Tropical Life Sciences Research, 27(2), 25–36, 2016

Toxicity of Chlorophyllin against *Lymnaea acuminata* at Different Wavelengths of Visible Light

Divya Chaturvedi and Vinay Kumar Singh*

Malacology Laboratory, Department of Zoology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur-273 009, UP, India

Published date: 17 August 2016
To cite this article: Divya Chaturvedi and Vinay Kumar Singh. (2016). Toxicity of chlorophyllin against *Lymnaea acuminata* at different wavelengths of visible light. *Tropical Life Sciences Research* 27(2): 25–36. doi: 10.21315/tlsr2016.27.2.3
To link to this article: http://dx.doi.org/10.21315/tlsr2016.27.2.3

Abstrak: Fasciolosis merupakan penyakit bawaan air dan makanan yang disebabkan oleh fluk hati *Fasciola hepatica* dan *Fasciola gigantica*. Penyakit ini menular di pelbagai bahagian di dunia ini. Siput Lymnaeidae and Planorbidae ialah perumah perantara bagi fluk ini. Pengurusan populasi siput menjadi kaedah terbaik bagi mengawal fasciolosis kerana gastropod merupakan rantaian yang paling lemah dalam kitaran hayat trematod. Klorofil boleh diekstrak dari sebarang tanaman hijau. Klorofilin disediakan daripada bayam dalam etanol 100% dengan menggunakan pelbagai jenis bahan kimia yang berbeza. Klorofil yang diperoleh daripada bayam bertukar menjadi klorofilin larut air. Dalam kajian yang dijalankan ini, ketoksikan klorofilin bagi menghapuskan siput *Lymnaea acuminata* bergantung kepada masa dan kepekatan. Ketoksikan klorofilin yang diekstrak dan yang tulen di bawah cahaya matahari secara berterusan selama 4 jam adalah tinggi dengan kepekatan maut (LC_{50}) 331.01 mg/L dan 2.60 mg/L, masing-masing, berbanding dengan pendedahan kepada cahaya matahari selama 8 jam yang bukan secara berterusan dengan LC_{50} 357.04 mg/L dan 4.94 mg/L, masing-masing. Ketoksikan klorofilin yang diesktrak dapat diketahui dengan kehadiran cahaya nampak monokromatik yang berbeza. Ketoksikan yang paling tinggi didapati dalam cahaya kuning (96 jam, LC_{50} 392.77 mg/L) dan yang paling rendah dalam cahaya hijau (96 jam, LC_{50} 833.02 mg/L). Kewujudan klorofilin dalam kombinasi dengan radiasi solar atau cahaya nampak monokromatik yang berbeza panjang gelombangnya mungkin menjadi satu penawar pendam bagi menghapuskan siput *L. acuminata*. Klorofilin terbukti lebih toksik dalam kehadiran cahaya matahari. Klorofilin adalah selamat dari segi ekologi dan lebih berekonomi berbanding molusisid sintetik yang berpotensi mengawal insiden fasciolosis di negara-negara membangun.

Kata kunci: Fasciolosis, *Fasciola gigantica*, *Lymnaea acuminata*, Klorofilin, Cahaya Monokromatik

Abstract: Fasciolosis is a water and food-borne disease caused by the liver fluke *Fasciola hepatica* and *Fasciola gigantica*. This disease is widespread in different parts of the world. Lymnaeidae and Planorbidae snails are the intermediate hosts of these flukes. Snail population management is a good tool to control fasciolosis because gastropods represent the weakest link in the life-cycle of trematodes. Chlorophyll can be extracted from any green plant. Chlorophyllin was prepared from spinach in 100% ethanol by using different types of chemicals. The chlorophyll obtained from spinach was transformed into water-

*Corresponding author: vinaygkpuniv@gmail.com

soluble chlorophyllin. In the present paper, toxicity of chlorophyllin against the snail *Lymnaea acuminata* was time and concentration dependent. The toxicity of extracted and pure chlorophyllin at continuous 4 h exposure of sunlight was highest with lethal concentration (LC_{50}) of 331.01 mg/L and 2.60 mg/L, respectively, than discontinuous exposure of sunlight up to 8 h with LC_{50} of 357.04 mg/L and 4.94 mg/L, respectively. Toxicity of extracted chlorophyllin was noted in the presence of different monochromatic visible lights. The highest toxicity was noted in yellow light (96 h, LC_{50} 392.77 mg/L) and the lowest in green light (96 h, LC_{50} 833.02 mg/L). Chlorophyllin in combination with solar radiation or different wavelength of monochromatic visible lights may become a latent remedy against the snail *L. acuminata*. It was demonstrated that chlorophyllin was more toxic in sunlight. Chlorophyllin is ecologically safe and more economical than synthetic molluscicides which have the potential to control the incidence of fasciolosis in developing countries.

