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Original Article

Chlorophyllin Treatment Against the Snail *Lymnaea acuminata*: A new tool in Fasciolosis Control

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ABSTRACT

Objective: To observe the toxicity of chlorophyllin against *Lymnaea acuminata* to control fasciolosis caused by liver fluke *fasciola gigantica*, very prominent in eastern region of Uttar Pradesh. **Materials and Methods:** Ten snails *Lymnaea acuminata* were placed in a glass aquarium containing 3 L of dechlorinated tap water. These snails were treated with different concentrations of chlorophyllin. Chlorophyll was extracted from spinach with the help of macerated leaves and kept for 2 h in 100% ethanol at 55°C. **Results:** The results of the experiment showed that the photodynamically active chlorophyllin, at low concentration was able to kill the snails under exposure of solar radiation in summer season instead of winter season. In winter, extracted chlorophyllin toxicity against *L. acuminata* in sunlight (96 h LC_{50} 91.82 mg L^{-1}) /laboratory condition (96 h LC_{50} 921.93 mg L^{-1}) was less than pure chlorophyllin in sunlight (96 h LC_{50} 12.05 mg L^{-1}) /laboratory condition (96 h LC_{50} 19.22 mg L^{-1}), respectively. In summer, pure chlorophyllin was more toxic in sunlight (96 h LC_{50} 3.90 mg L^{-1}) than laboratory condition (96 h LC_{50} 7.18 mg L^{-1}). Pure chlorophyllin is more than five times toxic than synthetic molluscicides. Treatment of chlorophyllin caused no toxic effect against the fish (*Colisa fasciatus*). The result presented in this paper is found very beneficial and ecologically safe, as a photodynamic substance chlorophyllin, which found in every green plant. **Conclusion:** Phytotherapy of snails by photodynamic water soluble chlorophyllin to control fasciolosis can be used as potent molluscicides with low cost and easily biodegradable.

Key words: Fasciolosis, *Lymnaea acuminata, Fasciola gigantica,* Plant molluscicide, Chlorophyllin, Photodynamic reaction.

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INTRODUCTION

Fasciolosis is a zoonotic disease and third most prevalent disease in the world.[1-3] Human fasciolosis and it outbreaks in the last two decades have changed the status of fasciolosis from a zoonosis to an emerging health problem in tropics.[4,5] *Fasciola gigantica* is the causative agent of fasciolosis in eastern Uttar Pradesh, India.[6,7] Snails are the important links in transmission of fasciolosis. The snail *Lymnaea acuminata* is the intermediate host of *F. gigantica*.[8,9] An obvious preventive method to reduce the incidence of fasciolosis is to control the population of carrier snail. Indiscriminate use of synthetic molluscicides such as Carbamates and organophosphates has created several environmental hazards.[10] So that, plant molluscicides are advocated as they are easily biodegradable, cheap and easier to handle by native users.[11,12]

Chlorophyll is found in all green plants. Chlorophyll product chlorophyllin is extremely toxic against mosquito larvae in sunlight.[13,14] Recently, Singh and Singh[7] noted the cercaricidal activity of chlorophyllin against *Fasciola gigantica* larvae. The present study reports the molluscicidal activity of chlorophyllin against host snail *L. acuminata*.

MATERIALS AND METHODS

Pure Compound

Chlorophyllin is purchased from Sigma Chemical Co. USA.

Experimental Animal

Adult *L. acuminata* of average size (2.25 ± 0.30 in length) were collected locally from ponds, lakes and low-lying submerged fields of the district Gorakhpur, UP, India. Gorakhpur lies between latitude 26° 46´ N and longitude 83° 22´ E at an altitude of 95 meter above the sea level. The collected snails were kept in glass aquarium containing de-chlorinated tap water for 72 h for acclimatization. The animals were kept in de-chlorinated tap water at room temperature (22-25°C). The pH, dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 7.1-7.3; 6.5-7.3 mg L^{-1}; 5.2-6.3 mg L^{-1} and 102-105 mg L^{-1}, respectively. Water was changed once every 24 h and dead animals were removed to prevent the water from being contaminated by decaying tissue.

