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Research Article
Effect of Photodynamic Product Chlorophyllin on Certain Biochemical Parameter in *Lymnaea acuminata*: Causative Agent of Fasciolosis

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Abstract

Background and Objective: Chlorophyll and chlorophyll derivatives are photosensitizer, which are capable to oxidize and reduce other molecules. The most extensively studied derivative of chlorophyll for their photodynamic activity against insect larvae is chlorophyllin, water-soluble derivative that can be obtained by removal of the phytol tail from the chlorophyll. The aim of present experiment to describe the certain biochemical/enzymatic changes in the gonadal tissues/nervous tissues of *Lymnaea acuminata* with the treatment of 20 and 60% of 24 h LC_{50} of extracted chlorophyllin in sunlight at 24 and 96 h in winter and summer season. **Materials and Methods:** Chlorophyll was extracted from spinach with the help of macerated leaves for 2 h in 100% ethanol at 55°C. Different biochemical assay estimation with sub-lethal concentration (20 and 60% of 24 h LC_{50}) of chlorophyllin were performed in treated and control group in winter sunlight and summer sunlight. **Results:** Among all the biochemical parameters the maximum reduction/inhibition was noted in the activity of DNA and AChE against *L. acuminata* in summer sunlight. Treatment of 60% of 24 h LC_{50} of extracted chlorophyllin at 96 h in sunlight caused maximum reduction in DNA (12.19% of control) in summer season. Exposure of 60% of 24 h LC_{50} of chlorophyllin for 96 h caused maximum inhibition in enzyme AChE activity (14.36% of control) in nervous tissues of snail in summer sunlight. **Conclusion:** Maximum variation in DNA and AChE activity by chlorophyllin in the gonadal tissue and nervous tissue of *L. acuminata* may be responsible for their molluscicidal activity and more effective in summer than winter season (sunlight condition).

Key words: Snail, fasciolosis, *F. gigantica*, spinach, chlorophyllin, biochemical parameters, enzyme

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Globally fasciolosis is the disease caused by two parasites *Fasciola hepatica* and *F. gigantica* (a liver fluke) Anuracpreeda *et al.*[1]. The secondary host of liver fluke *F. gigantica* is a hermaphroditic mollusc *Lymnaea acuminata* belonging to family Lymnaeidae inhabiting freshwater ponds and ditches[2]. Fasciolosis is a disease caused low fertility, reduced meat and milk yield in infected cattle population Tripathi *et al.*[3], Cwiklinski *et al.*[4]. Incidence of endemic fasciolosis is very common in the eastern region of the state of Uttar Pradesh in India[5] and it may directly transmit disease or they may serve as intermediate host/vectors for parasites of animals and human Borkakati *et al.*[6]. It has been reported that chlorophyllin is potent molluscicides Chaturvedi and Singh[7] Singh *et al.*[8]. Singh and Singh[5] reported cercaricidal activity of chlorophyllin against *Fasciola gigantica* larvae. Definitely no mortality is observed on condition that the larvae are not exposed to light, because chlorophyllin is not effective without light radiation Mahmoud *et al.*[9], Richter *et al.*[10], Hader *et al.*[11]. Recently, Singh *et al.*[8] have observed the mortality with photodynamically active chlorophyllin treatment against the snail *Lymnaea acuminata.*

The present experiment was aimed to study the effect of biochemical/enzymatic action of chlorophyllin in summer and winter season in the gonadal tissue/nervous tissue of snail *Lymnaea acuminata*, to explore the mode of action of photodynamic product chlorophyllin in the snail body.

MATERIALS AND METHODS

Preparation of chlorophyllin: Chlorophyllin was prepared by the method of Wohllebe *et al.*[12].

Biochemical estimation: Adult snails were collected locally and allowed to acclimatize at 25°C for 72 h. Batches of 20 snails in water were exposed to sub-lethal concentration (20 and 60% of 24 h LC_{50}) of chlorophyllin in sunlight condition in summer and winter season, respectively. Six batches were prepared for each concentration. Control aquaria contained only dechlorinated tap water without treatment. After 24 and 96 h (8 h duration in sunlight) and of the treatment the snails were removed from the aquaria and washed with fresh water. The gonadal tissue/nervous tissue were dissected out, placed on filter paper to remove the adherent water and weigh. Different biochemical assay estimation such as protein, amino acid, DNA, RNA and enzyme AChE were performed in treated as well as control group in winter sunlight and summer sunlight for 24 and 96 h. This experiment was done in winter season (November-February) and summer season (March-June) in the year 2015-2016. The ethical committee constituted by Department of Zoology DDU, Gorakhpur University Gorakhpur has been approved.

Estimation of protein: Quantitative assessment of protein was made according to procedure of Lowry *et al.*[13].