Keywords: Fasciolosis, *Fasciola gigantica*, *Lymnaea acuminata*, Chlorophyllin, Monochromatic Light

INTRODUCTION

Fasciolosis is one of the most debilitating zoonotic diseases. Fresh water snail *Lymnaea acuminata* is the vector of liver flukes *Fasciola gigantica*, which causes endemic fasciolosis in cattle populations in Northern parts of India (Singh & Agarwal 1981; Singh & Singh 2016). About 94% buffaloes slaughtered in Gorakhpur, UP, India, are infected with *F. gigantica* (Singh & Agarwal 1981; Sunita *et al.* 2016). No continent is free from fasciolosis (Soliman 2008). The epidemiology of human fasciolosis has changed in recent years and significantly increased in the last two decades (Mas-Coma *et al.* 2005, 2009). Snail control is one of the best methods to eliminate fasciolosis. Molluscicide is still in the centre of efforts to control fasciolosis. Synthetic molluscicides have been widely used for the effective control of carrier snails (Singh *et al.* 2010; Singh *et al.* 2012). Now it has been realised that synthetic molluscicides are toxic to non-target animals and have a long term detrimental effect on the aquatic environment (Shafer *et al.* 2005; Upadhyay & Singh 2011). The molluscicides of plant origin are gaining importance than their synthetic counterparts (Singh *et al.* 1996). The derivates of chlorophyll is a very promising substance for snail control. Chlorophyllin can be easily extracted from any green plant. It is very economical and biodegradable. Molluscicidal activity of chlorophyllin has been reported against *Lymnaea stagnalis, Biomphalaria spp.* and *Physa marmorata* (Mahmoud *et al.* 2013). Previously, it has been reported that chlorophyllin is a potent larvicide (Wohllebe *et al.* 2009). Recently, it has been reported by Singh and Singh (2015) that the combination of monochromatic visible light with chlorophyllin has effective larvicidal activity against *F. gigantica*. Visible light spectrum initiates the orientation and locomotion of exposed snails towards a light source (Sakakibara *et al.* 2005). It was also noted that the *L. acuminata* snail monitors the intensity variation of visible light (Tripathi *et al.* 2013, 2014). Kumar and Singh (2015) reported that the toxic effect of chlorophyllin against *Lymnaea acuminata* in the presence of red light and sunlight. The aim of this present study is to evaluate the photo-toxicodynamic activity of extracted/pure chlorophyllin

against fresh water snail *L. acuminata* in sunlight and in different wavelengths of monochromatic visible light.

MATERIALS AND METHODS

Pure Compound

Chlorophyllin was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

Experimental Animal

The fresh water snail, *L. acuminata* (2.35±0.30 cm in length) was collected locally from ponds, lakes, and low lying submerged fields in Gorakhpur and was used as the test animals. The collected snails were acclimatised for 72 h in laboratory conditions. The experimental animals were kept in a glass aquarium containing dechlorinated tap water at room temperature (22°C–25°C). The pH, dissolved oxygen, free carbon dioxide, and bicarbonate alkalinity were 7.1–7.3, 6.5–7.3 mg/L, 5.2–6.3 mg/L, and 102–105 mg/L, respectively. Dead animals were removed immediately to prevent the water from being contaminated by decaying tissue.

Preparation of Extracted Chlorophyllin

Preparation of chlorophyllin was done according to the method of Wohllebe *et al.* (2011). Chlorophyll was isolated from spinach using 100% ethanol (for about 2 h at 55°C). Then, $CaCO_3$ (about 1 mg/gm plant material) was added as a buffer, to prevent the transformation of chlorophyll into pheophytin. Before adding benzene the extract was irradiated with solar radiation for 1–2 h. The extract was filtered and 50 mL benzene was added. After addition of benzene, the mixture was shaken well and as a result the chlorophyll moved into the lipophilic benzene phase. The two phases were separated in separatory funnel and about 1.0 mL methanolic KOH was added to 50 mL of the benzene phase. Upon agitation 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. After separation of the methanolic KOH phase and the benzene phase, most of the chlorophyllin was found in the KOH phase. The extract was stored in a dark flask at room temperature. Only fresh chemicals were used in the course of these experiments.

Design of Photo Toxicity Experiments

Light experiment was set up according to the method of Tripathi *et al.* (2013). Xenon arc lamp (500 W) was used as the visible light source. Spectral response from 400 nm to 650 nm were produced with the help of the interference colour filters. Exposure of monochromatic light at different wavelengths and fixed intensity (500 Wm^{-2}) was used to study their effects on snail's mortality.