Preparation of Chlorophyllin

Chlorophyllin was prepared by the method of Wohllebe *et al.*[14] Chlorophyll was extracted from spinach with the help of macerated leaves for 2 h in 100%

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ethanol at 55ºC. To avoid transformation of chlorophyll into pheophytin by the acidic content of the cell vacuoles 1.0 mg $CaCO_3$/ g 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).

Toxicity-Determination

Toxicity experiments were done according to the method of Singh and Agarwal.[15] Ten experimental snails were placed in a glass aquarium containing 3 L of de-chlorinated tap water. The experiment was setup with two groups and in the I group the control 1 snails were kept in laboratory condition (winter/summer) after 4 h of dark incubation for 96 h with no chlorophyllin, and control 2 put into the sunlight condition (winter/summer) and all the condition was same as in control 1. Further in the II group the treatment 1 were kept in laboratory condition (mercury light intensity 150W/m^2, winter/summer) and treatment 2 in sunlight condition (light intensity 900W/m^2 in winter and 1200W/m^2 in summer). Light intensity was measure with the help of digital lux meter (Mextech LX-1010B) in flux. Thereafter, it has been converted in irradiance W/m^2. Snail mortality was recorded at every 24 h upto 96 h. Each treatment was replicated six times. Dead animals were removed immediately from the aquarium to avoid any contamination of the water. Snail mortality was confirmed by the contraction of the body within the shell and absence of any response to a needle probe. Toxicity determinations were also studied against non-target animal fish *Colisa fasciatus*. A group III of ten fishes were taken in a 6 L dechlorinated tap water and all the conditions were same as in group I and group II. The fishes were treated with 24 h Lethal Concentration$_{90}$ (LC_{90} against *L. acuminata*) for 96 h in the same experimental condition as in snail treatment.

The slope value of the probit line was also estimated. This program ran chi-square tests 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 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 with the heterogeneity factor as correction factor when the value of Pearson's chi-square statistics 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 (LeOra software) of Robertson *et al*[16] was used to test equality and parallelism of the slope of the probit lines was calculated by using the probit analysis programme. The regression co-efficient was determined between exposure time and different values of LC_{50} by the method of Sokal and Rohlf[17].

RESULTS

The toxicity of chlorophyllin against *L. acuminata* was concentration and time- dependent. In winter, toxicity of extracted and pure chlorophyllin in laboratory condition (96 h LC_{50} Extracted- 921.93 mg L^{-1}, Pure- 19.22 mg L^{-1}) was lower than the sunlight (96 h LC_{50} extracted- 91.82 mg L^{-1}, Pure- 12.05 mg L^{-1}). Whereas, in summer toxicity of extracted and pure chlorophyllin in laboratory condition (96 h LC_{50} Extracted- 257.11 mg L^{-1}, Pure- 7.18 mg L^{-1}) was lower than the sunlight (96 h LC_{50} extracted- 24.95 mg L^{-1}, Pure- 3.90 mg L^{-1}) (Table 1 and 2).

Toxicity of extracted chlorophyllin against *L. acuminata* was 1.75 to 3.68 times higher in summer than winter. Whereas, pure chlorophyllin toxicity was 2.67 to 4.84 times higher in summer than winter. The toxicity of pure chlorophyllin in laboratory condition (96 h LC_{50} 7.18 mg L^{-1}) was less effective than sunlight (96 h LC_{50} 3.90 mg L^{-1}) (Table 1 and 2). No mortality in snails was noted in group III control 1 and contr.ol 2 as well as fish treated with 24 h LC_{90} against *L. acuminata*. No mortality in fish population may be either due to rapid detoxification of chlorophyllin in fish body or the concentration range used for the snails is safe against fish.

