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Toxic Effects of Crude Aqueous Stem Bark and Leaf Extracts of *Phyllanthus niruri* on *Lymnaea acuminata*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Many aquatic snails like *Lymnaea* (Radix) *acuminate* Lamarck are well known carriers or vectors of larvae of trematodes and are known causing a harmful disease, fascioliasis. Thus, use of plant origin molluscides is being in practice as an alternative approach to prevent the transmission of snail-borne parasitic diseases. The aim of the present study is to evaluate the toxic effect and potency of the molluscicidal activity of aqueous stem bark and leaf extracts of the plant *Phyllanthus niruri* (a tropical small herb, commonly known as a Bhumi Amla), (family-Phyllanthaceae), on various tissues of the vector snail *Lymnaea acuminata*. After being exposed for 24 hours to sublethal doses i.e. 107.156µM and 214.312 µM (40% and 80% of 24h LC_{50} of *L.acuminata*), of the crude aqueous stem bark extracts and 95.97 µM and 191.93 µM (40% and 80% of 24h LC_{50} of *L.acuminata*), of the crude aqueous leaf extracts of *P.niruri* respectively, biochemical parameters were assessed in various snail tissues. Sub-lethal doses (40% and 80% of $LC_{50}$of 24h) of aqueous stem bark and leaf extracts of this plant show significant (P<0.05) alteration in the carbohydrates and nitrogenous

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metabolismsin nervous, hepatopancreas, and ovotestis tissues of the vector snail *Lymnaea acuminata* in time and dose dependent manner. After the withdrawal of toxic aqueous extracts, the snail tissues recovered in part after 7days completely. This study therefore signifies that, the aqueous extracts of stem bark and leaf of *P. niruri* may be used as a potent source of molluscicides; being less expensive, easily available, easily soluble in water.

Keywords: Snail; fascioliasis; metabolism; enzyme activity; phyllanthus niruri.

1. INTRODUCTION

Snails belong to the phylum Molluscs which comprise the second largest group of invertebrates. Many aquatic snails are well known carriers or vectors of larvae of trematodes and are thus known for causing a number of diseases, among which fascioliasis and schistosomiasis are responsible for causing immense harm to man and his domestic animals. For *Fasciola hepatica* and *Fasciola gigantica, Lymnaea acuminata* and *Indoplanorbis exustus* serve as vectors [1]. This snail is the intermediate host of *Fasciola hepatica* which causes endemic fascioliasis in cattle and sheep in the Northern section of India. Snail-borne parasitic diseases are serious parasitic infections that continue to be a major public health concern around the world, particularly in impoverished areas. Snails are the transmission vectors and intermediate hosts for a number of parasitic diseases that have affected millions of humans in about 90 different countries. Thus, an alternative approach to prevent the transmission of snail-borne parasitic diseases can be to focus on the elimination or control of snails. Synthetic chemicals biodegrade slowly, and preliminary evidence suggests that some populations of snail hosts may have developed resistance to them. The hazardous nature of synthetic pesticides has prompted the scientists to find less disruptive, newer techniques in controlling pests. Heavy use of synthetic pesticides have caused high rate of toxicity levels in water bodies, owing to their bioaccumulation and long time persistent nature. Plant molluscicides are currently receiving more attention from national and international institutions in the hopes that they will prove to be less expensive and more readily available than synthetic chemicals. As a result, plant molluscicide research has become multidisciplinary.

Plant molluscicides have been studied since the 1930s, when Archibald and Wagner proposed for the planting of the desert palms *Balanites aegyptiaca* and *B. maughamii* along the Sudan's and Southern Africa's waterways, respectively [2]. Through cytotoxicity and molluscicidal activity assays, the leaves and stem-bark of *Cassia renigera* were assessed against *Lymnaea acuminate* Lamarck adults. All other leaf extracts were shown to be effective besides the Petroleum ether extract [3]. In another research, the molluscicidal activity of the essential oils of *Cymbopogon nervatus* and *Boswellia papyrifera* was evaluated and confirmed against the snail *Lymnaea acuminata*, as plant origin molluscicides [4]. The concentration and time dependent molluscicidal efficacy of *Solanum surattense* against *L. acuminata*, has also been reported recently [5]. Being environment safe, Ipomoea batatas leaf extracts have been investigated for having potency in controlling golden apple snails especially methanol leaf extracts [6]. For controlling the snail species, *Monacha obstructa*, the aqueous plant extracts of S. nigrum has been found as an efficient molluscicide and thus can be readily use in integrated control programs as alternative of chemical pesticides [7]. In a recent study, the lethal concentration (LC_{50}) of *Brucea javanica* extract against freshwater snail *Oncomelania hupensis lindoensis* have been investigated, which has again turned out to be a promising molluscicide [8].

