlorophyllin + FCU (1:1 ratio) in sunlight against redia larva (8 h LC_{50}: 0.03 mg/mL) was more pronounced than using just chlorophyllin (8 h LC_{50}: 0.06 mg/mL). Toxicity of chlorophyllin + FCU in sunlight against redia (8 h LC_{50}: 0.03 mg/mL) was higher than against cercaria (8 h LC_{50}: 0.06 mg/mL). The larvicidal activity of chlorophyllin in sunlight (redia/cercaria larvae: 8 h LC_{50}: 0.06 mg/mL) was more pronounced than under laboratory conditions (redia: 8 h LC_{50}: 22.21 mg/mL/, cercaria 8 h LC_{50}: 96.21 mg/mL). Toxicity of FCU against both larvae was lower than that of chlorophyllin and chlorophyllin + FCU. Chlorophyllin and its formulations + FCU were 357.4 to 1603.5 times more effective against redia/cercaria larvae in sunlight than under laboratory conditions. The present study has shown that chlorophyllin formulations may be used as potent larvicides against fasciolosis.

KEYWORDS: Cercaria; Chlorophyllin; *Lymnaea acuminata*; Redia; *Fasciola gigantica*.

INTRODUCTION

Fasciolosis is a well-known zoonotic disease. The snail *Lymnaea acuminata* is the intermediate host of the liver flukes *Fasciola gigantica*[1], which causes endemic fasciolosis in the cattle population of the Eastern region of the state of Uttar Pradesh in India[2]. Human fasciolosis has been reported in 51 countries from five continents[3]. Human fasciolosis in the last two decades has changed its current status from a zoonosis to an emerging health problem[1,4]. An obvious solution to reduce the incidence of fasciolosis is to destroy the vector snails[5] or to kill the larvae of *Fasciola* inside the snail, therefore interrupting the life cycle of *Fasciola*[6]. Killing of the *Fasciola* larvae in the body of the snail without killing the snail will be a new tool in fasciolosis control.

Bioactive plant products have been given much attention because they are ecologically safe and culturally more acceptable than their synthetic counterparts[5]. The chlorophyll derivative chlorophyllin is gaining widespread acceptance among researchers as a natural larvicide, which is based on its photodynamic activity[7,8]. Erzinger[7] has reported that chlorophyllin is extremely toxic against mosquito larvae in sunlight. Chlorophyllin is the most widely used plant product against larvae of insects[3,8,9,10]. Chlorophyllin is known to have larvicidal activity, which cause necrosis/apoptosis in *Chaoborus crystallinus* larvae[9] and it is apparently effective against certain parasites of fish[9,11]. Abdel Kader & El-Tayeb[12] recommended that chlorophyll derivatives can be successfully applied to control vector borne diseases such as malaria, filariasis and dengue. Earlier, Tripathi *et al*.[13] noted that freeze dried cow urine (FCU) kept for 15 days in sunlight is a potent molluscicide against the vector snail *L. acuminata*.

Snail *L. acuminata* is the intermediate host of *Fasciola gigantica* in Eastern Uttar Pradesh, India[14]. Although snail control is one of the best methods to control fasciolosis, snails are still a bioindicator[15], as well as an important component of the aquatic ecosystem. The frequent use of molluscicides in the aquatic ecosystem for snail control also affects the non target organisms sharing the same habitat. Redia and cercaria larvae of *F. gigantica* are different developmental stages in the life cycle. If these larval stages will be killed by biolarvicides at sublethal concentrations inside the snail body, the incidence of fasciolosis can be reduced without killing the snails.

The present investigation reports *in vitro* larvicidal activity of chlorophyllin and FCU against redia and cercaria larvae of *F. gigantica*.

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MATERIALS AND METHODS

Animals

Adult *Lymnaea acuminata* snails (average length 25-27.2 mm), collected locally from lakes and low-lying submerged fields in Gorakhpur, were used as the test animals. Cercariae shedding infected snails were separated. The snails were kept in aquarium water for 24 hours, under laboratory conditions. Each infected snail was dissected in a glass Petri dish containing 10 mL of dechlorinated water at 23-24 °C by using the method of Sunita & Singh[6]. After opening the mantle of the snails a large number of redia and cercaria larvae emerged from the body of snails and they were placed in the Petri dish.

