Assessment the Effect Of Photodynamic Chlorophyllin On Biochemical Changes In The Cerebral Ganglion Of Snail *Lymnaea Acuminata*

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ABSTRACT

Purpose: The snail *Lymnaea acuminata* is the intermediate host of liver fluke *Fasciola gigantica* which caused endemic fasciolosis in the eastern Uttar Pradesh. Aim of the present study is to describes the effect of sublethal treatment (40% and 80% of 4h LC_{50}) of chlorophyllin on the level of different biochemical parameters viz. protein, amino acids, DNA/RNA, acetylcholinesterase, acid and alkaline phosphatase in the nervous tissue of *Lymnaea acuminata*.

Methods: Chlorophyll can be extracted from any green plant. In present study, chlorophyll obtained from spinach was transformed into water-soluble chlorophyllin in 100% ethanol by using different types of chemicals. 20 experimental snails were kept in glass aquarium containing 3l of dechlorinated tap water at 22-25°C. Snails were exposed to sublethal concentrations 40% and 80% of 4 h LC_{50} of the chlorophyllin in sunlight.

Result: Treatment of 80% of 4 h LC_{50} of chlorophyllin caused maximum reduction in protein (50.19% of control), amino acid (40.60% of control), DNA (56.14% of control), RNA (51.98% of control) and enzymes acetylcholinesterase (45.06% of control), alkaline phosphatase (56.54% of control) and acid phosphatase (63.17% of control) activity in nervous tissue of *L. acuminata.* Changes in the biochemical parameters in the nervous tissue of *L. acuminata* were time and concentration dependent. Withdrawal experiments showed that these changes were reversible.

Conclusions: Phytotherapy of snails by photodynamic chlorophyllin is a new approach to control fasciolosis in developing countries.

Keywords: *Lymnaea acuminata*; Protein; Nucleic acids; Amino acid; Photodynamic product;Chlorophyllin; Enzymes

INTRODUCTION

Fasciolosis is a global veterinary and human disease [1], [2]. It is caused by the digenetic trematode *Fasciola hepatica* and *F. gigantica;* having two hosts, a final mammalian hosts and a molluscan intermediate hosts [3], [4]. These flukes infect a wide range of mammals especially cattle and sheep [5], [6]. Incidence of fasciolosis in the cattle population is very common in the eastern region of the state of Uttar Pradesh in India [7]. An effective method to reduce the incidence of fasciolosis is to control the population of vector snails and thereby, break the life-cycle of these flukes [4], [8]. The continuous and indiscriminate use of synthetic pesticides for pest control has created the problem of acute and chronic toxicity to man and other non-target animals. Alternatively, new researches in different parts of the world have been focused on the plant derived molluscicides to reduce harmful snail number [9], [10]. Plant-derived molluscicides in general have an advantage over synthetic products, because natural products have eco-friendly, biodegradable and hence are less likely to accumulate in the environment [11], [12]. Earlier, it has been reported that chlorophyllin have potent larvicidal activity against *F. gigantica* and also potential molluscicidal activity against *Lymnaea acuminata* [13], [14]. The mechanism by which chlorophyllin cause snail death is not known. The aim of the present study is to evaluate the effect of sublethal treatment of chlorophyllin on different biochemical changes viz. protein, amino acid, nucleic acids

(DNA/RNA), acetylcholinesterase, alkaline and acid phosphatase in the nervous tissue of *Lymnaea acuminata*. Withdrawal experiments were also performed to study the reversibility of the effect on the snails.

MATERIALS AND METHODS

Preparation of chlorophyllin

Preparation of chlorophyllin was done according to the method of Wohllebe et al., [15] as modified by Singh and Singh [13]. Chlorophyll was isolated from spinach using 100% ethanol (for about 2 h at 55ºC). Then, $CaCO_3$ (about 1mg/g plant material) was added as a buffer, it prevent the transformation of chlorophyll into pheophytin. The extract was subsequently filtered using Whatman qualitative filter papers (Whatman International Ltd, UK) and 50 ml petroleum benzene was added. After shaking the mixture 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 KOH phase. The extract was stored in a dark flask at room temperature. However, only fresh chemicals were used in the course of these experiments.

