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# Phytotherapy of chlorophyllin exposed *Lymnaea acuminata*: A new biotechnological tool for fasciolosis control

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ABSTRACT

Phytotherapy of chlorophyllin formulations against *Fasciola gigantica* infected *Lymnaea acuminata* under sunlight exposure was highly toxic against redia and cercaria larvae. Binary combinations (1:1 ratio) of chlorophyllin (CHL) + freeze dried cow urine (FCU) were more toxic against cercariae (8 h $LC_{50}$: 9.6 mg $L^{-1}$) than single treatment with chlorophyllin (8 h $LC_{50}$: 12.6 mg $L^{-1}$) in sunlight. The larvicidal activity of sunlight exposed CHL against rediae (8 h $LC_{50}$: 13.5 mg $L^{-1}$) and cercariae (8 h $LC_{50}$: 12.6 mg $L^{-1}$) was more pronounced than laboratory conditions CHL treatment (rediae- 8 h $LC_{50}$: 305.9 mg $L^{-1}$; cercariae- 8 h $LC_{50}$: 765.4 mg $L^{-1}$). Larvicidal activity of FCU was less than CHL and CHL + FCU against both redia and cercaria. Chlorophyllin and its formulations were more toxic against redia and cercaria larvae in sunlight than laboratory conditions. CHL and its different formulations may be used as potent larvicides against *Fasciola gigantica* larvae. Chlorophyllin formulations will be economical, ecologically sounder and their use in aquatic environment will be safe.



## 1. Introduction

The freshwater snail *Lymnaea acuminata* acts as an intermediate host of liver fluke *Fasciola gigantica*, which causes endemic fasciolosis in cattle population of northern India (Singh and Agarwal, 1981). Fasciolosis is one of the most important zoonotic disease worldwide (Piedrafita et al., 2010; Imani-Baran et al., 2012) with economic losses of approximately US $ 3.2 billion per annum (Spithill et al., 1999). The control of fasciolosis may be achieved by either killing the host snail or *Fasciola* larvae inside the snail (Sunita et al., 2013; Singh and Singh, 2015). A number of chemically diverse groups of plant molluscicides have been isolated and identified for effective killing of snails (Singh et al., 1996; Jaiswal and Singh, 2008). Although the control of snail populations is one of the important methods for fasciolosis control in India (Kumar and Singh, 2009), snails are an important component of aquatic ecosystem. Release of molluscicides in aquatic system for snail control affects other non-target organism. One of the possible approaches to eradicate this problem is to interrupt the life cycle of parasitic trematode. The best method to combat fasciolosis is to kill the infective stages of the larvae of *Fasciola* i.e. redia and cercaria inside the snail body (Sunita and Singh, 2011). Wohllebe et al. (2009) have reported that chlorophyll product chlorophyllin was able to kill mosquito larvae in the presence of solar radiation. *In vitro* treatment of chlorophyllin is highly toxic against redia and cercaria larva (Singh and Singh, 2016).

In Indian Ayurveda and Greco Arabic systems of medicine several workers have noted that cow urine possess insecticidal, fungicidal, antimicrobial, anthelmintic and molluscicidal activity (Shastri, 1998; Khanuja et al., 2002; Adane and Gautam, 2003;

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Kekuda et al., 2010; Kumar et al., 2011). Cow urine extract of *Azadirachta indica* was evaluated for its antimicrobial activity against multi drug resistant pathogens (Rajapandiyan et al., 2011). The efficacy of cow dung and cow urine separately and in combination in the control of root-knot nematode in tomato exhibit potent nematicidal activity (Abubakar et al., 2004). The results of nematicidal activity of cow urine and cow urine distillate showed a dose dependent activity in terms of causing paralysis and death of worms (Krupanidhi et al., 2008). Earlier, in our laboratory we have studied the molluscicidal activity of cow urine along with plant derived molluscicides against vector snail *Lymnaea acuminata* (Kumar et al., 2011; Tripathi et al., 2006).