The slope values were steep and separate estimations of $LC_{50,}$ based on each of the six replicates, were found within the 95% confidence limits of LC_{50}. The t-ratio values were greater than 1.96 indicating a significant regression of each dose response line. The heterogeneity factor was less than 1.0, demonstrating the log-dose-probit lines are within the 95% confidence limits and thus the model fitted our data. Value of g less than 0.5 indicated that mean was within the limit at all probability levels of 90, 95 and 95%.

DISCUSSION

The results of the present study indicate that the chlorophyllin extracted from spinach is a potent source of plant molluscicides. Toxicity of chlorophyllin is time and concentration dependent, as evident from negative

Table 1: Toxicity of chlorophyllin in laboratory condition against *Lymnaea acuminata*

Exposure time	Treatment	Winter LC_{50} (LCL-UCL)				Summer LC_{50} (LCL-UCL)			
		Nov 22°C	Dec 19°C	Jan 16°C	Feb 21°C	March 29°C	Apr 39°C	May 32°C	Jun 39°C
24h	Ext Chl	958.96 (951.76-969.07)				545.59 (465.99-743.74)			
	Pure Chl	62.26 (46.45-113.65)				12.84 (11.06-17.08)			
48h	Ext Chl	944.07 (937.44 - 951.37)				421.57 (373.85-504.13)			
	Pure Chl	40.11 (30.10-70.84)				9.49 (8.51-11.04)			
72h	Ext Chl	933.42 (926.67-939.79)				319.51 (284.81-355.74)			
	Pure Chl	29.06 (21.69-44.43)				8.26 (7.42-9.31)			
96h	Ext Chl	921.93 (915.07-927.68)				257.11 (223.08-285.07)			
	Pure Chl	19.22 (14.12-24.38)				7.18 (6.40-7.93)			

Each experiment was replicated six times. Toxicity measured at intervals of 24 h upto 96 h. Concentrations given are the final concentration (w/v) in the glass aquarium water. The t-ratio was >1.96, g-value <0.5 and heterogeneity factor <1.0 at all probability level (90, 95 and 99) slope value is ± SE of six replicates. Significant ($P < 0.05$) negative regression was observed between exposure time and LC_{50} of treatments. Ts- testing significant of the regression coefficient- Ext Chl- -0.623^{+} and 1025^{++} in winter; -5.792^{+} and 1066^{++} in summer. Pure Chl- - 0.954^{+} and 137.5^{++} in winter; -0.144^{++} and 22.53^{++} in summer.

+: linear regression between x and y; ++ non linear regression between log x and log y.

Abbreviations: Chl, chlorophyllin; Pure Chl, pure chlorophyllin. LCL, lower confidence limit; UCL, upper confidence limit;

Table 2: Toxicity of chlorophyllin in sunlight against *Lymnaea acuminata*

Exposure time	Treatment	Winter LC_{50} (LCL-UCL)				Summer LC_{50} (LCL-UCL)			
		Nov 22°C	Dec 19°C	Jan 16°C	Feb 21°C	March 29°C	Apr 39°C	May 32°C	Jun 39°C
24h	Ext Chl	95.55 (94.93-96.36)				52.27 (45.38-67.61)			
	Pure Chl	51.80 (39.53-87.21)				10.75 (9.41-13.57)			
48h	Ext Chl	94.64 (93.36-94.79)				41.16 (36.73-48.33)			
	Pure Chl	33.42 (26.68-46.78)				7.91 (6.70-9.40)			
72h	Ext Chl	93.17 (92.51-93.77)				31.20 (27.69-34.75)			
	Pure Chl	22.29 (17.16-28.48)				6.44 (5.31-7.32)			
96h	Ext Chl	91.82 (91.17-92.36)				24.95 (21.48-27.72)			
	Pure Chl	12.05 (9.06-14.55)				3.90 (2.34-4.84)			

Each experiment was replicated six times. Toxicity measured at intervals of 24 h upto 96 h. Concentrations given are the final concentration (w/v) in the glass aquarium water. The t-ratio was >1.96, g-value <0.5 and heterogeneity factor <1.0 at all probability level (90, 95 and 99) slope value is ± SE of six replicates. Significant ($P < 0.05$) was observed between exposure time and LC_{50} of treatments. Ts- testing significant of the regression coefficient- Ext Chl- -0.064^{+} and 102.1^{++} in winter; - 0.522^{+} and 101.7^{++} in summer. Pure Chl- -0.787^{+} and 121.8^{++} in winter; -0.123^{+} and 22.67^{++} in summer.