A glass aquarium (70 cm diameter and 15 cm height) was filled with 3 L of dechlorinated tap water. Ten *L. acuminata* snails were placed in each

aquarium. Then, all the 10 snails were exposed to different concentrations of extracted chlorophyllin and monochromatic visible light. Each treatment of extracted chlorophyllin and monochromatic visible light was replicated six times. Experiment was done at a room temperature of 22°C–25°C.

Toxicity Experiment

Toxicity experiments were done according to the method of Singh and Agarwal (1984). A total of 10 snails were placed in a glass aquarium containing 3 L of dechlorinated tap water. In all 3 sets (treated/control) of experiment, 10 snails were used at different concentrations of extracted/pure chlorophyllin (Table 1). For the first set of experiments, snails were treated with extracted chlorophyllin (600 mg/L, 700 mg/L, 800 mg/L, and 900 mg/L) in laboratory conditions (no exposure to sunlight or monochromatic visible light). Mortality of snails in laboratory condition was recorded at 24 h up to 96 h. In the second set of experiments, the extracted and pure chlorophyllin (200 mg/L, 300 mg/L, 500 mg/L, and 700 mg/L, and 2 mg/L, 4 mg/L, 6 mg/L, and 8 mg/L, respectively) was continuously exposed for 4 h in sunlight (10:00 a.m. to 2:00 p.m.). Mortality was recorded at every 1 h interval up to 4 h, and in another experiment the extracted and pure chlorophyllin (200 mg/L, 300 mg/L, 400 mg/L, and 500 mg/L and 3 mg/L, 5 mg/L, 7 mg/L, and 9 mg/L, respectively) was discontinuously exposed for 8 h in sunlight and a break of 1 h was given after each 2 h exposure to sunlight up to 8 h. Mortality was recorded at every 1 h interval up to 8 h. During the break period, the animals were placed under laboratory condition for 1 h. The third set of experiments was set up to observe the mortality of extracted chlorophyllin (300 mg/L, 500 mg/L, 700 mg/L, and 900 mg/L) treated snails in the presence of different wavelength of monochromatic visible lights in place of sunlight, while other controlled variables were held constant. Mortality of snails in laboratory condition was recorded at 24 h up to 96 h. For the first, second, and third sets of experiments, three types of control groups, I, II, and III, were also set up. In control group I no treatment of chlorophyllin was given in laboratory condition. In control group II, snails were only exposed to sunlight without any treatment of chlorophyllin. In control group III, snails were only exposed to monochromatic visible lights in laboratory condition without chlorophyllin treatment. Each treatment (treated/control) was replicated 6 times. Dead animals were removed from the aquarium immediately to avoid any contamination of water. Snail mortality was established by the contraction of the body within the shell and absence of response to a needle probe was taken as evidence of death.

The experiment was designed to observe the effect of extracted chlorophyllin in laboratory conditions (Table 2). Thereafter, the second set of experiments was designed to observe any effect of continuous exposure of sunlight or interrupted exposure of sunlight against chlorophyllin treated snails (Tables 3 and 4). In another experiment, the effect of different monochromatic visible lights on extracted chlorophyllin toxicity against *L. acuminata* was recorded (Table 5). All the above experiments indicate that continuous exposure of chlorophyllin in sunlight causes more toxicity against snails than laboratory condition. Interruption of light exposure also affects toxicity of chlorophyllin

whereby the use of chlorophyllin in a cloudy environment will certainly affect the chlorophyllin's activity against snails. Toxicity observed in different spectral bands also indicate that variation in wavelength of visible light has a significant effect on the toxicity of chlorophyllin. Longer wavelength range red and yellow was more effective against chlorophyllin treated snails.

The lethal concentration values, lower and upper confidence limits (LCL, UCL), slope values, t-ratio, g-values, and heterogeneity factors were calculated using POLO computer software (Petaluma, CA, USA) (Robertson *et al.* 2007). The regression co-efficient between exposure time and different values of lethal concentration (LC_{50}) was determined (Sokal & Rohlf 1996).

Table 1: Concentration of extracted and pure chlorophyllin used in toxicity experiment against *L. acuminata*.

Experimental design	Chemical	Concentration (mg/L)
Continuous exposure of sunlight	Ext Chl	200, 300, 500, 700
	Pure Chl	2, 4, 6, 8
Discontinuous exposure of sunlight	Ext Chl	200, 300, 400, 500
	Pure Chl	3, 5, 7, 9
Different spectra of light	Ext Chl	300, 500, 700, 900
Laboratory condition	Ext Chl	600, 700, 800, 900

Note: Ext Chl - extracted chlorophyllin; Pure Chl - pure chlorophyllin.