Phyllanthus niruri is an annual herb that grows wild along India's coasts. It has been employed in Indian ayurvedic systems since ancient times (about 2000 years), however it has a relatively short life span. *P.niruri* is a weed that belongs to the Phyllanthus genus, which contains 600-700 species with slight differences. It is an erect annual herb, which grows about 40 - 70cm in height and has ascending herbaceous branching; it shows glabrous and branching at the base. In the Indian ayurvedic system, *Phyllanthus niruri* plant extract is prescribed as medicine for a variety of ailments, including leprosy, anaemia, asthma, bronchitis, and urinary issues. Many plants belonging to this family phyllanthaceae have been extensively studied for their various pharmacological and molluscicidal activities. *Phyllanthus niruri* extract has been stated to block the calcium oxalate crystals formation [9]

and formation of stone in urolithiasis [10,11]. 20 crude extracts from nine African medicinal plants used in Kinshasa, Congo, with *Phyllanthus niruri*, has confirmed its anti-malarial activity [12, 13]. *Phyllanthu acidus* has been identified to have the molluscicidal activity of against *Pomacea canaliculata* (golden apple snail) [14]. The slugs treated with 8 ppm of *Phyllanthus niruri* extracts, resulted in the loss of the compact arrangement of prostate gland tubules, thus indicating the molluscicidal properties of the *P. niruri* extracts [15].

The aim of the present study was to report the effect of sub lethal exposure of aqueous crude extracts of stem and leaves of the plant *Phyllanthus niruri* on the carbohydrate and nitrogenous metabolism as well as on the metabolic enzyme systems of different tissues of the target snail species *Lymnaea acuminata,* hence confirming the molluscicidal nature of *P. niruri.*

2. MATERIALS AND METHODS

2.1 Test Plant

The plants under investigation, *Phyllanthus niruri* (Fig.1) were collected easily, during the rainy season, from the Botanical Garden and Identified by Faculty of Department of Botany Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur, U.P. India. *Phyllanthusniruri* (commonly called bhumi amla), belongs to the family Phyllanthaceae.

Fig. 1. *Phyllanthus niruri*(Photograph by Author)

2.2 Preparation of Aqueous Stem bark and Leaf Crude Extracts

Fresh Stem bark and leaves from *Phyllanthus niruri* were collected, minced with distilled water, homogenized for 5 minutes, and then centrifuged at 1000xg for around 10 minutes. The molluscicidal activity of the obtained supernatant was tested.

2.3 Test Animals

The target organisms for this research study, adult freshwater snails, *Lymnaea* (Radix) *acuminata* Lamarck (Fig. 2) (2.5 ± 0.9cm in shell height), were collected from pool alongside the campus of Veer Abdul Hameed P.G. College, Medical Road, Gorakhpur district (U.P). To acclimatize to laboratory settings, the collected creatures were maintained in glass aquariums with de-chlorinated tap water. Every 24 hours, the water in the aquariums was changed. Any dead animals were routinely removed to keep the water from being contaminated.

Fig. 2. *Lymnaea acuminata(Photograph by Author)*

2.4 Experimental Conditions

Experimental conditions of water were calculated using APHA/WPCF method [17]. Accordingly, the parameters and their values determined were as follows:

Atmospheric temperature: 29.0-30.0^{0}C, Water temperature: 19.0-21.0^{0}C, pH of water: 7.2-7.4, Dissolved Oxygen: 6.9-7.4, Free carbon dioxide: 4.6-6.7, Bicarbonate alkalinity: 110.0- 111.0.

Treatment protocol for dose- response relationship: *Lymnaea acuminata*, the freshwater snail under investigation, was kept in glass aquaria containing 3L de-chlorinated tap water. Each aquarium contained 30 experimental animals. *Lymnaea acuminata* was exposed for 24 h to sub-lethal doses, 107.156µM and 214.312µM (40% and 80% of 24h LC_{50} of *L. acuminata*), of the crude aqueous stem bark extracts and 95.97 µM and 191.93 µM (40% and 80% of 24h LC_{50} of *L. acuminata*), of the crude aqueous leaf extracts of *P.niruri*. Similar conditions, but without any treatment, were given to the control animals.