Preparation of chlorophyllin

Chlorophyllin was prepared by the method of Wohllebe *et al*.[10]. Chlorophyll was isolated from spinach using 100% ethanol (for about 2 h at 55 ^{0}C). To avoid the transformation of chlorophyll into pheophytin by the acid content of the cell vacuoles, 1 mg of $CaCO_3$/g of plant material was added as a buffer. The extract was subsequently filtered and petroleum benzene was added. After the homogenization of the mixture, the chlorophyll has turned into the lipophilic benzene phase. The two phases were separated using a separatory funnel and about 1.0 mL of methanolic KOH was added to the 50 mL of benzene phase. Using agitation, the chlorophyll has contacted the methanolic KOH and has been transformed into water soluble chlorophyllin by saponification and the consequent cleavage of the ester bond located between the chlorophyllin and the phytol tail. The phytol tail is responsible for the lipophilic property of the chlorophyll. Chlorophyll is found as chlorophyllin in the KOH phase.

Preparation of freeze dried cow urine (FCU)

Freeze dried cow urine was collected and prepared by the method of Tripathi *et al*.[13]. Geer cow urine was kept in sunlight (8 h/day), under laboratory conditions, for 15 days. After 15 days the FCU sample was freeze dried in a lyophilizer. Freeze dried fractions of the cow urine were used in w/v treatment.

Toxicity determination

In vitro: *In vitro* toxicity experiments were performed by the method of Sunita & Singh[6]. Six Petri dishes were set for each concentration of chlorophyllin formulations. Ten experimental larvae (redia/cercaria) were kept in different Petri dishes containing 10 mL of dechlorinated tap water. Treatment of different chlorophyllin formulations were made directly in the Petri dishes that were kept in the dark for 4 h. Thereafter, these Petri dishes were exposed to normal daylight in laboratory conditions, and outside directly exposed in sunlight. Mortality of larvae was observed after 2 h, 4 h, 6 h, and 8 h of treatment. The number of dead and live larvae was recorded by using a stereomicroscope. No locomotion was considered as dead larvae. The cross checking of cercariae mortality was examined by the use of the vital stains viz Janus green B and Neutral red. In the control group I, no chlorophyllin treatment was given and samples were kept in sunlight. In the control group II, chlorophyllin treatment was given and samples were kept in the dark. In the control group III, no chlorophyllin treatment was given and samples were kept in the dark. In all the control groups, chlorophyllin concentration was the same as used in the corresponding treated group. Concentration-mortality data for each group of larvae were analyzed using the probit analysis program, POLO-PC (LeOra Software) by Robertson *et al*.[16] to estimate the LC_{50} of chlorophyllin formulations and the 95% confidence intervals for these concentrations. The slope of probit lines was also estimated. This program performed the chi-square test to estimate the data goodness-of-fit for the probit model. If the model fits, the calculated chi-square value is lower than the chi-square table value considering the appropriate degree of freedom. If the model does not fit, the LC_{50} value for the particular population may not be reliably estimated and is adjusted according to the heterogeneity factor (observed chi-square values divided by the degrees of freedom). This program uses the heterogeneity factor as a correction factor when the Pearson's chi-square statistic value is significant at $p = 0.05$. The index of significance for potency estimation (g-value) was used to calculate the potency 95% confidence intervals (relative potency is equivalent to the tolerance ratio). The parallelism of the probit regression lines implies a constant relative potency at all levels of response. POLO-PC was used to test the equality and the parallelism of the probit lines slope. The coefficient analysis regression between the exposure time and different values of LC_{50} was determined by the method of Sokal & Rohlf[17].

RESULTS

In vitro toxicity of chlorophyllin and its different FCU formulations against redia/cercaria were dependent on time and concentration. Toxicity of chlorophyllin + FCU in sunlight was higher against redia than against cercaria larvae, whereas toxicity of chlorophyllin against both the larvae was the same (Fig. 2). Laboratory treatments with chlorophyllin, chlorophyllin + FCU and FCU alone were more toxic against redia larva than against cercaria (Fig. 1).

The rediacidal activity of chlorophyllin (8 h LC_{50}: 0.06 mg/mL), chlorophyllin + FCU (8 h LC_{50}: 0.03 mg/mL) and FCU (8 h LC_{50}:0.11