Table 2: Toxicity of extracted chlorophyllin in laboratory condition against *L. acuminata*.

Exposure period (h)	Treatment	LC_{50} mg/L (w/v)	LCL	UCL	Slope value	t-ratio	g-value	Heterogeneity
24	Ext Chl	882.71	831.01	981.00	7.68± 1.45	5.27	0.13	0.18
48	Ext Chl	795.51	755.58	851.20	7.46± 1.35	5.52	0.12	0.27
72	Ext Chl	728.41	687.15	768.60	7.31± 1.32	5.54	0.12	0.26
96	Ext Chl	666.56	629.89	695.48	9.79± 1.44	6.75	0.08	0.48

Notes: Six batches of 10 snails were exposed to different concentrations. Mortality was determined at 24 h to 96 h. Concentrations given are the final concentration (w/v) in the glass aquarium water. Ext Chl - extracted chlorophyllin; Pure Chl - pure chlorophyllin; LCL - lower confidence limit, UCL - upper confidence limit. Significant negative regression ($p<0.05$) was observed between exposure time and LC_{50} treatments. Ts - testing significance of the regression coefficient - Ext Chl -3.72^{+} to -2.23^{+} and 265.8^{++} to 1268^{++} was observed. $^{+}$Linear regression between X and Y. $^{++}$Non-linear regression between X and Y.

Table 3: Toxicity of chlorophyllin against *L. acuminata* with continuous exposure (10:00 a.m. – 02:00 p.m.) of sunlight.

Exposure period (h)	Treatment	LC_{50} mg/L (w/v)	LCL	UCL	Slope value	t-ratio	g-value	Heterogeneity
1	Ext Chl	938.16	715.53	1688.32	2.36±0.50	4.68	0.17	0.28
	Pure Chl	11.23	8.30	22.81	2.03±0.47	4.31	0.20	0.21
2	Ext Chl	597.05	492.40	827.10	2.18±0.43	5.08	0.14	0.15
	Pure Chl	7.43	5.85	11.76	1.75±0.39	4.40	0.19	0.22
3	Ext Chl	492.68	408.85	645.86	2.04±0.41	4.94	0.15	0.25
	Pure Chl	4.17	3.38	5.04	2.06±0.38	5.38	0.13	0.31
4	Ext Chl	331.01	287.98	374.68	3.21±0.44	7.22	0.07	0.48
	Pure Chl	2.60	1.96	3.13	2.43±0.40	6.03	0.10	0.59

Notes: Six batches of 10 snails were exposed to different concentrations. Mortality was determined at 1 h up to 4 h. Concentrations given are the final concentration (w/v) in the glass aquarium water. Ext Chl - extracted chlorophyllin, Pure Chl - pure chlorophyllin, LCL - lower confidence limit, UCL - upper confidence limit. Significant negative regression (p<0.05) was observed between exposure time and LC_{50} of treatments. Ts - testing significant of the regression coefficient - Ext Chl $–343.9^{+}$ to $–41.22^{+}$ and 0.0^{++} to 1973^{++} and Pure Chl $–4.47^{+}$ to $–1.35^{+}$ and 0.0^{++} to 27.00^{++} was observed. $^{+}$Linear regression between X and Y. $^{++}$Non-linear regression between X and Y.

Table 4: Toxicity of chlorophyllin against *L. acuminata* with discontinuous (1 h interval) exposure in sunlight.

Exposure period (h)	Treatment	LC_{50} mg/L (w/v)	LCL	UCL	Slope value	t-ratio	g-value	Heterogeneity
2	Ext Chl	686.57	562.90	1098.42	4.12± 0.93	4.42	0.19	0.94
	Pure Chl	12.19	9.51	22.06	2.64± 0.61	4.30	0.20	0.25
4	Ext Chl	556.74	481.10	733.07	3.90± 0.74	5.26	0.13	0.67
	Pure Chl	8.83	7.30	12.87	2.32± 0.51	4.53	0.18	0.18
6	Ext Chl	451.68	403.14	538.18	4.24± 0.67	6.27	0.11	0.79
	Pure Chl	6.50	5.60	7.86	2.56± 0.49	5.21	0.14	0.31
8	Ext Chl	357.04	320.26	403.29	4.74± 0.6	7.39	0.11	0.58
	Pure Chl	4.94	4.41	5.46	4.02± 0.52	7.61	0.06	0.73

Notes: Six batches of 10 snails were exposed to different concentrations. Mortality was determined at 2 h to 8 h each with 1 h intervals. Concentrations given are the final concentration (w/v) in the glass aquarium water. Ext Chl - extracted chlorophyllin, Pure Chl - pure chlorophyllin, LCL - lower confidence limit, UCL - upper confidence limit. Significant negative regression (p<0.05) was observed between exposure time and LC_{50} treatments. Ts - testing significant of the regression coefficient - Ext Chl $–66.85^{+}$ to $–42.51^{+}$ and 0.0^{++} to 1268^{++} and Pure Chl $–1.81^{+}$ to $–0.59^{+}$ and 0.0^{++} to 25.10^{++} was observed. $^{+}$Linear regression between X and Y. $^{++}$Non-linear regression between X and Y.