Table 1. Sub-lethal doses of stem bark and leaf extracts of *Phyllanthus niruri* plant used for biochemical estimation in the freshwater snail *Lymnaea acuminata* [16]

Plant	Plant parts	Doses (µM)	
		40% of LC_{50} of 24h	80% of LC_{50} of 24h
Phyllanthusniruri	Stem bark	107.156	214.312
	Leaf	95.97	191.93

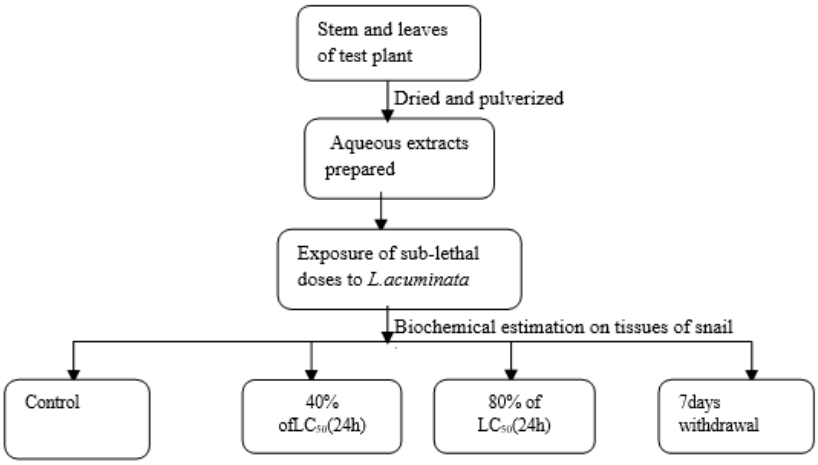

Chart 1. Study protocol

The test animals were taken out of the aquariums once the therapy was finished and given a freshwater wash. The tissues of *L. acuminate* ovotestis (OT), hepatopancreas (HP) and nervous systems (NT) were rapidly removed and placed in an ice tray for biochemical testing.

Lymnaea acuminata were exposed for 24 hours to sub-lethal doses of crude aqueous stem bark and leaf extracts of *P.niruri*, i.e. 214.312 µM and191.93 µM(80% of 24 h LC_{50} of *L. acuminata*) respectively, and then were switched to extract-free water to determine the effects of withdrawal from treatment. For the following seven days, this water was replaced every 24 hours. Following that, biochemical parameters were assessed in various snail tissues. Each experiment was replicated at least six times and the values have been expressed as means ±SE of six replicates. Student's t' test and analysis of variance were applied to locate significant changes [18].

2.5 Biochemical Estimation

Following biochemical estimations were done:

Protein: computed using the Lowry et al. (1951) technique and a standard of bovine serum albumin. In 10% TCA, homogenates (5mg mL-1, w/v) were created [19].

Total free aminoacid: calculated using the Spies (1957) technique. In order to estimate amino acid content, homogenates (10 mg mL^{-1}, w/v) were produced in 95% ethanol, centrifuged at 6000 g, and then utilized [20].

Nucleic acids (DNA and RNA): calculated using the Schneider (1957) method with the reagents diphenylamine and orcinol respectively. For estimation, homogenates (1 mg mL^{-1}, w/v) were produced. The DNA and RNA concentrations have both been given as g mg^{-1} tissue [21].

Glycogen: measured using the anthrone method developed by Van Der Vies in 1954 [22] and modified by Mahendru and Agarwal in 1982 [23] for the snail *L. acuminata*. In the current experiment, 5 mL of cold, 5% TCA were used to homogenise 50 mg of tissue. 1.0 mL of the filtrate from the homogenate's filtering was utilized for the test.

Pyruvate: based on estimates from Friedemann and Haugen [24]. In 10% TCA, homogenate (50 mg mL^{-1}, w/v) was made. Sodium pyruvate was used as the benchmark.

Lactate: calculated in accordance with Barker and Summerson [25], which Huckabee [26] amended. In 10% cold TCA, homogenate (50 mg mL^{-1}, w/v) was produced, use sodium lactate as a reference.

***Protease**:* based on an estimate from Moore and Stein [27]. Cold distilled water was used to make the homogenate (50 mg mL^{-1}, w/v), and the optical density was assessed at 570 nm. The enzyme's activity was measured in mol of tyrosine equivalents per milligramme of protein per hour.

Acid and Alkaline phosphatase: Approximated by the Bergmeyer (1967) technique, which Singh and Agarwal [28,29] modified. In ice-cold 0.9% saline, tissue homogenates (2% w/v) were made, and they were centrifuged at 5000 g and 0°C for 15 min. At 420 nm, optical density was evaluated in comparison to a simultaneously manufactured blank. The enzyme's activity was measured in terms of the amount of *p*-nitrophenol produced per mg of protein per minute.