Phytotherapy of snail *L. acuminata* to kill the *Fasciola* larvae inside the snail body is a new biotechnological tool in fasciolosis control programme. In the present study *in vivo* larvicidal activity of chlorophyllin formulations against *Fasciola gigantica* redia and cercaria larvae is evaluated to observe whether chlorophyllin/cow urine singly and in binary combinations are effective against parasite larvae inside the snail body or not. If these formulations were effective against both host and parasite, what will be the concentration difference in between their treatments?

## 2. Materials and methods

### 2.1. Test material

#### 2.1.1. Animals

Adult *Lymnaea acuminata*, each (2.7 ± 0.3 cm in length) were collected locally from the field, Mahesra Lake and low lying submerged areas of Gorakhpur district of Uttar Pradesh, India. Cercariae shedding infected snails were identified according to morphological characteristics (large size, swollen foot, appeared yellowish in colour, slow locomotion, shedding cercaria were appeared at the mouth of snails, shell morphology is changed) as described by of Largue et al. (2007) and Sunita et al. (2013). The infected snails were allowed to acclimatize for 24 h in laboratory conditions. All the experiments were performed in laboratory conditions so that animals should be acclimatized to this condition. Without stable similar conditions, response of snails against different treatments would not be accurate. Thereafter, snails were treated with different formulations of chlorophyllin and cow urine as given in Section 2.1.4. Each infected snail was dissected in glass Petri dish containing 10 mL of dechlorinated water at 23 °C–24 °C. After opening the mantle of the snails a large number of redia and cercaria larvae emerged outside the body of snails in Petri dish. These larvae survived up to 48 h in laboratory conditions. With the help of micropipette redia and cercaria larvae were separated and thereafter, number of live and dead larvae was counted. The pH of the water was 7.1–7.5, and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.3–7.4 mg $L^{-1}$, 5.2–6.4 mg $L^{-1}$ and 103–105 mg $L^{-1}$, respectively. Snail *L. acuminata* and *F. gigantica* were identified by Zoological Survey of India (ZSI), Kolkata (Sunita et al., 2013).

#### 2.1.2. Preparation of chlorophyllin

Chlorophyllin was prepared by the method of Wohllebe et al. (2011). Chlorophyll was isolated from spinach using 100% ethanol (for about 2 h at 55 °C). To avoid transformation of chlorophyll into pheophytin by the acidic content of the cell vacuoles, $CaCO_3$ (about 1 mg $g^{-1}$ plant material) was added as a buffer. The extract was subsequently filtered and petroleum benzene was added. After shaking the mixture chlorophyll moved into lipophilic benzene phase. The two phases were separated in a separatory funnel and about 1 mL methanolic KOH was added to 50 mL of benzene phase. On agitation the chlorophyll came into contact with the methanolic KOH and was transformed to water soluble chlorophyllin (cleavage of the ester bond between the chlorophyllin and the phytol tail by saponification). The phytol tail is responsible for the lipophilic property of chlorophyll. Chlorophyll is found as chlorophyllin in the KOH phase. However, only fresh chemicals were used at the time of experiment.

#### 2.1.3. Collection of cow urine

Preparation of cow urine was done by the method of Tripathi et al. (2006). Geer breed cow urine was collected in sterilized bottles, from green grass grazing 3–5 years old healthy cows. No other food supplements were given to the cows. Freeze dried fractions of cow urine were used in w/v treatments. Freeze dried powder of cow urine (1 mL urine = 30–35 mg freeze dried) kept for 15 days in 8 h/day sunlight. Formulations of cow urine with chlorophyllin were tested against the larvae of *Fasciola gigantica.*

**Table 1**
Concentrations of different formulations of chlorophyllin used in toxicity determination against *F. gigantica* larvae (rediae and cercariae) in infected snails.