Table 5: Toxicity of chlorophyllin in the presence of different spectra of light against *L. acuminata.*

Exposure period (h)	Treatment	Different spectra of light	LC_{50} mg/L (w/v)	LCL	UCL	Slope value	t-ratio	g-value	Heterogeneity
24	Ext Chl	Green	1428.47	1060.01	3264.72	2.71±0.69	3.91	0.25	0.37
		Violet	1427.16	1051.04	3338.91	2.55±0.65	3.89	0.25	0.32
		Blue	1090.88	881.95	1721.74	2.72±0.58	4.63	0.17	0.20
		Orange	1009.97	819.65	1567.96	2.47±0.54	4.57	0.18	0.29
		Red	905.52	744.63	1341.32	2.32±0.51	4.51	0.18	0.22
		White	708.74	609.36	883.19	2.55±0.49	5.11	0.14	0.17
		Yellow	619.73	548.31	711.90	3.19±0.51	6.25	0.09	0.23
48	Ext Chl	Green	1273.84	920.19	3510.62	1.86±0.53	3.51	0.31	0.20
		Violet	1328.08	953.03	3682.09	1.92±0.53	3.56	0.30	0.23
		Blue	864.70	712.37	1269.62	2.23±0.50	4.41	0.19	0.14
		Orange	775.75	663.90	997.24	2.61±0.51	5.12	0.14	0.43
		Red	721.37	620.75	901.36	2.59±0.50	5.17	0.14	0.29
		White	574.21	492.87	673.09	2.62±0.48	5.38	0.13	0.25
		Yellow	533.09	465.93	604.48	3.14±0.49	6.30	0.09	0.35
72	Ext Chl	Green	1148.51	831.01	3488.41	1.61±0.49	3.23	0.36	0.12
		Violet	1017.82	784.95	2018.16	1.86±0.50	3.72	0.27	0.22
		Blue	764.53	649.07	1000.67	2.43±0.50	4.86	0.16	0.20
		Orange	630.78	541.77	761.17	2.51±0.48	5.15	0.14	0.37
		Red	556.12	470.96	655.76	2.47±0.48	5.13	0.14	0.31
		White	494.64	418.94	568.22	2.77±0.48	5.69	0.11	0.32
		Yellow	468.70	408.55	524.83	3.50±0.50	6.88	0.08	0.41
96	Ext Chl	Green	833.02	633.91	1927.15	1.41±0.47	3.01	0.42	0.13
		Violet	826.81	670.98	1278.36	1.95±0.48	4.01	0.23	0.30
		Blue	621.33	527.88	758.46	2.35±0.48	4.87	0.16	0.31
		Orange	505.02	421.30	588.74	2.50±0.48	5.21	0.14	0.37
		Red	472.42	403.63	536.33	3.05±0.49	6.17	0.10	0.33
		White	432.66	368.54	488.57	3.32±0.50	6.53	0.09	0.54
		Yellow	392.77	342.63	436.07	4.28±0.56	7.60	0.06	0.60

Notes: Six batches of 10 snails were exposed to different concentrations. Mortality was determined at every 24 h up to 96 h. Concentrations given are the final concentration (w/v) in the glass aquarium water. Ext Chl - extracted chlorophyllin, LCL - lower confidence limit, UCL - upper confidence limit. Significant negative regression (p<0.05) was observed between exposure time and LC_{50} of treatments. Ts - testing significant of the regression coefficient of green light -13.31^{+} to -2.62^{+} and 0.0^{++} to 2520^{++}, violet light -13.73^{+} to -3.86^{+} and 0.0^{++} to 2629^{++} , blue light -9.46^{+} to -3.10^{+} and 0.0^{++} to 1889^{++}, orange light -10.12^{+} to -3.71^{+} and 0.0^{++} to 1887^{++}, red light -9.05^{+} to -3.14^{+} and 0.0^{++} to 1685^{++}, white light -5.89^{+} to -1.67^{+} and 0.0^{++} to 1188^{++}, yellow light -3.63^{+} to -2.58^{+} and 0.0^{++} to 1018^{++} was observed. $^{+}$Linear regression between X and Y. $^{++}$Non-linear regression between X and Y.