Lactic dehydrogenase(LDH): calculated using the Sigma Diagnostics approach [30].In 1 mL of 0.1 M phosphate buffer, pH 7.5, homogenates (50 mg mL^{-1}, w/v) were produced and incubated for 5 minutes in an ice bath. The enzyme's activity is given as mol of pyruvate reduced per minute per milligramme of $protein^{-1}$.

Ssuccinic dehydrogenase(SDH): using the Arrigoni and Singer [31] approach, measure. In 1 mL of 0.5 M potassium phosphate buffer, pH 7.6, homogenate (50 mg mL^{-1}, w/v) was produced for 5 min in an ice bath. At 600 nm, the optical density was determined. The enzyme's activity is given as mol of dye decreased per minute per milligramme of $protein^{-1}$.

Cytochrome oxidase: recorded using the Cooperstein and Lazarow [32]technique . In order to determine the enzyme activity, homogenates (50 mg mL^{-1}, w/v) were produced in 1 mL of 0.33 M phosphate buffer, pH 7.4, for 5 min in an ice bath. The enzyme activity was then expressed in arbitrary $units min^{-1}$ mg $protein^{-1}$.

Acetylcholinesterase (AChE): calculated using the Ellman et al. (1961) technique [33]. After homogenising (50 mg mL^{-1}) in 0.1 M phosphate buffer, pH 8.0, for 5 min in an ice bath, the sample was centrifuged at 1000 g for 30 min at -4 °C.

3. RESULTS

3.1 Effect on Freshwater Target Snail

Data of sub-lethal doses of 40% and 80% of LC_{50} (107.156µM and 214.312 µM) of aqueous stem bark extract and (95.97 µM and 191.93 µM) of aqueous leaf extract exposure, and their recovery after 7th day withdrawal experiment of treatment, to the freshwater snail *L. acuminata* are given in Tables 2 to 5. Exposure of snails to sub-lethal doses of aqueous stem and leaves extracts for 24h caused significant alterations in the nitrogenous and carbohydrate metabolism in different body tissues of the freshwater snail *L. acuminata*.

Exposure of snails to sub-lethal doses of aqueous stem and leaves extracts for 24h caused significant alterations in the nitrogenous and carbohydrate metabolism in different body tissues of the freshwater snail *L. acuminata*.

Effect of aqueous stem bark extract of *Phyllanthus niruri* exposure and withdrawal effect on tissues of *L. acuminata*: Total protein and nucleic acids (DNA and RNA) levels were significantly reduced, while the free amino acid level was significantly enhanced in all body tissues after exposure to sublethal doses. Acid and alkaline phosphatase activities were significantly reduced, while the protease activity was increased after exposure. Total protein levels were reduced to 39%, 41%, and 40%, DNA level was reduced to 61%, 62%, and 58% , RNA level was reduced to 55 %, 50%, and 52% ,total free amino acid levels were induced to 143%, 137%, and 141% of controls after treatment with sublethal doses of 214.312 µM (80% of 24h LC_{50}), of aqueous stem bark extract of *Phyllanthusniruri*respectively, in nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*. The activity of acid phosphatase was inhibited to 65%, 72%, and 73% ,the activity of alkaline phosphatase was reduced to 56%, 62%, and 59% ,the protease activity was increased to 143%, 161%, and 150% ,the glycogen level was reduced to 52%, 57%, and 46% ,the pyruvate level was reduced to 53%, 59%, and 50%, the lactate level was increased to 150%, 159%, and 171% , the lactic dehydrogenase activity was reduced to 65%, 60%, and 66%. The activity of cytochrome oxidase was reduced to 61%, 56%, and 59%, the acetylcholinesterase activity was reduced to 64%, 67%, and 71% and the succinic dehydrogenase activity was increased to 158%, 153%, and 155% of controls after treatment with sublethal doses of214.312 µM (80% of 24h LC_{50}), of aqueous stem bark extract of *Phyllanthusniruri*, in nervous, hepatopancreas, and ovotestis tissue of *L. acuminata,* respectively (Table 2 and 3).