Free amino acid: Estimation of total free amino acids in the gonadal tissues of snails were made according to method of Spies[14] as modified by Singh and Agarwal[15]. The tissues were homogenized in 96% ethanol (10:1 W/V) in an electrical and centrifuged (8000 g × 20 min). In 0.1 mL of supernatant, 0.1 mL distilled water and 2.0 mL of ninhydrin reagent (1.0 gm ninhydrin in 25 mL of absolute ethanol to 0.44 gm of stannous chloride in 25 mL of citrate buffer pH, 5.0) were added. The reaction mixture was kept in boiling water bath for exactly 15 min. Two milliliters of 50% ethanol was added to above after cooling. A violet colour developed which was measured at 575 nm. Standard curves using the same procedure was drawn with known amount of glycine. Free amino acids have been expressed as μg mg^{-1} tissue.

Estimation of nucleic acids: Estimation of nucleic acids (DNA and RNA) in gonadal tissues of snail were performed by the methods of Schneider[16] using diphenylamine and orcinol reagents, respectively.

Acetylcholinesterase (AChE) activity: Acetylcholinesterase activity was measured according to the method of Ellman *et al.*[17] as modified by Singh *et al.*[18]. Fifty milligram of nervous tissue of *Lymnaea acuminata* was taken from around the buccal mass homogenized in 0.1 M phosphate buffer pH 8.0 for 5 min in an ice bath and centrifuged at 1000 g for 30 min at 4°C. The supernatant was used as an enzyme source. Enzyme activity was measured in 10 mm path length cuvette using an incubation mixture consisting of 0.1 mL of enzyme source, 2.9 mL of 0.1 M buffer, 0.1 mL of chromogenic agent DTNB (5,5′-ithio-bis-2 nitrobenzoate) and, 0.02 ml of freshly prepared ATChI (acetylthiocholine iodide) solution in distilled water. The change in optical density at 412 nm was recorded for 3 min after every 30 sec interval at 25°C. Enzyme activity was expressed as μmole sulphydril ('SH') hydrolyzed min^{-1} mg^{-1} protein.

Statistical analysis: The data have been expressed as Mean±SE of at least six replicates. Student's t-test was applied between control and treated groups to analyze significant changes ($p<0.05$) variations.

RESULTS

In sublethal 20 and 60% of 24 h LC_{50} (for 24 and 96 h exposure) treatment of chlorophyllin in sunlight condition during winter and summer season, respectively caused significant ($p<0.05$) reduction in the activities of protein, amino acid, DNA, RNA and inhibition in AChE in the gonadal tissue and in the nervous tissue of *Lymnaea acuminata*, respectively. Among both the condition 20 and 60% of 24 h LC_{50} (for 24 and 96 h exposure) the maximum reduction/ inhibition was observed in 60% of 24 h LC_{50} at 96 h in summer sunlight.

Exposure of 60% of 24 h LC_{50} of chlorophyllin for 96 h caused maximum reduction in protein (30.39% of control) and in amino acid (13.87% of control) in summer sunlight in gonadal tissue of *L. acuminata* (Table 1). Whereas, exposure of 60% of 24 h LC_{50} of chlorophyllin for 96 h caused maximum reduction in DNA (12.19% of control) and in RNA (56.27% of control) in summer sunlight in gonadal tissue of *L. acuminata* (Table 2). Further, exposure of 60% of 24 h LC_{50} of chlorophyllin for 96 h caused maximum inhibition in AChE activity (14.36% of control) in summer sunlight in the cerebral ganglion of *L. acuminata* (Table 3).

DISCUSSION

There was a significant ($p<0.05$) change in the endogenous level of protein, amino acid, DNA, RNA and enzyme AChE in gonadal tissue/nervous tissue by

Table 1: Effect of sub-lethal exposure (20 and 60% of 24 h LC_{50}) of chlorophyllin on the levels of protein (μg mg^{-1}) and amino acid (μg mg^{-1}) in the gonadal tissue of snail *Lymnaea acuminata*

		Protein		Amino acid	
Name of molluscicide	Treatment (mg L^{-1})	24 h	96 h	24 h	96 h
Winter control		0.300±0.0004 (100)	0.306±0.0004 (100)	14.60±0.11 (100)	14.45±0.11 (100)
	20% 24 h LC_{50} (19.11)	0.196±0.0002* (65.33)	0.116±0.0006* (38.66)	14.03±0.05* (96.09)	10.05±0.07* (68.83)
Ext Chl winter (sunlight)	60% 24 h LC_{50} (57.33)	0.183±0.0007* (61.00)	0.100±0.0008* (33.33)	3.34±0.20* (22.87)	2.20±0.08* (15.06)
Summer control		0.227±0.001 (100)	0.228±0.001 (100)	12.25±0.10 (100)	12.36±0.10 (100)
	20% 24 h LC_{50} (10.55)	0.101±0.0006* (44.49)	0.080±0.0003* (35.24)	11.02±0.23* (89.95)	8.70±0.0.05* (71.02)
Ext Chl summer (sunlight)	60% 24 h LC_{50} (31.35)	0.096±0.0004* (42.29)	0.069±0.0003* (30.39)	2.60±0.003* (21.22)	1.70±0.003* (13.87)