RESULTS

The molluscicidal activity of extracted/pure chlorophyllin was tested at different times of exposure to various light spectra and chlorophyllin concentrations against the host snail *L. acuminata* (Table 1). A significant (p<0.05) negative regression was observed between the exposure time and the LC_{50} of the treatments (Tables 2–5). The toxicity of extracted chlorophyllin at 96 h, LC_{50} was 666.56 mg/L in laboratory condition (Table 2). Continuous 4 h exposure of sunlight in experimental aquarium containing chlorophyllin caused significant mortality in snails. Toxicity of extracted and pure chlorophyllin at continuous 4 h exposure of sunlight (4 h, LC_{50} 331.01 mg/L and 2.60 mg/L, respectively) was more than break of 1 h after each 2 h exposure of sunlight up to 8 h (8 h, LC_{50} 357.04 mg/L and 4.94 mg/L, respectively) (Tables 3 and 4). Toxicity experiment was conducted in laboratory condition with extracted chlorophyllin in the presence of monochromatic visible light. The highest toxicity in yellow light (96 h, LC_{50} 392.77 mg/L) and lowest in green light (96 h, LC_{50} 833.02 mg/L) was observed (Table 5). There was no mortality in control groups I, II, and III. It was observed that both extracted/pure chlorophyllin was more toxic in sunlight than in laboratory conditions.

The slope values were steep and the separate estimation of the LC_{50} based on each of the six replicates was within the 95% confidence limits of LC_{50}. The t-ratio was greater than 1.96, the heterogeneity factor was less than 1.0, and the g-value was less than 0.5 at all probabilities (90, 95, and 99) levels (Tables 2–5).

DISCUSSION

Chlorophyllin, a product of chlorophyll is a very effective photodynamic substance to control host *L. acuminata.* Recently, the toxicity of chlorophyllin in different wavelengths of visible light against redia and cercaria larvae of *F. gigantica* has been reported by Singh and Singh (2015). The larvicidal efficiency of chlorophyllin was demonstrated by Wohllebe *et al.* (2009) from spinach against mosquito larvae (*Chaoborus crystallinus*). Even at low concentrations, chlorophyllin was able to kill mosquito larvae and other small animals within a few hours in sunlight (Wohllebe *et al.* 2009). Chlorophyllin was also able to kill the protozoan parasite *Ichthyophthirius multifiliis* (Fouquet) of fresh water fish species (Wohllebe *et al.* 2012; Hader *et al*. 2016). Erzinger *et al.* (2011) also noted that mosquito larvae were killed by chlorophyllin.

Results of the present study indicate that the chlorophyllin extracted from the spinach is a potent molluscicide. Chlorophyllin toxicity against *L. acuminata* was time and concentration dependent, by the negative regression between exposure period and LC_{50} values of the different treatments. The time-dependent toxic effect of tested plant products may be due to the uptake of active compounds by the snails, which progressively accumulated in the body with an increase time in the exposure period. It is also possible that the active compound could change into more toxic forms in the aquarium water or in the snail's body

due to the sunlight. Four hours of continuous exposure of sunlight treatment was more effective as compared to the intermittent sunlight treatment of 2 h exposure followed by 1 h break. In sunlight, solubilised chlorophyllin transferred its excitation energy to oxygen, which produced singlet oxygen and other reactive oxygen species (ROS), which have the potential to kill the vector organism (He & Hader 2002; Tominaga *et al.* 2004).

In another set of toxicity test, extracted chlorophyllin treatments were given in combination of various wavelengths of monochromatic visible light at fixed intensity (500 Wm^{-2}). Table 5 shows that the yellow light spectrum recorded the highest toxicity of extracted chlorophyllin against *L. acuminata* (96 h, LC_{50} 392.77 mg/L) than other wavelengths of light. Higher toxicity of extracted chlorophyllin was also noted in other monochromatic visible lights than extracted chlorophyllin treatment in laboratory conditions (96 h, LC_{50} 666.56 mg/L) without any exposure to monochromatic visible lights. Chlorophyllin is a photodynamic substance (Wohllebe *et al.* 2009). Consequently, toxicity of the chlorophyllin was higher in sunlight or in monochromatic visible light than in laboratory conditions. All the monochromatic visible lights have sufficient energy, which can elicit the response of the photodynamic product of chlorophyllin. Absorbed photon produces ROS. Even though the toxicity of chlorophyllin in laboratory conditions is also noted against *L. acuminata*, exposure to sunlight and monochromatic light of any wavelengths caused comparatively higher mortality of snails within a few hours. It was also reported that the retina of snails contain only one type of photo pigment, rhodopsin; they can differentiate only gradation of light intensity but not the colour of light (Chernorizov & Sokolov 2010). Earlier, it has been reported that *Lymnaea stagnalis*'*s* eye has two types of ocular photoreceptors and three types of statocyst hair cells (Sakakibara *et al.* 2005). Type-A photoreceptor had more spectral sensitivity between 480 nm and 500 nm. Type-T photoreceptor had a much broader spectral sensitivity between 450 nm and 600 nm. At low intensity, Type-A photoreceptor and at higher intensity, Type-T photoreceptor are more sensitive. Probably, it seems that in the treatment of extracted chlorophyllin in exposure to the same intensity of visible monochromatic light, there is a significant variation in their toxicity as evident from different LC_{50} of chlorophyllin. Obviously, it also indicates that variation in wavelength of light has significant effect on mortality, as evident from higher toxicity of extracted chlorophyllin in yellow light.