Table 2. Changes in total protein, total free amino acid, nucleic acid (DNA and RNA) (µg mg^{-1}), activities of protease (µg mol of tyrosine equivalents mg protein^{-1} h^{-1}), acid and alkaline phosphatase (amount of *p*-nitrophenol formed from (30) min^{-1} mg protein-^{1}) level in nervous (NT), hepatopancreas (HP),and ovotestis (OT) tissues of *Lymnaea acuminata* after exposure to sub-lethal doses of (40)% and(80)% of LC_{50} of 24h of aqueous stem bark extract of *Phyllanthus niruri*and after 7th day of withdrawal experiment

Parameter	Tissue	Control	40% of LC_{50}(24h)	80% of LC_{50}(24h)	7thday of withdrawal
Protein	NT	36.07±0.034(100)	23.10±0.190 (64)	14.22±0.210(39)	34.02±0.013(94)
	HP	28.07±0.330 (100)	15.10±0.030(54)	11.43±0.008 (41)	26.98±0.198 (96)
	OT	29.28±0.040(100)	15.27±0.010(52)	11.69±0.010(40)	28.02±0.010(96)
Amino acid	NT	28.58±0.008(100)	36.81±0.400^{+}(128)	40.81±0.019 (143)	30.71±0.012 (107)
	HP	18.01±0.004(100)	22.67±0.014(126)	24.63±0.940 (137)	19.51±0.004 (108)
	OT	23.83±0.005(100)	31.03±0.020(130)	33.61±0.049 (141)	24.32±0.025(102)
DNA	NT	23.65±0.084(100)	18.49±0.300^{+} (78)	14.45±0.099 (61)	22.08±0.075(93)
	HP	13.62±0.031(100)	11.08±0.041(81)	8.44±0.017 (62)	12.91±0.004 (94)
	OT	18.13±0.009(100)	15.06±0.028(83)	10.58±0.011 (58)	17.72±0.052 (97)
RNA	NT	19.62±0.019 (100)	13.72±0.051(70)	10.85±0.013(55)	19.12±0.014(97)
	HP	9.78±0.390 (100)	6.81±0.004^{+} (70)	4.82±0.009(50)	9.41±0.004(96)
	OT	12.80±0.020(100)	9.79±0.016 (76)	6.71±0.20(52)	12.60±0.006 (98)
Protease	NT	1.53±0.013(100)	2.14±0.008^{+}(139)	2.20±0.012 (143)	1.62±0.008^{+}(105)
	HP	0.71±0.004 (100)	0.95±0.004^{+}(133)	1.15±0.004^{+} (161)	0.74±0.007 $^{+}$ (104)
	OT	1.01±0.003(100)	1.36±0.008$^{+(}$134)	1.52±0.006 $^{+}$(150)	1.10±0.008^{+} (108)
Acid phosphatase	NT	1.61±0.008 (100)	1.36±0.010 (84)	1.04±0.010 (65)	1.52±0.012 (94)
	HP	0.76±0.006 (100)	0.71±0.004^{+} (93)	0.55±0.004 $^{+}$(72)	0.74±0.005^{+} (97)
	OT	0.82±0.004 (100)	0.73±0.006^{+} (89)	0.60±0.006^{+} (73)	0.75±0.009^{+} (92)
Alkaline phosphatase	NT	2.05±0.030 (100)	1.42±0.008^{+}(69)	1.14±0.008 $^{+}$ (56)	1.98±0.014 (96)
	HP	0.92±0.252(100)	0.61±0.004^{+} (66)	0.57±0.004 $^{+}$ (62)	0.90±0.006^{+}(97)
	OT	1.22±0.010(100)	0.76±0.006^{+}(62)	0.72±0.006 $^{+}$(59)	1.140±0.10 (94)

- $^{+}$, *Significant (P<0.05) Student's 't' test was applied between treated groups and withdrawal groups.*
- *Values are mean ± SE of six replicas.*
- *Values in parenthesis are percent change with control taken as 100%.*

Table 3. Changes in glycogen (mg g^{-1}), pyruvate (µmol g^{-1}),lactate (mg g^{-1}), activities of LDH (µmol pyruvate reduced min^{-1} mg protein^{-1}), SDH (µmol of dye reduced min^{-1} mg protein^{-1}), cytochrome oxidase (arbitrary units min^{-1} mg protein^{-1}), AChE (µmol of sulfohydryl min^{-1} mg protein^{-1}) in nervous (NT), hepatopancreas (HP),and ovotestis (OT) tissues of *Lymnaeaacuminata* after exposure to sub-lethal doses of (40)% and(80)% of LC_{50} of 24h of aqueous stem bark extract of *Phyllanthusniruri*and after 7th day of withdrawal experiment