| Larvicides/target | Sunlight conditions (mg $L^{-1}$) | Laboratory conditions (mg $L^{-1}$) |
|---|---|---|
| CHL-redia | 20, 30, 50, 60 | 200, 300, 500, 900 |
| CHL + FCU-redia | 10, 20, 30, 50 | 30, 50, 70, 120 |
| FCU-redia | 500, 600, 700, 1100 | 700, 900, 1100, 1500 |
| CHL-cercaria | 10, 20, 30, 50 | 600, 700, 900, 1100 |
| CHL + FCU-cercaria | 10, 20, 30, 50 | 300, 600, 700, 900 |
| FCU-cercaria | 50, 600, 700, 1000 | 600, 800, 1100, 1200 |

CHL-chlorophyllin.
FCU-freeze dried cow urine.

*2.1.4. Preparation of freeze dried cow urine (FCU)*

Geer cow urine was kept in sunlight (temperature- 25–30 °C, duration- 8 h/day, intensity- 1200 $Wm^{-2}$) laboratory conditions (temperature- 22–24 °C, duration- 8 h, intensity- 150 $Wm^{-2}$) for 15 days. After 15 days it was freeze dried in lyophilizer. Binary combinations (1:1) of chlorophyllin with FCU kept for 15 days in sunlight (8 h/day) and laboratory condition were prepared at the time of treatment.

*2.2. Toxicity determination*

*2.2.1. In vivo*

*In vivo* toxicity of single and binary combinations (1:1 ratio) of chlorophyllin with cow urine (Table 1) were determined against larvae of *F. gigantica* in infected snail *Lymnaea acuminata* by the method of Sunita and Singh (Sunita and Singh, 2011). Each concentration (Table 1) of chlorophyllin singly and with cow urine was given (w/v) directly in 3 L of aquarium water containing 10 infected snails in sunlight (temperature- 25–30 °C, duration- 8 h/day, intensity- 1200 $Wm^{-2}$) and laboratory conditions (temperature- 22–24 °C, duration- 8 h, intensity- 150 $Wm^{-2}$). Each treatment of a single concentration was replicated six times. Thereafter, 2 infected snails out of 10 of each treatment were dissected after 2 h, 4 h, 6 h, and 8 h. Six replicates of ten redia and cercaria larvae were separated in a glass Petri dish to observe the mortality under microscope. The numbers of live and dead redia/cercaria were counted with the help of stereomicroscope. Mortality of redia/cercaria was established by immediate arrest of locomotion/movement. To ensure death of larvae the experiment was continuously monitored up to 8 h in all the treatments. In control experiments chlorophyllin treatments were not given. Twenty treated infected snails were kept in separate aquaria and morphological changes (size of body, colour of shell, swelling of foot, locomotion) were observed for next 15 days. Physical parameters of water such as temperature, pH, dissolved oxygen and carbon dioxide were estimated by the method of American Public Health Association (APHA) (American Public Health Association (APHA), 2005).

Concentration-mortality data for each group of larvae were analysed using the probit analysis programme, POLO-PC (LeOra Software), programmed by Robertson et al. (2007) 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 programme ran chi-square test for goodness-of-fit of the data to the probit model. If the model fits, the calculated value of chi-square is less than the chi-square table value for the appropriate degrees of freedom. If the model does not fit, the $LC_{50}$ value for the particular population may not be reliably estimated and is adjusted with the heterogeneity factor (observed chi-square values divided by degrees of freedom). The programme uses heterogeneity factor as a correction factor when the value of Pearson's chi-square statistic is significant at $p = 0.05$. The index of significance for potency estimation (g-value) was used to calculate 95% confidence intervals for potency (relative potency is equivalent to tolerance ratio). Parallelism of the probit regression lines implies a constant relative potency at all levels of response. POLO-PC was used to test equality and parallelism of the slope of the probit lines. The regression coefficient analysis between exposure time and different values of $LC_{50}$ were determined by the method of Sokal and Rohlf (Sokal and Rohlf, 1973).