The steep slope value indicates that a small increase in the concentration of molluscicide caused higher mortality. A t-ratio value greater than 1.96 indicates that the regression is significant (p<0.05). A heterogeneity factor value less than 1.0 denotes that in the replicate test of random samples, the concentration response is limited and thus, the model fits the data adequately. The index of significance of the potency estimation g indicates that the value of the mean is within the limit at all probability levels (90, 95, and 99) since it is less than 0.5.

CONCLUSION

In conclusion, the present study demonstrated that the treatment of photodynamically active chlorophyllin in solar light or in different wavelengths of visible light has significant toxicity effects on vector snail *L. acuminata*. This is an investigative research work by means of plant extracts to control snail population. Chlorophyllin is a photodynamic product of chlorophyll and chlorophyll is present in all green plants. Therefore, the production of chlorophyllin is inexpensive, easy, and environmentally safe and sound. It is a promising approach to control water-borne diseases. This molluscicide might be a valuable, ecologically safe tool against vector snails which has the potential to replace the synthetic molluscicides and control the incidence of fasciolosis in developing countries. For proper utilisation of chlorophyllin as molluscicides, further studies are required to elucidate the mechanism of action in the snail's body.

ACKNOWLEDGEMENT

The authors are grateful to Prof. D. K. Singh, Department of Zoology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur, India for his valuable suggestions in preparation of the manuscript.

REFERENCES

Chernorizov A M and Sokolov E N. (2010). Mechanisms of achromatic vision in invertebrates and vertebrates: A comparative study. *The Spanish Journal of Pschycology* 13(1): 18–29. doi.org/10.1017/S1138741600003656

Erzinger G S, Wohllebe S, Vollrath F, Souza S C, Richter P, Lebert M and Hader D P. (2011). Optimizing conditions for the use of chlorophyll derivatives for photodynamic control of parasites in aquatic ecosystems. *Parasitology Research* 109(3): 781–786. doi.org/10.1007/s00436-011-2322-7

Hader D P, Schmidl J, Hilbig R, Oberle M, Wedekind H and Richter P. (2016). Fighting fish parasites with photodynamically active chlorophyllin. *Parasitology Research* 115(6): 2277–2283.

He Y Y and Hader D P. (2002). Involvement of reactive oxygen species in the UV-B damage to the cyanobacterium *Anabaena* sp. *Journal of Photochemistry Photobiology B: Biology* 66(1): 73–80. doi.org/10.1016/S1011-1344(01)00278-0

Kumar N and Singh V K. (2015). Bait formulations of chlorophyllin against infected/uninfected *Lymnaea acuminata* in red and sunlight. *International Journal of Zoological Research* 11(5): 207–214.

Mahmoud M S, Richter P, Shalaby H A M, Kandil O M and Hader D P. (2013). Molluscicidal activity of chlorophyll extraction against the freshwater snails. *Journal of Coastal Life Medicine* 1(2): 85–88.

Mas-Coma S, Bargues M D and Valero M A. (2005). Fascioliasis and other plant borne trematode zoonoses. *International Journal of Parasitology* 35(11–12): 1255–1278. doi.org/10.1016/j.ijpara.2005.07.010

Mas-Coma S, Valero M A and Bargues M D. (2009). *Fasciola* Lymnaeids and human fascioliasis with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. In D Rollinson and S I Hay (eds.). *Advances in Parasitology*. Burlington: Academic Press, 69, 141–146. doi.org/10.1016/s0065-308x(09)69002-3

Robertson J L, Russell R M, Preisler H K and Savin N E. (2007). *Bioassay with arthropods, POLO computer programme for analysis of bioassay data,* 2nd ed. London: CRC Press, 1–224.