Parameter	Tissue	Control	40% of LC_{50}(24h)	80% of LC_{50}(24h)	7thday of withdrawal
Glycogen	NT	14.36±0.012(100)	9.66±0.086 (67)	7.43±0.012(52)	13.62±0.008^{+}(94)
	HP	4.94±0.015(100)	3.76±0.008^{+} (76)	2.84±0.020(57)	4.74±0.006^{+} (96)
	OT	7.07±0.008 (100)	5.09±0.010 (72)	3.25±0.166 (46)	6.71±0.004^{+} (95)
Pyruvate	NT	7.24±0.014(100)	4.53±0.012 (63)	3.84±0.043 (53)	7.16±0.008^{+} (99)
	HP	3.02±0.004 (100)	2.06±0.027 (68)	1.77±0.004 $^{+}$(59)	2.91±0.00 (96)
	OT	4.36±0.004(100)	3.25±0.006^{+}(74)	2.16±0.008^{+} (50)	4.17±004^{+} (95)
Lactate	NT	8.41±0.008 (100)	10.84±0.012(129)	12.63±0.019 (150)	9.11±0.012^{+} (108)
	HP	3.20±0.004 (100)	4.03±0.004^{+} (126)	5.09±0.008^{+} (159)	3.57±0.004 $^{+}$(111)
	OT	5.38±0.006 (100)	7.47±0.042 (138)	9.23±0.012 (171)	5.89±0.008^{+}(109)
LDH	NT	0.34±0.008 (100)	0.26±0.008^{+} (76)	0.22±0.008^{+} (65)	0.313±0.006^{+} (92)
	HP	0.15±0.004 (100)	0.11±0.008 $^{+}$ (73)	0.09±0.008^{+} (60)	0.143±0.004(93)
	OT	0.12±0.006 (100)	0.09±0.003^{+} (75)	0.08±0.006 (66)	0.11±0.004^{+}(92)
SDH	NT	28.78±0.013 (100)	41.91±0.024 (145)	45.58±0.008 (158)	29.53±0.013(103)
	HP	19.42±0.007 (100)	26.64±0.007 (137)	29.85±0.012 (153)	19.96±0.008^{+} (102)
	OT	16.44±0.052(100)	23.12±0.017 (140)	25.46±0.001 (155)	17.09±0.006^{+}(104)
Cytochrome oxidase	NT	15.63±0.012 (100)	11.02±0.008^{+} (70)	9.58±0.008^{+} (61)	15.06±0.008^{+} (97)
	HP	9.76±0.006 (100)	6.70±0.025 (67)	5.51±0.004^{+} (56)	9.41±0.004 (96)

Parameter	Tissue	Control	40% of LC_{50}(24h)	80% of LC_{50}(24h)	7thday of withdrawal
	OT	12.57±0.004 (100)	7.87±0.006^{+} (63)	7.35±0.011 (59)	12.44±0.007^{+} (98)
AChE	NT	1.28±0.010 (100)	1.01±0.016 (79)	0.82±0.008^{+}(64)	1.21±0.008^{+}(95)
	HP	0.42±0.006 (100)	0.32±0.133 (76)	0.28±0.005^{+} (67)	0.40±0.004^{+} (96)
	OT	2.43±0.006 (100)	2.15±0.005^{+} (88)	1..71±0.006^{+} (71)	2.36±0.021 (97)

- $^{+}$*, Significant (P<0.05) Student's 't' test was applied between treated groups and withdrawal group.*
- *Values are mean ± SE of six replicas.*
- *Values in parenthesis are percent change with control taken as 100%.*

3.2 Withdrawal Effect

Following recovery was found in the tissues of snail on exposure to 80%of LC_{50} for 96h, as the levels of total protein increased (94% in nervous tissue, 96% in hepatopancreas and 94% in ovotestis), while recovery in total free amino acid (107%, 108% ,102%), DNA (93%, 94%, 97%), RNA (97%, 96%, 98%) , glycogen (94%, 96%, 95%), pyruvate (99%, 96%, 95%), lactate (108%, 111%, 109%) , AChE (95%, 96%, 97%), Cytochrome oxidase (97%, 96%, 98%), Protease (104%, 103%, 101%), Acid phosphatase (94%, 97%, 92%), Alkaline phosphatase (96%, 97%, 94%), LDH (92%, 93%, 92%) and SDH (103%,102%, 104%), were found in nervous tissue, hepatopancreas and ovotestis respectively, in comparison to the control.