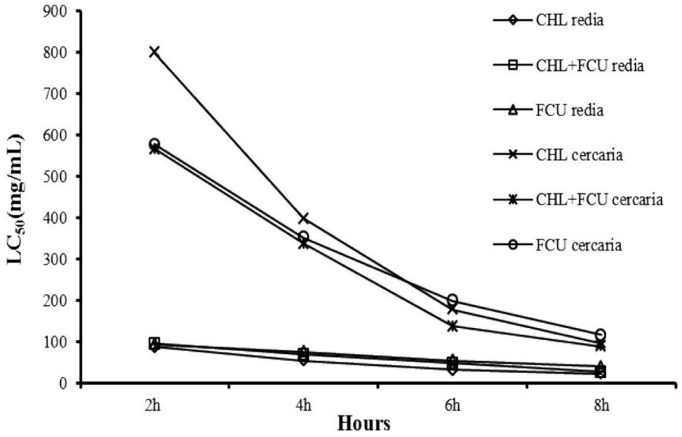

Fig. 1 - *In vitro* toxicity (LC_{50}: mg/mL) of different chlorophyllin formulations (CHL), freeze dried cow urine (FCU) against redia and cercaria larvae of *F. gigantica* under laboratory conditions. The concentration given is the final concentration (W/V) in glass aquarium water. A significant negative regression ($p < 0.05$) was observed between the exposure time and LC_{50} of formulations against redia. Ts-testing - significant regression coefficient: chlorophyllin- 8.59^{++}, chlorophyllin + cow urine- 21.11^{+}, cow urine- 22.55^{+}. + Linear regression between X and Y. ++ Non- linear regression between log X and Y. Cercaria (Ts- testing - significant regression coefficient), chlorophyllin - 7.51^{++}, chlorophyllin + cow urine - 5.84^{++}, cow urine - 6.79^{+}. + Linear regression between X and Y; ++ Nonlinear regression between log X and log Y.

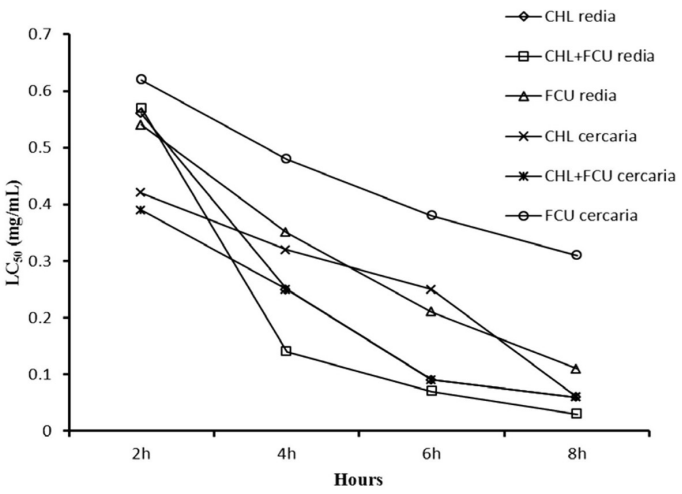

Fig. 2 - *In vitro* toxicity (LC_{50}- mg/mL) of different chlorophyllin formulations (CHL), freeze dried cow urine (FCU) against redia and cercaria larvae of *Fasciola gigantica* in sunlight. The concentration given is the final concentration (W/V) in the glass aquarium water. A significant negative regression ($p < 0.05$) was observed between exposure time and LC_{50} of formulations against redia. Ts-testing -significant regression coefficient: chlorophyllin - 16.62^{++}; chlorophyllin+ cow urine - 8.54^{++}; cow urine-10.03^{+}. $^{+}$ Linear regression between X and Y; ++ Nonlinear regression between log X and log Y. Cercaria (Ts-testing - significant regression coefficient): chlorophyllin - 6.47^{+}; chlorophyllin+ cow urine - 5.64^{+}; cow urine - 9.30^{++}. + Linear regression between X and Y; ++ Nonlinear regression between log X and log Y.

mg/mL) in sunlight was 740.3, 442.2 and 357.4 times higher than under laboratory conditions (8 h LC_{50}: 22.21 mg/mL, 8 h LC_{50}: 26.53 mg/ mL, 8 h LC_{50}: 39.32 mg/mL), respectively (Figs. 1and 2).

The cercaricidal activity of chlorophyllin (8 h LC_{50}: 0.06 mg/mL), chlorophyllin + FCU (8 h LC_{50}: 0.06 mg/mL) and FCU (8 h LC_{50}: 0.31 mg/ mL) were 1603.5, 1460.6 and 374.6 times higher than under laboratory conditions (8 h LC_{50}: 96.21 mg/mL). No mortality of redia and cercaria larvae was observed in control group I (no chlorophyllin treatment and samples kept in sunlight), II (chlorophyllin treatment was given and samples were kept in the dark) and III (no chlorophyllin treatment and samples kept in the dark).

The slope values were steep and found within the 95% confidence limits of LC_{50}. The t-ratio was higher than 1.96 and the heterogeneity factor was less than 1.0. The g-value was less than 0.5 at all probability levels, i.e., 90, 95 and 99. There was a significant negative regression ($p < 0.05$) between the exposure time and the LC_{50} of treatments.