+: linear regression between x and y; ++ nonlinear regression between log x and log y.

Abbreviation: Chl, chlorophyllin; Pure Chl, pure chlorophyllin. LCL, lower confidence limit; UCL, upper confidence limit;

regression between exposure period and LC_{50} of chlorophyllin. Earlier, Wohllebe *et al*[18] have noted that chlorophyllin can kill the different stages of the protozoan parasite *Ichthyophthirius mulftifiliis* in fresh water fish species. It is reported that chlorophyllin was able to kill four different species, a small crustacean (*Daphnia similis*), a unicellular alga (*Euglena gracilis*) and two species of fish (*Astyanax bimaculatus* and *Cyprynus carpio*) the vector of parasitic diseases.[19] Although it has been reported[19] that chlorophyllin is toxic against *Astyanax bimaculatus* and *Cyprynus carpio*, yet in our observation it is not toxic against *C. fasciatus*. Both extracted and pure chlorophyllin are more effective in summer than winter. Water in winter season holds more oxygen[20] Singh *et al.*[21] reported that in 2010 summer water temperature was in between 20°C to 35°C in year 2010. The reported dissolved oxygen concentration in this water was 2.0 to 4.0 mg L^{-1}, which caused higher mortality of snails. Earlier it was reported in my own laboratory[22] that in warm water dissolved oxygen concentration in summer season is low (2.0 - 4.0 mg L^{-1}) and in winter season it is high (5.0 – 7.0 mg L^{-1}). In the present experiment when we have treated the same conditions while toxicity the chlorophyllin, it may be possible that their effect along with toxic singlet oxygen production in the body of the snail after the treatment of chlorophyllin enhances the snail mortality.

Whereas in winter, when water temperature and corresponding dissolved oxygen concentration was in between 18°C to 25°C and was 5.0 to 7.3 mg L^{-1}, respectively[21]. Higher toxicity of chlorophyllin in sunlight in comparison to laboratory condition is due to the more production of toxic singlet oxygen. Photodynamic reactions of chlorophyllin lead to the formation of the highly reactive singlet oxygen, which can react with various biomolecules.[23] The toxicity of pure chlorophyllin is much more than extracted chlorophyllin because it is isolated from extraction of spinach. And it has high concentration of chlorophyll in comparison to extracted chlorophyllin which consist other component such as carotene, xanthophylls, and many other components too.[24] The HPLC (High performance liquid chromatography) was done in my own laboratory by Chaturvedi and Singh[25] identification of active components in the extracted and pure chlorophyllin.

Chlorophyllin is a derivative of chlorophyll,[26] in which magnesium atom at the centre of the ring is replaced with copper and phytol tail is lost.[27] Due to loss of phytol ring chlorophyllin is more soluble and stable in water than chlorophyll.[28] Chlorophyllin is accumulated in the intestine of exposed mosquito larvae and cercaria larva due to its higher solubility.[29,7] Recently, Chaturvedi and Singh,[30] also observed the toxicity of chlorophyllin against *Lymnaea acuminata* at different wavelengths of visible light. Photosensitization of this chlorophyllin produces reactive oxygen substances causing cell death.[31] Very earlier, Singh *et al.*[32] was studied the larvicidal activity of photodynamic product pheophorbide a in different wavelength of light as well as in sunlight against cercaria larvae. The 96 h LC_{50} of toxicity of pure chlorophyllin (3.90 mg L^{-1}) in sunlight is lower than active molluscicidal component *Papain* (9.74 mg L^{-1}), *Quercetin* (65.91 mg L^{-1}), *thymol* (10.71 mg L^{-1}), and *Citral* (16.68 mg L^{-1})[33-35] and synthetic molluscicides *phorate* (15 mg L^{-1}), *carbaryl* (14.4 mg L^{-1}) and *formothion* (8.5 mg L^{-1}).[36] Extracted and pure chlorophyllin can be used as safe molluscicides as there was no mortality in fish even at 24 h LC_{90} (against *L. acuminata*).