Effect of aqueous leaf extract of *Phyllanthus niruri* exposure and withdrawal effect on tissues of *L. acuminata*: Total protein and nucleic acids (DNA and RNA) levels were significantly reduced, while the free amino acid level was significantly enhanced in all body tissues after exposure to sublethal doses of aqueous leaf extract of *Phyllanthus niruri*. Acid and alkaline phosphatase activities were significantly reduced, while the protease activity was increased after exposure. Total protein levels were reduced to 37%, 39%, and 40 %,the DNA level was reduced to 55%, 59%, and 62% , the RNA level was reduced to 51 %, 47 %, and 46%, total free amino acid levels were induced to 142%, 132%, and 139% , the activity of acid phosphatase was inhibited to 55%, 52%, and 57% , the activity of alkaline phosphatase was reduced to 54%, 55%, and 42% , the protease activity was increased to 135%, 132%, and 139% of controls after treatment with sublethal doses of 191.93µM (80% of 24h LC_{50}), of aqueous leaf extracts of *Phyllanthus niruri*, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*, respectively (Table 3 - 4).Glycogen and pyruvate levels were significantly reduced, while the lactate level was significantly enhanced after exposure to sub-lethal doses in all body tissues. Lactic dehydrogenase (LDH), cytochrome oxidase, and acetylcholinesterase (AChE) activities were significantly reduced, while the succinic dehydrogenase (SDH) activity was increased after exposure. The glycogen level was reduced to 42%, 45%, and 51% , the pyruvate level was reduced to 47%, 50%, and 47% , the lactate level was increased to 148 %, 142%, and 159%, the lactic dehydrogenase activitywas reduced to 55%, 57%, and 54%, the activity of cytochrome oxidase was reduced to 59%, 49%, and 56%, the acetylcholinesterase activity was reduced to 55%, 54%, and 59% and the succinic dehydrogenase activity was increased to 137%, 132%, and 138% of controls after treatment with sublethal doses of191.93 µM(80% of 24h LC_{50}), of aqueous leaf extract of *Phyllanthusniruri*, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata* (Table 4 and 5).

3.3 Withdrawal Effect

Following recovery was found in the tissues of snail on exposure to 80%of LC_{50} for 96h, as the levels of total protein increased (94% in nervous tissue, 95% in hepatopancreas and 94% in ovotestis), while recovery in total free amino acid (107%, 106% ,103%), DNA (93%, 94%, 91%) , RNA (97%. 95%, 98%) , glycogen (94%, 96%, 94%), pyruvate (97%, 94%, 94%), lactate (107%, 110%, 108%) , AChE (93%, 96%, 95%), Cytochrome oxidase (96%, 96%, 97%), Protease (104%, 101%, 107%), Acid phosphatase (96%, 97%, 92%), Alkaline phosphatase (92%, 95%, 93%), LDH (91%, 93%, 91%) and SDH (102%,103%, 109%), were found in respectively, in nervous tissue,hepatopancreas and ovotestis respectively comparison to the control.(Table 4 and 5).

Table 4. Changes in total protein, total free amino acid, nucleic acid (DNA and RNA) levels (μmg^{-1}), level activities of protease (μmol of tyrosine equivalents mg protein^{-1} h^{-1}) acid and alkaline phosphatase (amount of *p*- nitrophenol formed from (30)min^{-1} mg protein-^{1}) in nervous (NT), hepatopancreas (HP) and ovotestis (OT) tissues of *Lymnaeaacuminata* after exposure to sub-lethal doses of (40)% and(80)% of LC_{50} of 24h of aqueous leaf extract of *Phyllanthusniruri*and after 7th day withdrawal experiment