Sakakibara M, Aritaka T, Iizuka A, Suzuki H, Horikoshi T and Lukowiak K. (2005). Electrophysiological responses to light of neurons in the eye and statocyst of *Lymnaea stagnalis*. *Journal of Neurophysiology* 93(1): 493–507. doi.org/10.1152/jn.00692.2004

Shafer T J, Meyer D A and Crofton K M. (2005). Developmental neurotoxicity of pyrethroid insecticides: Critical review and future research needs. *Environment Health Perspectives* 113(2): 123–136. doi.org/10.1289/ehp.7254

Singh A, Singh D K, Misra T N and Agarwal R A. (1996). Molluscicide of plant origin. *Biological Agriculture and Horticulture* 13(3): 205–252. doi.org/10.1080/01448765.1996.9754782

Singh D J and Singh D K. (2016). Phytotherapy of chlorophyllin exposed *Lymnaea acuminata*: A new biotechnological tool for fasciolosis control. *Parasite Epidemiology and Control* 1(2): 20–25.

____. (2015). Toxicity of chlorophyllin in different wavelengths of visible light against *Fasciola gigantica* larvae. *Journal of Photochemistry and Photobiology B: Biology* 144: 57–60. doi.org/10.1016/j.jphotobiol.2015.01.016

Singh D K and Agarwal R A. (1984). Correlation of the anticholinesterase and molluscicidal activity of the latex of *Euphorbia royleana* Bioss. on *Lymnaea acuminata. Journal of Natural Products* 47(4): 702–705. doi.org/10.1021/np50034a023

Singh D K, Singh V K and Kumar P. (2012). Pestiferous gastropods and their control. Germany: Lambert Academic Publishing GmbH and Co.

Singh O and Agarwal R A. (1981). Toxicity of certain pesticides to two economic species of snail in Northern India. *Journal of Economic Entomology* 74(5): 568–571. doi.org/10.1093/jee/74.5.568

Singh V, Singh V K and Singh D K. (2010). Seasonal variation in the toxicity of carbaryl to the snail *Lymnaea acuminata*: Implications for dosing regimes. *Australasian Journal of Ecotoxicology* 16: 1–8.

Sokal R R and Rohlf F J. (1996). *Introduction to biostatistics.* San Francisco: W. H. Freeman and Co., 271–273.

Soliman M F M. (2008). Epidemiological review of human and animal fasciolosis in Egypt. *Journal of Infection in Developing Countries* 2(3): 182–189. doi.org/10.3855/jidc.260

Sunita K, Habib M, Kumar P, Singh V K, Husain S A and Singh D K. (2016). Inhibition of acetylcholinesterase and cytochrome oxidase activity in *Fasciola gigantica* cercaria by phytoconstituents. *Acta Tropica* 154: 19–24.

Tominaga H, Kodama S, Matsuda N, Suzuki K and Watanabe M. (2004). Involvement of reactive oxygen species (ROS) in the induction of genetic instability by radiation. *Journal of Radiation Research* 45(2): 181–188. doi.org/10.1269/jrr.45.181

Tripathi A P, Singh V K and Singh D K. (2014). Attraction of *Lymnaea acuminata* against the visible photo stimulant: A new tool in fasciolosis control. *Research* 1: 743. doi.org/10.13070/rs.en.1.743

____. (2013). Orientation of the snail *Lymnaea acuminata* to different wavelength of visible light. *The Ecoscan* 4(spl.): 235–240.

Upadhyay A and Singh D K. (2011). Molluscicidal activity of *Sapindus mukorossi* and *Terminalia chebula* against the fresh water snail *Lymnaea acuminata*. *Chemosphere* 83: 468–474. doi.org/10.1016/j.chemosphere.2010.12.066

Wohllebe S, Richter P and Hader D P. (2012). Chlorophyllin for the control of *Ichthyophthirius multifiliis* (Fouquet). *Parasitology Research* 111(2): 729–733. doi.org/10.1007/s00436-012-2893-y

Wohllebe S, Richter R, Richter P and Hader D P. (2009). Photodynamic control of human pathogenic parasites in aquatic ecosystems using chlorophyllin and pheophorbid as photodynamic substances. *Parasitology Research* 104(3): 593–600. doi.org/10.1007/s00436-008-1235-6

Wohllebe S, Ulbrich C, Grimm D, Pietsch J, Erzinger G, Richter R, Lebert M, Richter P R and Hader D P. (2011). Photodynamic treatment of *Chaoborus crystallinus* larvae with chlorophyllin induces necrosis and apoptosis. *Photochemistry and Photobiology* 87(5): 1113–1122.