Each value is Mean±SE of six replicates, Value in parentheses is percent change with control taken as 100%, *Significant ($p<0.05$) when't' test was applied in between treated and control groups

Table 2: Effect of sub-lethal exposure (20 and 60% of 24 h LC_{50}) of chlorophyllin on the levels of DNA (μg mg^{-1}) and RNA (μg mg^{-1}) in the gonadal tissue of snail *Lymnaea acuminata*

		DNA		RNA	
Name of molluscicide	Treatment (mg L^{-1})	24 h	96 h	24 h	96 h
Winter control		23.51±0.56 (100)	23.45±0.56 (100)	37.52±0.17 (100)	37.91±0.17 (100)
	20% 24 h LC_{50} (19.11)	9.74±0.19* (41.42)	4.90±0.19* (20.84)	35.10±0.18* (92.58)	29.47±0.15* (77.73)
Ext Chl winter (sunlight)	60% 24 h LC_{50} (57.33)	6.81±0.005* (28.96)	3.75±0.13* (15.95)	24.26±0.12* (63.99)	21.14±0.08* (55.76)
Summer control		23.85±0.36 (100)	23.78±0.36 (100)	37.07±0.11 (100)	37.10±0.11 (100)
	20% 24 h LC_{50} (10.55)	8.19±0.16* (34.33)	3.62±0.14* (15.21)	25.62±0.13* (69.11)	22.25±0.17* (60.00)
Ext Chl summer (sunlight)	60% 24 h LC_{50} (31.35)	4.29±0.32* (17.98)	2.908±0.0003* (12.19)	21.93±0.24* (59.15)	20.86±0.13* (56.27)

Each value is Mean±SE of six replicates, Value in parentheses is percent change with control taken as 100%, *Significant ($p<0.05$) when't' test was applied in between treated and control groups

Table 3: Effect of sub-lethal exposure (20 and 60% of 24 h LC_{50}) of chlorophyllin on the levels of AChE (μg mg^{-1}) in the nervous tissue of snail *Lymnaea acuminata*

		AChE μmole 'SH' hydrolyzed min^{-1} mg^{-1} protein	
Name of molluscicide	Treatment (mg L^{-1})	24 h	96 h
Winter control		0.904±0.001 (100)	0.905±0.001 (100)
	20% 24 h LC_{50} (19.11)	0.407±0.0005* (45.02)	0.381±0.0003* (42.14)
Ext Chl winter (sunlight)	60% 24 h LC_{50} (57.33)	0.371±0.0005* (41.03)	0.230±0.0004* (25.44)
Summer control		1.357±0.0003 (100)	1.346±0.0003 (100)
	20% 24 h LC_{50} (10.55)	0.392±0.001* (28.88)	0.333±0.0003* (24.53)
Ext Chl summer (sunlight)	60% 24 h LC_{50} (31.35)	0.264±0.0003* (19.45)	0.195±0.0005* (14.36)

Each value is Mean±SE of six replicates, Value in parentheses is percent change with control taken as 100%, *Significant ($p<0.05$) when't' test was applied in between treated and control groups

chlorophyllin treated snails. Among all the biochemical parameters the maximum reduction/inhibition was noted in the activity of DNA and AChE against *L. acuminata* in summer sunlight. It seems that there is a cumulative effect of all biochemical parameters such as protein, amino acid, RNA and specifically DNA in the gonadal tissue of *L. acuminata* which affect may be direct or indirect through Caudodorsal cells which release Ovulatory hormone present in the nervous system of brain of *L. acuminata* Roubos *et al*.[19]. Amino acid is the chief substance of building block for structural proteins as well as enzymes and critical importance in energy metabolism of molluscs. Glutamic acid is an amino acid which represents the amino acid nitrogen pool for amino acid transferases to provide intermediates for TCA cycle (Tricarboxylic acid cycle) Nabih *et al*.[20]. The amino acid level significantly decreased in the gonadal tissue of snails exposed to different treatments in sunlight condition as compared to the snails with no any treatment. As a result of the diminution in free amino acid, there is a significant reduction in the levels of protein. A significant decrease in protein content might be mainly by reason of increase in messenger RNA deprivation and probable cause of hypoalbuminemia Metwally *et al*.[21]. Reduction in levels of protein in gonadal tissue of the treated snails may also be caused by the reduced synthesis of RNA along with DNA falls on treatment with chlorophyllin. Data emerging from the result section demonstrate that treatment following with sublethal concentration of chlorophyllin showed a sharp decline in the levels of RNA with the fact that RNA is an obligate precursor to protein synthesis has led to its use as indicator of growth rate Robbins *et al*.[22]. As a result of which changes in RNA content are often reflected by a change in protein synthesis rate Schlechtriem *et al*.[23]. A significant reduction is observed in total protein content in soft tissues of *Biomphalaria alexandrina* snails treated with *Azadirachta indica* plant extract Bakry *et al*.[24]. Reduction in level of nucleic acids viz. DNA and RNA was also considered in the treated snails in comparison to the control ones.