Bioassay

Adult snail *Lymnaea acuminata* (2.60±0.30cm in length) were collected locally and allowed to acclimatize in laboratory condition for 72 h at 25°C. 20 experimental snails were kept in glass aquarium containing 3l of dechlorinated tap water at 22-25°C. Six aquaria were set up for each concentration. Snails were exposed to sublethal concentrations 40% and 80% of 4 h LC_{50} of the chlorophyllin in sunlight. Control aquarium contained only equal volume of dechlorinated tap water without treatment. Different biochemical assay were performed viz. protein, nucleic acids, amino acids, acetylcholinesterase, alkaline and acid phosphatase in the nervous tissue of *L. acuminata.* After 4 h of the treatment snails were washed out for the measurement of different enzyme activity. Nervous tissue was removed and placed on ice cubes. Afterwards, the nervous tissue was placed on filter paper to remove the adherent water and weight. Enzyme activity was performed in treated as well as control group of test animals.

Withdrawal Experiments

In order to study the effect of withdrawal from treatment, the snails were first exposed to 40% and 80% of 4 h LC_{50} of chlorophyllin in sunlight for 4 h following which they were transferred for next 24 h to fresh water. After 24 h biochemical parameters were estimated.

Biochemical Estimations

Protein

Quantitative estimation of protein was made according to the method of Lowry et al., [16]. The nervous tissue was homogenized (1.0 mg/ml, w/v) in 10% TCA using an electrical homogenizer for 5 min. Standard curves were prepared with different concentrations of bovine serum albumin. Results have been expressed as µg/mg tissue.

Nucleic acids

Estimation of nucleic acids (DNA/RNA) was performed by the method of Schneider [17] using diphenylamine and orcinol. Homogenate (1.0 mg/ml, w/v) of nervous tissue was prepared in 5% TCA at 90°C. The resulting suspension was centrifuged (5000g×20min) and clear supernatant solution was taken for the nucleic acids estimation. Standard curve were drawn at different concentration of calf thymus DNA to determine standard. Yeast RNA was used to determine standard. Results have been expressed as µg/mg tissue.

Free amino acids

Method of Spies [18] was used to estimate the total free amino acids. The nervous tissue homogenate (1.0 mg/ml, w/v) was prepared in 96% ethanol. Glycine was used to determine the standard. Free amino acids have been expressed as µg/mg tissue.

Acetylcholinesterase (AChE)

Acetylcholinesterase activity was measured according to the method of Ellman et al., [19] as modified by Singh and Agarwal [20]. Fifty milligram of nervous tissue of *Lymnaea acuminata* was taken around the buccal mass and homogenized in 1.0 ml of 0.1M phosphate buffer pH 8.0 for 5 min. in an ice bath then centrifuged at 1000 g for 30 min. at 4°C. The supernatant was used as an enzyme source. Enzyme using an incubation mixture

consisting of 0.1 ml of enzyme source, 2.9 ml of 0.1M buffer pH 8.0, 0.1 ml of chromogenic agent DTNB (5,5-dithio-bis-2-nitrobenzoic acid), 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 second interval at 25°C. Enzyme activity has been expressed as µ mole "SH" hydrolyzed/ min/mg protein.

Acid and alkaline phosphatase (ACP/ALP)

Phosphatases were measured by the method of Bergmeyer [21] modified by Singh and Agarwal [22]. Tissue homogenate (2%, w/v) was prepared in ice cold 0.9% NaCl and centrifuged at 5000 g for 20 min. at 4°C. The enzyme activity in the supernatant was assayed using 4-nitrophenyl phosphate disodium as substrate. The yellow color developed due to the formation of p-nitro-phenol, was determined colorimetrically at 420 nm. The acid (ACP) (pH 4.8) and alkaline (ALP) (pH 10.3) phosphatases activity have been expressed as µmole substrate hydrolysed/30min/mg protein.

Statistical analysis

Each experiment was replicated at least 6 time and values were expressed as mean±SE of six replicates. Student's t-test was applied to locate significant changes in between control and treated groups [23].

RESULTS

There was a significant ($p<0.05$) decrease in protein, amino acid, nucleic acids, acetylcholinesterase, acid and alkaline phosphatase levels in the nervous tissue of snail *L. acuminata* exposed to 40% and 80% of 4 h LC_{50} for 4h in sunlight with the plant derived molluscicide, chlorophyllin.