## 3. Results

Larvicidal activity of chlorophyllin and its different formulations with FCU in *in vivo* condition was time and concentration dependent. *In vivo* toxicity of chlorophyllin + FCU against redia larvae in laboratory conditions (8 h $LC_{50}$: 43.2 mg $L^{-1}$) was higher than single treatment of CHL (8 h $LC_{50}$: 305.9 mg $L^{-1}$) (Fig. 1). Toxicity of FCU against cercaria in laboratory conditions was lowest (8 h $LC_{50}$: 1136.2 mg $L^{-1}$) (Fig. 1). Toxicity of CHL + FCU in laboratory conditions was 5.1 times higher against rediae (8 h $LC_{50}$: 43.2 mg $L^{-1}$) than cercariae (8 h $LC_{50}$: 220.7 mg $L^{-1}$) (Fig. 1). However, toxicity of single chlorophyllin against redia larvae

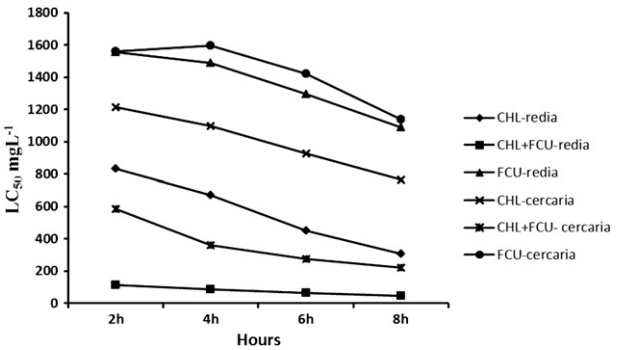


**Fig. 1.** Regression analysis of different formulations of chlorophyllin (CHL), freeze dried cow urine (FCU) against redia and cercaria larvae of *F. gigantica* under laboratory conditions. Significant negative regression ($p < 0.05$) was observed between exposure time and $LC_{50}$ of formulations against redia. (Ts-testing significant of the regression coefficient, chlorophyllin: $-1.24^{++}$, chlorophyllin + FCU: $-1.21^{+}$, FCU: $-2.38^{+}$. +, Linear regression between X and Y. ++, nonlinear regression between log X and log Y cercaria. (Ts-testing significant of the regression coefficient, chlorophyllin: $-3.18^{+}$, chlorophyllin + FCU: $-0.30^{++}$, FCU: $-2.29^{+}$. +, Linear regression between X and Y. ++, nonlinear regression between log X and Y).

(8 h $LC_{50}$: 305.9 mg $L^{-1}$) in laboratory conditions was 2.5 times more toxic than cercariae (8 h $LC_{50}$: 765.4 mg $L^{-1}$) (Fig. 1). In laboratory conditions toxicity of CHL + FCU against redia larvae (8 h $LC_{50}$: 43.2 mg $L^{-1}$) was highest (Fig. 1).

*In vivo* toxicity of chlorophyllin in sunlight against redia (8 h $LC_{50}$: 13.5 mg $L^{-1}$) was 22.57 times higher than laboratory conditions (8 h $LC_{50}$: 305.9 mg $L^{-1}$) (Figs. 1 and 2). Toxicity of FCU in sunlight against rediae (8 h $LC_{50}$: 657.7 mg $L^{-1}$) was 1.6 times higher than laboratory conditions (8 h $LC_{50}$: 1086.4 mg $L^{-1}$) (Figs. 1 and 2). Toxicity of binary combinations of CHL + FCU in sunlight against rediae (8 h $LC_{50}$: 11.2 mg $L^{-1}$) was 3.83 times higher than laboratory conditions (8 h $LC_{50}$: 43.2 mg $L^{-1}$) (Figs. 1 and 2). Cercaricidal activity of binary combinations of CHL + FCU in sunlight was 1.31 times higher (8 h $LC_{50}$: 9.6 mg $L^{-1}$) than single treatment with CHL (8 h $LC_{50}$ 12.6 mg $L^{-1}$) (Fig. 2). Toxicity of FCU against cercaria in sunlight was lowest (8 h $LC_{50}$ 614.9 mg $L^{-1}$) (Fig. 1). Cercaricidal activity of binary combinations of CHL + FCU in sunlight (8 h $LC_{50}$ 9.6 mg $L^{-1}$) was 22.97 times higher than laboratory conditions (8 h $LC_{50}$ 220.79 mg $L^{-1}$) (Figs. 1 and 2). Toxicity of chlorophyllin against cercariae was 60.75 times more in sunlight conditions (8 h $LC_{50}$ 12.6 mg $L^{-1}$) than laboratory conditions (8 h $LC_{50}$ 765.4 mg $L^{-1}$) (Figs. 1 and 2). Cercaricidal activity of FCU in sunlight (8 h $LC_{50}$ 614.9 mg $L^{-1}$) was 1.84 times higher than laboratory conditions (8 h $LC_{50}$ 1136.2 mg $L^{-1}$) (Figs. 1 and 2). Highest cercaricidal activity was noted in combination of CHL + FCU (8 h $LC_{50}$ 9.6 mg $L^{-1}$) (Fig. 2) in sunlight.