The photodynamic product chlorophyllin both are excited by light energy and produce the singlet oxygen and free radicals so these species sometimes severely damage nuclear membranes which promote "DNA damage" specifically Geiger *et al*.[25]. These species induces cellular apoptosis and damage the cell membranes and other structures of exposed cells, which immediately caused cell death[26,27]. DNA damaging is mostly caused by the hydroxyl radical (OH) Halliwell and Dizdaroglu[28]. The Caspases (C8-C9) trigger the activation of dozen or more effecter caspases that carry out death program through destruction of sub-cellular structures and genome Kroemer *et al*.[29]. The morphological manifestation of apoptosis can be recognized as degradation of various structures protein and DNA Godar[30]. So, the most common features of apoptosis are such as chromatin condensation, increase cytoplasmic Ca^{2+} concentration and most specifically fragmentation of nucleus leads to DNA damage[31]. There is a significant reduction in the level of DNA treated with 60% of 24 h LC_{50} of chlorophyllin in summer season (sunlight condition).

So, on conclusion it can be stated that the reduction in the level of DNA in summer season (sunlight condition) may be directly or indirectly affected by Caudodorsal cells as well as due to the photodynamic property or nature of chlorophyllin.

AChE is very essential enzyme, which is governed from the cells mediated through the brain of snail *L. acuminata*. There is correlation between AChE and Ovulatory hormones by which both are affected with each other so, due to the reduction in the level of DNA may also inhibit the activity of AChE in the nervous tissue of snail *L. acuminata*[32] Acetylcholinesterase is fundamental enzyme in transmission of nerve impulse in both vertebrates and in invertebrates which inhibition result in an increase the acetylcholine (ACh) level that causes permanent stimulation of the neural system and can lead to paralysis and finally death Upadhyay and Singh[33], Kumar *et al*.[34]. The mode of action of these compounds is to check the action of the parasite. The excessive build-up of the neurotransmitter acetylcholinesterase enzyme (AChE) plays an important role in nerve impulse transmission in all types of animals showing to various natural/unnatural stimulants might be due to interaction with the sulphydryl group of the enzyme AChE[35]. AChE is reported in a number of developmental stages of the parasite, i.e., in cercaria and some adult worms Sunita *et al*.[36,37]. Significant inhibition of AChE activity was noted in cercaria larva in each months of year 2011-2012 exposed to 60% of 4 h LC_{50} of citral, ferulic acid, umbelliferone, azadirachtin and allicin Sunita *et al*.[36,37]. *Terminalia arjuna* and the *Tamarindus indica* by its active constituents inhibit the acetylcholinesterase and phosphatase activity in the cerebral ganglion of *Lymnaea acuminata* Soni *et al*.[38]. Very recently effect of Chlorophyllin on *Biomphalaria alexandrina* Snails and *Schistosoma mansoni* Larvae studied by Elhadad *et al*.[39].

It can be concluded that maximum variation in DNA and AChE activity by chlorophyllin in the gonadal tissue and nervous tissue of snail *Lymnaea acuminata* may be responsible for their molluscicidal activity.

CONCLUSION

In conclusion, it can be affirmed from the photodynamic assets of chlorophyllin, has the competence to perform as a molluscicidal as well as larvicidal agent. Reduction/inhibition of DNA and AChE can be used as biomarkers because activities of both biochemical parameter and enzyme were significantly altered in treated snails. Though, the death of snail could not only by the single nervous tension of all these biochemical parameters and enzymes but also the seasonal changes and other metabolic changes in the snail *Lymnaea acuminata*, contributed to the demise of them. Consequently, these effects would be benefit in their exploitation against fresh water snails to control fasciolosis.

SIGNIFICANCE STATEMENTS

The present study revealed potent molluscicidal constituents from any green plant or conclusively, it can be stated that this photosensitive product chlorophyllin containing plant derived molluscicides can significantly alter the biochemical parameters and ultimately the developmental process of snail *Lymnaea acuminata* in winter and summer season. The depiction emerges from this study finally stated that summer season sunlight condition is suitable period for the control of the snail at threshold level which eventually diminish this incidence and needs the widespread research to successfully control fasciolosis.

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