The levels of amino acids, DNA and RNA in the nervous tissue of control groups of *L. acuminata* were 45.91 µg/mg, 33.86 µg/mg and 44.26 µg/mg, respectively. Maximum reduction in total free amino acid (40.60% of control), RNA (51.98% of control) and DNA (56.14% of control) levels were observed in the nervous tissue of snails exposed to 80% of 4 h LC_{50} of chlorophyllin for 4h in sunlight, respectively (Table 1).

Significant decrease ($p < 0.05$) in protein levels were observed in the nervous tissue of *L. acuminata* exposed to 40% and 80% of 4 h LC_{50} of chlorophyllin. The level of protein in the nervous tissue of *L. acuminata* of control group was 0.251 µg/mg. Maximum reduction (50.19% of control) in the protein level was observed in the nervous tissue of snail exposed to 80% of 4 h LC_{50} of chlorophyllin for 4h in sunlight (Table 1).

Significant inhibition ($p < 0.05$) in acetylcholinesterase activity were observed in the nervous tissue of *L. acuminata* exposed to 40% and 80% of 4 h LC_{50} of chlorophyllin. The level of acetylcholinesterase in the nervous tissue of *L. acuminata* of control group was 1.62 µ mole "SH" hydrolyzed/min/mg protein. Maximum inhibition (45.06% of control) in the AChE activity was observed in the nervous tissue of snail exposed to 80% of 4 h LC_{50} of chlorophyllin for 4h in sunlight (Table 2).

The levels of acid and alkaline phosphatase in the nervous tissue of control groups of the snail *L. acuminata* were 12.49 µmole substrate hydrolyzed/30min/mg protein and 3.59 µmole substrate hydrolyzed/30min/mg protein, respectively. Maximum inhibition in acid phosphatase level (63.17% of control) and alkaline phosphatase (56.54% of control) levels were observed in the nervous tissue of the snail exposed to 80% of 4 h LC_{50} of chlorophyllin for 4h in sunlight, respectively (Table 2).

After 24 h in fresh water the withdrawal snails showed a significant ($p < 0.05$) recovery in the levels/activity of protein, amino acids, nucleic acids, acetylcholinesterase, acid and alkaline phosphatase in the nervous tissue of *L. acuminata* (Table 3). Treatment with 40% of 4 h LC_{50} of chlorophyllin showed maximum recovery in the nervous tissue of withdrawn snails after 24 h withdrawal period. The protein level recovered up to 88.44% of control, amino acids 77.02% of control, DNA 79.46% of control, RNA 84.61% of control, acetylcholinesterase 87.50% of control, acid phosphatase 92.01% of control and alkaline phosphatase activities recovered up to 90.25% of control. After 24 h, maximum recovery was noted in acid phosphatase activity in the nervous tissue of *L. acuminata* withdrawn to fresh water.

DISCUSSION

It is evident from the result section that the sublethal exposure to 40% and 80% of 4 h LC_{50} of chlorophyllin caused a significant change in certain biochemical parameters in the nervous tissue of *L. acuminata*. It indicates that the biochemical changes in the nervous tissue are the cause of mortality of these snails. Earlier, it had been reported that direct release of chlorophyllin in aquarium water have significant molluscicidal activity against *L. stagnalis, Biomphalaria* spp., *Physa marmorata* and *L. acuminata* [24], [14]. Mode of entry of molluscicide into the snail's body is through the body surface which cause effective killing of snails. It was reported by Kumar et al., [25] that there was a depletion of amino acids and reduction of protein and nucleic acids level in the ovotestis of *L. acuminata* when active molluscicidal components of *Ferula asafoetida, Syzygium aromaticum*

and *Carum carvi* was directly released in the aquarium. Amino acids level in the nervous tissue of snail exposed to chlorophyllin was significantly lower than control. It indicates that they also interfere with the biosynthesis of amino acids in the cell [25], [26]. Reduction in protein levels may be due to the direct interference of the chlorophyllin with the protein biosynthesis. The synthesis of protein in any of a tissue can be affected in two ways by a chemical, (I) it either affects the RNA synthesis at the transcription stage or (II) it somehow affects the uptake of amino acids in the polypeptide chain. Both these possibilities may account for the lower protein content in the affected tissue. In the first case, the RNA synthesis would be inhibited resulting in reduced RNA as well protein content. In the second case, only the protein content would be affected [27].