Infected snails were morphologically identified having swollen foot, yellowish shell colour, slow locomotion and cercaria shedding character (Sunita et al., 2013; Largue et al., 2007). When treated infected snails were observed for next 15 days they looked like uninfected snails because there was significant reduction in swelling of foot, and darkness of shell colour and no cercariae shedding. No mortality was noted in treated snails in 15 days of observation. When these infected snails were dissected out after 15 days no redia/cercaria larvae were observed.

The slope values were steep and separate estimation of $LC_{50}$, based on each of the six replicates were found within 95% confidence limits of $LC_{50}$. The t-ratio values were higher than 1.96. Heterogeneity factor was less than 1.0. The g-value was less than 0.5. There was significant negative regression ($p < 0.05$) between exposure time and $LC_{50}$ of the treatments.

## 4. Discussion

The results of the present study clearly indicate that *in vivo* toxicity of chlorophyllin and its formulations with FCU act as potential larvicides. Their larvicidal activities are time and concentration dependent as evident from negative regression between exposure period and $LC_{50}$ of different formulations. These formulations are able to kill the redia and cercaria larvae of *Fasciola* in the snail body, without killing the snails. Chlorophyllin is a photodynamic substance, which caused necrosis and apoptosis in the intestine of *Chaoborus crystallinus* larvae (Wohllebe et al., 2009; Wohllebe et al., 2011). Chlorophyllin was toxic against fish ectoparasite *Ichthiophthirius multifiliis*, *Ichthyobodo*, *Dactylogyrus*, *Trichodina*, *Argulus* (Kumar et al., 2011; Hader et al., 2016) and certain microbes (Kreitner et al., 2001; Lopez-Carballo et al., 2008). Earlier, it was noted that exposure of visible day light caused significant mortality in *Chaoborus crystallinus* larvae prior exposure to chlorophyllin (Erzinger et al., 2011). Sublethal laboratory/sunlight treatments with chlorophyllin singly and with FCU caused significant mortality of *Fasciola* larvae in exposed infected snails. Binary combinations of CHL + FCU (1:1 ratio) in sunlight are more toxic against cercariae than rediae. Generally photodynamic substances are not toxic in darkness, but are activated by light and transformed to a reactive singlet state. On reaction with light, reactive cytotoxic singlet oxygen is produced (Kessel and Smith, 1989; DeRosa and Crutchley, 2002). Probably this singlet oxygen is the cause of higher chlorophyllin toxicity in sunlight than laboratory treatment. It has been noted that solubilized chlorophyllin is toxic enough to damage and kill developmental stages of certain pests/vectors (He and Hader, 2002; Tominaga et al., 2004; Richter et al., 2014). Earlier, Abdel-Kader et al. (Abdel-Kader et al., 1999) has reported that chlorophyllin exclusively kills mosquito larvae without affecting the other non-target organism in water body.