DISCUSSION

The results of the present study have clearly demonstrated that the larvicidal activity of chlorophyllin and FCU formulations is time and concentration-dependent, as evidenced by the negative regression between the exposure period and LC_{50}. Sunlight-exposed chlorophyllin transfers its excitation energy to oxygen and produces singlet reactive oxygen species. These products have the potential to kill the developmental stages of pests/vectors[18, 19]. The *in vitro* treatment of chlorophyllin with FCU in laboratory/sunlight caused significant mortality of *Fasciola* larvae. The larvicidal activity of sunlight treated chlorophyllin+FCU is hundred times more toxic than treatment under laboratory conditions. This difference may be due to a higher production of singlet oxygen in light-exposed chlorophyllin, which can easily cross the outer covering of redia and cercaria in sunlight[20,21,22]. Abdel-Kader *et al*.[23] and Erzinger *et al*.[8] noted that chlorophyllin can exclusively kill mosquito larvae, while other organisms sharing the same habitat were not affected. Erzinger *et al*.[8] reported that the chlorophyllin uptake by mosquito larvae is higher at high temperatures but chlorophyllin does not influence the photodynamic toxicity. The incidence of fasciolosis infection is higher in the summer. Sunita *et al*.[24] and Singh *et al.*[14,25] have shown that the number of *F. gigantica* larvae in host snails is maximum when the water temperature is higher during the summer. Consequently, there is a higher infection index of *Fasciola* larvae and also a higher uptake of chlorophyllin in warm waters during the summer, resulting in a higher photodynamic toxicity against *Fasciola* larvae. Externally applied chlorophyllin was toxic against *Culex and Chaoborus* larvae at concentrations of 6.88 and 24.18 mg/L, respectively[9]. The chlorophyllin formulation required to kill 50% of redia and cercaria larvae was 0.03 and 0.06 mg/10mL, respectively. When chlorophyll derivatives were applied on 250,000 m^2 of infected swamps and sand pits, a 0.1-100 µM concentration of chlorophyllin has killed 85-100% of *Anopheles gambiae* larvae[12]. The chlorophyllin treatment against *Aedes* (2.34 mg/L) and *Anopheles* (5.88 mg/L) mosquito species was very effective[26,27]. A comparison of the larvicidal activity of chlorophyllin with phyto larvicides demonstrates that chlorophyllin is more toxic than ferulic acid (0.10 mg/mL), umbelliferone (0.18 mg/L) extracted from *Ferula asafoetida*[28,29], citral (6.08 mg/mL) extracted from *Zingiber officinale*[2] against *Fasciola gigantica* cercaria larvae. The use of chlorophyllin is safe because it is non toxic to humans and do not have any toxic effect on animals with non-transparent body[6].

In the Indian Ayurveda and the Greco Arabic medical system, several workers have noted that cow urine possess insecticidal, fungicidal, antimicrobial, anthelmintic and molluscicidal activity[30,31,32,33]. The cow urine can act as a bioenhancer in the pharmaceutical composition of antibiotics, increasing their effects (US patent 6410059 B1; 2002)[32]. FCU is more toxic against redia and cercaria larvae in sunlight than under laboratory conditions. Tripathi *et al*.[13] have noted that sunlight-exposed FCU is more toxic against *Lymnaea acuminata* than under laboratory conditions. However, it can be stated that FCU is toxic against both, host snails and *Fasciola* larvae.

The light attenuation in the water column is required for the photodynamic activity of chlorophyllin[34]. As the cercaria shedding host snails are found in shallow water areas of ponds and lakes, the light attenuation problem is not relevant for larvae of *F. gigantica* exposed to dark-incubated chlorophyllin.

The steep slope value indicates that a small increase in the concentration of different larvicides caused higher larvae mortality. A t-ratio value greater than 1.96 indicates that the regression is significant. The heterogeneity factor value less than 1.0 indicates that in the replicate test of random samples, the concentration response is limited and thus the model fits the data adequately. The significance index of the potency estimation g indicates that the mean value is within the limit at all probability levels (90, 95 and 99, respectively), since it is lower than 0.5[16].

The results of the present study have shown that chlorophyllin and chlorophyllin-FCU formulations are potent larvicides. Both chlorophyllin and FCU can be used against fasciolosis in endemic areas as they are

able to kill the *Fasciola* larvae. It has also been shown that *in vivo* phytotherapy of snails using chlorophyllin and FCU may specifically kill redia and cercaria in the body of snails without killing the snails. The use of chlorophyllin is safe as it is not toxic to humans, is cost-effective, ecologically sound and culturally more acceptable to native users. Nevertheless, further studies are required on the *in vivo* toxicity of these formulations against redia/cercaria larvae in the body of snails, providing a possible photo/phytotherapy technique, and at the same time not targeting other organisms that share the same aquatic habitat. This biotechnological tool can be effective to control fasciolosis without killing the snail *L. acuminata*, which is one of the most important bio indicators of healthy aquatic ecosystems.

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