Chlorophyll derivates such as chlorophyllin is a photodynamically active substance [28], [29]. By simple chemical modifications hydrophobic chlorophyll can be transformed into water soluble chlorophyllin. Due to water solubility, chlorophyllin can be applied in aquatic environments. In general, photosensitizers are molecules which are excited by light [30]. Photodynamic reactions with oxygen lead to the formation of the highly reactive singlet oxygen, which can react with various biomolecules [31]. In addition, photosensitizers such as chlorophyll derivates are capable to oxidize and reduce other molecules. In the excited state chlorophyll is a strong reductant, but in the subsequent oxidized state chlorophyll is a strong oxidant, which may oxidize other biomolecules. As a result, reactive oxygen species (ROS), such as superoxide or hydrogen peroxide are formed posing strong oxidative stress to the cells. Excessive oxidative stress result in damage to cell membranes, proteins, DNA and other cell structures [32], [14].

Oxygen is one of the important ecological factors, essential for many metabolic processes that are vital to aerobic life [33]. Oxygen is used for various biochemical reactions; because of its two lone electrons of parallel spins, the molecular oxygen is stable. However, oxygen generates reactive oxygen species (ROS) by successive transfer of electrons which has highly cytotoxic effects [34], [35]. The ROS have strong reactivity and can potentially interact with all other cellular components such as protein, DNA and amino acids [36], [37]. Formation of activated oxygen can have extremely detrimental consequence not only for phospholipids but also protein, nucleic acids and inhibition of vital enzymes [38], [39], [40]. The amino acids most sensitive are those having sulphydryl groups such as methionine and tryptophan [41]. The nucleic acids are also targets for the free oxygenated radicals. The damage is not specific, simple or double cuts, formation of basic sites, covalent bond between DNA or DNA and proteins [36], [42]. The DNA damages are mainly caused by the hydroxyl radical 'OH' [43]. 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 reduction in DNA levels change in RNA level is certainly not surprising. The effects of chlorophyllin were investigated at the cellular level by using the transparent mosquito larvae *Chaoborus crystallinus* [15]. Reduction in the DNA levels in the nervous tissue of *L. acuminata* may be due to the genotoxic effect of chlorophyllin.

AChE is widely used as a biomarker in various toxicological studies [44]. Seasonal variation in AChE activity in the nervous tissue of the calm *Tapes philippinarum* [44] and snail *Cronia contracta* were noted in different Goa cost of India [45]. The enzyme AChE play an important role in animals exposed to various natural/unnatural stimulants and in nerve impulse transmission in both vertebrate and invertebrates [46]. Acetylcholinesterase inhibition results in the accumulation of acetylcholine at the nerve synapses, so that the post synaptic membrane is in a state of permanent stimulation producing paralysis, ataxia and general lack of co-ordination in neuromuscular system and eventual death [47], [25]. The results section clearly indicates that treatment with chlorophyllin caused a significant inhibition in AChE activity in the nervous tissue of *L. acuminata*. Any agonistic or antagonistic activity at the nerve cell signal and at the synapses will usually affect the physiology of the corresponding animal substantially. The causes of these effects might be due to interaction with the sulphydryl group of the enzyme AChE [48], [49], [50]. Usually inhibition of alkaline phosphatase activity was more pronounced than acetylcholinesterase. Animal behavior is a neurotropically regulated phenomenon which is mediated by neurotransmitter substances such as Ach [51]. The enzyme AChE is found in the synaptic regions and mediates transmission of impulses by breaking acetylcholine into acetic acid and choline [52]. The acetylcholine at neural and neuromotor regions upon accumulation causes hyper-excitability [53] which in turn might also influence behavior pattern of animals.

Alkaline phosphatase plays a critical role in protein synthesis [54], shell formation [55] and other secretory activities [56] in gastropod, its inhibition may result in reduction of protein level [22], [49]. It also plays an important role in transport of metabolites across the membrane [57]. The enzyme alkaline phosphatase was comparatively less inhibited than AChE and more inhibited than acid phosphatase. Acid phosphatase (ACP), a lysosomal enzyme [58] which plays an important role in autolysis, pathological necrosis and overall catabolism [59]. Acid phosphatase activity was reduced significantly although its increased concentration when may cause breakdown of existing protein [22].