Evidence from the steep slope shows values indicate that a small increase in the concentration of the different treatments causes a marked mortality in snails. A t- ratio value greater than 1.96 indicates that the regression is significant. Values of heterogeneity factor less than 1.0 denote that in the replicate test of random samples, the concentration response lines would fall within 95% confidence limits, and thus the model fits the data adequately. The index of significance of potency estimation g-value indicates that the value of the mean is within the limits at all probability levels (90, 95 and 99) less than 0.5.

CONCLUSION

It can be stated that water soluble chlorophyllin extracted from green plant leaves, can be used as potent molluscicides. Due to the photodynamic nature of chlorophyllin, it has the potential to control fasciolosis by killing the snails. Use of photodynamic chlorophyllin against snails, is one of the effective biotechnological tool for effective control of fasciolosis. The use of chlorophyllin is safe, as it is not toxic to fish sharing the same habitat.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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ABBREVIATIONS USED

Chl: Chlorophyllin; **Pure Chl:** Pure chlorophyllin; **LCL:** Lower confidence limit; **UCL:** Upper confidence limit.

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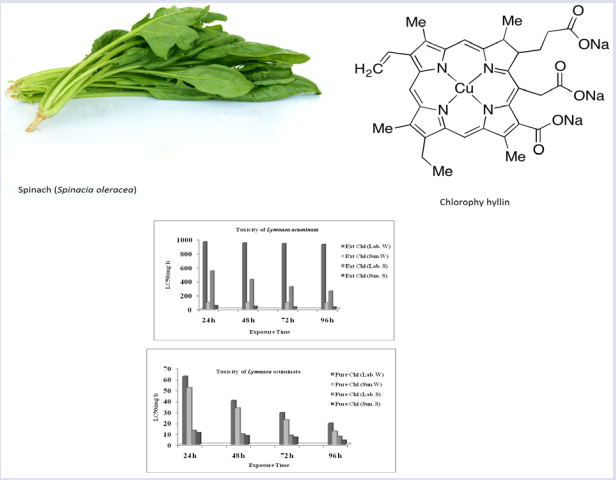
GRAPHICAL ABSTRACT



HIGHLIGHTS OF PAPER

- Water-borne disease fasciolosis also known as liver rot caused severe economic losses in Africa and Asia.
- The results of the experiment showed that the photodynamically active chlorophyllin, at low concentration was able to kill the snails under exposure of solar radiation in summer season instead of winter season.
- Pure chlorophyllin is more than five times toxic than synthetic molluscicides. It can be stated that water soluble chlorophyllin extracted from green plant leaves, can be used as potent molluscicides
- Phytotherapy of snails by photodynamic chlorophyllin is one of the effective biotechnological tool for effective control of fasciolosis

AUTHOR PROFILE

Kavita Singh: Research Scholar of Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur, UP, India. She completed her UG from St. Andrew's degree college and PG from DDU Gorakhpur University, Gorakhpur and enrolled for her doctorate studied under the supervision of Dr. Vinay Kumar Singh. She is gold medalist in M.Sc. Zoology from DDUGU in the year 2013 and also DST (Department of Science and Technology, New Delhi) INSPIRED FELLOW of 2014 (Inspire Fellowship Number-IF 140959). She has published 2 papers in an international journal and working on Effect of chlorophyllin/pheophorbide on the reproduction and development of snail Lymnaea acuminata.

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