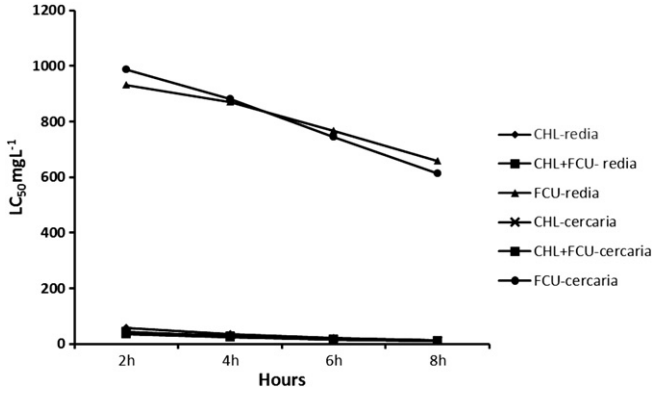


**Fig. 2.** Regression analysis of different formulations of chlorophyllin (CHL), freeze dried cow urine (FCU) against redia and cercaria larvae of *F. gigantica* under sunlight conditions. Significant negative regression ($p < 0.05$) was observed between exposure time and $LC_{50}$ of formulation against redia. (Ts-testing significant of the regression coefficient, CHL: $-0.41^{++}$, CHL + FCU: $-11.85^{++}$, FCU: $-2.42^{+\cdot}$ +, Linear regression between X and Y. ++, nonlinear regression between log X and log Y) cercaria. (Ts-testing significant of the regression coefficient, CHL: $-16.11^{++}$, CHL + FCU: $-1.65^{+}$, FCU: $-2.85^{+}$. +, Linear regression between X and Y. ++, nonlinear regression between log X and log Y).

Cow urine is potent molluscicides (Kumar et al., 2011). Tripathi et al. (Tripathi et al., 2006) has noted that toxicity of light exposed cow urine against *L. acuminata* was higher than laboratory condition. In the present study toxicity of 15 days sunlight exposed FCU is higher against both redia/cercaria larvae than laboratory condition. *In vivo* treatment with chlorophyllin and its different formulations against infected *L. acuminata* particularly kill redia and cercaria without affecting the snails itself as evident from the recovery of outer morphology of infected snail towards uninfected snails. Phytotherapy of infected snails under sunlight exposure kill the redia/cercaria larvae, as evident from the absence of larvae in treated snails after 15 days of treatment. In this way treated snails are cured from *Fasciola* infection. It is evident from the steep slope values that a small increase in the concentration of different treatments causes a marked mortality in the larvae of *Fasciola*. A t-ratio value greater than 1.96 indicates a significant regression of each concentration response line. Heterogeneity factor value less than 1.0 demonstrates that the log-dose-probit lines are within the 95% confidence limits and thus model fitted our data. The g value was less than 0.5 indicates that the mean was within the limit at all probability levels of 90, 95 and 99%.

In conclusion, it can be stated that the phytotherapy of infected snails with chlorophyllin and cow urine formulations under sunlight exposure is potential remedy against fasciolosis. Morphological recovery (shell colour from pale yellow to black, reduced foot swelling, comparatively fast locomotion) in treated snails towards the uninfected snails and no mortality within 15 days clearly indicates the eradication of *Fasciola* infection in snails. Snails are the bio-indicator of healthy aquatic system. Death of redia/cercaria in treated snail body is also an indication of strategic fasciolosis control without disturbing aquatic ecosystem. Chlorophyllin and FCU both are the natural products, easily available, ecologically safe and culturally more acceptable than synthetic larvicides. While calculating the economy we find that only 0.015 $ is required for killing of larvae inside the body of 120 snails in laboratory condition (cost of fresh spinach, and materials used during the preparation of chlorophyllin). It will be further required if we will apply control release system in field to combat fasciolosis. It will be a new biotechnological tool to reduce the fasciolosis.

## Conflict of interest declaration

The authors declare no conflict of interest.

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