Withdrawal experiment clearly shows the significant recovery in all biochemical parameters in the nervous tissue of *L. acuminata* which indicates the reversible effect of the molluscicide, chlorophyllin. Thus, the

reversibility in their mode of action added significant advantage in the use of photodynamic product chlorophyllin as a safer molluscicide for aquatic environment.

CONCLUSION

Conclusively, it can be stated from the phototoxico-dynamic property of chlorophyllin that it has the potential to act as a molluscicidal agent. However, the mortality of snail could not only by the single stress of these enzymes but also other metabolic changes in other organs of snail, contributed to the death of snail. Thus, reversibility of the effects would be an added advantage in their use against aquatic snails as they would cause only short-lived environmental toxicity, if any.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1: Treatment of sublethal concentrations (40% and 80% of 4h LC_{50}) of chlorophyllin on levels of protein, DNA/RNA and amino acids in the nervous tissue of *Lymnaea acuminata*.

Treatment	Concentration (mg/l) 4h LC_{50}	Protein (µg/mg)	DNA (µg/mg)	RNA (µg/mg)	Amino acids (µg/mg)
Control	_	0.251±0.0002 (100)	33.86±0.14 (100)	44.26±0.19 (100)	45.91±0.57 (100)
Chl in Sunlight	40% (132.40 mg/l)	0.155±0.0002* (61.75)	23.31±0.15* (68.84)	29.71±0.06* (67.12)	24.14±0.03* (52.58)
Chl in Sunlight	80% (264.80 mg/l)	0.126±0.0002* (50.19)	19.01±0.19* (56.14)	23.01±0.07* (51.98)	18.64±0.03* (40.60)

Values are mean±SE of six replicates. Values in parentheses are percent change with control taken as 100%; (*) significant ($p < 0.05$) when student's t-test was used for locating differences between experimental and control groups of test animals; abbreviation: Chl- Chlorophyllin.

Table 2: Treatment of sublethal concentrations (40% and 80% of 4h LC_{50}) of chlorophyllin on levels of Acetylcholinesterase, Acid Phosphatase and Alkaline Phosphatase in the nervous tissue of *Lymnaea acuminata.*

Treatment	Concentration (mg/l) 4h LC_{50}	Acetylcholinesterase (µmole 'SH' hydrolyzed /min/mg protein)	Acid Phosphatase (µmole substrate hydrolyzed/30min/mg protein)	Alkaline Phosphatase (µmole substrate hydrolyzed/30min/mg protein)
Control	_	1.62±0.002(100)	12.49±0.01(100)	3.59±0.01(100)
Chl in Sunlight	40% (132.40 mg/l)	0.88±0.002*(54.32)	8.98±0.01*(71.89)	2.25±0.003*(62.67)
Chl in Sunlight	80% (264.80 mg/l)	0.73±0.001*(45.06)	7.89±0.01*(63.17)	2.03±0.01*(56.54)

Values are mean±SE of six replicates. Values in parentheses are percent change with control taken as 100%; (*) significant ($p < 0.05$) when student's t-test was used for locating differences between experimental and control groups of test animals; Abbreviation: Chl- Chlorophyllin.

Table 3: Withdrawal treatment of sublethal concentrations (40% and 80% of 4h LC_{50}) of chlorophyllin in sunlight on levels of different biochemical parameters in the nervous tissue of *Lymnaea acuminata*.

Biochemical parameters	Control	40% of 4 h LC_{50}	80% of 4 h LC_{50}
Protein	0.225±0.0008(100)	0.199±0.0002*(88.44)	0.188±0.0002*(83.55)
DNA	18.94±0.34(100)	15.05±0.16*(79.46)	14.72±0.08*(77.71)
RNA	46.86±0.20(100)	39.65±0.04*(84.61)	38.19±0.04*(81.49)
Amino acid	40.04±0.12(100)	30.84±0.03*(77.02)	29.58±0.06*(73.87)
Acetylcholinesterase	1.04±0.005(100)	0.91±0.002*(87.50)	0.85±0.002*(81.73)
Acid Phosphatase	9.64±0.01(100)	8.87±0.002*(92.01)	8.57±0.004*(88.90)
Alkaline Phosphatase	4.31±0.01(100)	3.89±0.004*(90.25)	3.73±0.002*(86.54)

Values are mean±SE of six replicates. Values in parentheses are percent change with control taken as 100%; (*) significant ($p < 0.05$) when student's t-test was used for locating differences between experimental and control groups of test animals.