Parameter	Tissue	Control	40% of LC_{50}(24h)	80% of LC_{50}(24h)	7th day of withdrawal
Protein	NT	36.17±0.091 (100)	21.44±0.016 (59)	13.48±0.235(37)	33.97±0.008^{+}(94)
	HP	28.04±0.039(100)	13.10±0.068 (47)	10.86±0.08(39)	26.86±0.042(95)
	OT	29.65±0.026(100)	13.12±0.047 (44)	12.58±0.032(40)	27.9±0.010 (94)
Amino acid	NT	28.56±0.012(100)	35.35±0.072(124)	40.55±0.013(142)	30.66±0.008^{+}(107)
	HP	18.01±0.006(100)	21.90±0.033 (121)	23.86±0.080(132)	19.27±0.021(106)
	OT	23.83±0.004(100)	29.32±0.046(125)	32.59±0.061 (139)	23.98±0.006^{+}(103)
DNA	NT	23.64±0.012(100)	17.82±0.335 (73)	13.09±0.070 (55)	21.99±0.017(93)
	HP	13.65±0.039(100)	9.05±0.048 (66)	8.06±0.059 (59)	12.87±0.015(94)
	OT	18.36±0.004(100)	13.53±0.016 (73)	9.60±0.022(62)	16.75±0.004^{+}(91)
RNA	NT	19.65±0.016(100)	12.14±0.084(62)	10.01±0.017 (51)	19.06±0.008^{+}(97)
	HP	9.82±0.013 (100)	5.89±0.038(60)	4.69±0.027 (47)	9.39±0.004^{+}(95)
	OT	12.94±0.043(100)	8.11±0.010(63)	5.99±0.022(46)	12.71±0.004^{+}(98)
Protease	NT	1.54±0.013(100)	1.99±0.019 (129)	2.09±0.017(135)	1.61±0.036(104)
	HP	0.71±0.004(100)	0.85±0.009^{+}(120)	.94±0.016 (132)	0.72±0.01(101)
	OT	1.01±0.003(100)	1.27±0.008 (125)	1.41±0.010(139)	1.09±0.04 (107)
Acid phosphatase	NT	1.62±0.008(100)	1.24±0.12(76)	0.9±0.017 (55)	1.55±0.012 (96)
	HP	0.75±0.006 (100)	0.55±0.06 (73)	0.39±0.016(52)	0.73±0.004^{+} (97)
	OT	0.81±0.004(100)	0.58±0.012 (71)	0.56±0.100^{+}(57)	0.74±0.006^{+} (92)
Alkaline phosphatase	NT	2.04±0.031(100)	1.35±0.012(76)	1.10±0.016(54)	1.86±0.008^{+}(92)
	HP	0.94±0.006(100)	0.59±0.006^{+} (63)	0.51±0.004^{+}(55)	0.89±0.004^{+} (95)
	OT	1.20±0.008(100)	0.754±0.012 (61)	0.50±0.006^{+} (42)	1.12±0.012 (93)

- *+ , Significant (P<0.05) Student's 't' test was applied between treated groups and withdrawal groups.*
- *Values are mean ± SE of six replicas.*
- *Values in parenthesis are percent change with control taken as 100%.*

Table 5. Changes in glycogen (mg g^{-1}), pyruvate (μmol g^{-1}) ,lactate (mg g^{-1}) , activities of LDH(μmol pyruvate reduced min^{-1} mg protein^{-1}), SDH (μmol of dye reduced min^{-1} mg protein^{-1}), cytochrome oxidase (arbitrary units min^{-1} mg protein^{-1}), AChE (μmol of sulfohydryl min^{-1} mg protein^{-1}) in nervous (NT), hepatopancreas (HP),and ovotestis (OT) tissues of *Lymnaea acuminata* after exposure to sub-lethal doses of (40)% and(80)% of LC_{50} of 24h of aqueous leaf extract of *Phyllanthus niruri*and after 7th day of withdrawal experiment

Parameter	Tissue	Control	40% of LC_{50}(24h)	80% of LC_{50}(24h)	7th day of withdrawal
Glycogen	NT	14.48±0.08 (100)	8.28±0.056 (57)	6.04±0.014 (42)	13.56±0.012 (94)
	HP	4.94±0.015(100)	3.02±0.020 (61)	2.26±0.018(45)	4.77±0.003^{+} (96)
	OT	7.07±0.008(100)	4.05±0.013 (57)	3.58±0.029 (51)	6.68±0.006^{+}(94)
Pyruvate	NT	7.23±0.017(100)	4.05±0.065 (56)	3.40±0.014 (47)	7.06±0.008^{+}(97)
	HP	3.04±0.850(100)	1.80±0.062 (59)	1.55±0.008^{+}(50)	2.86±0.021 (94)
	OT	4.34±0.009(100)	2.58±0.049 (60)	2.04±0.009^{+}(47)	4.06±0.004 $^{+}$(94)
Lactate	NT	8.42±0.008(100)	10.41±0.026(123)	12.48±0.055(148)	9.09±0.008^{+}(107)
	HP	3.20±0.006(100)	3.85±0.058(120)	4.56±0.004^{+} (142)	3.55±0.004^{+}(110)
	OT	5.32±0.044 (100)	7.12±0.067(132)	8.46±0.049 (159)	5.83±0.008^{+}(108)
LDH	NT	0.33±0.008(100)	0.21±0.008(64)	0.18±0.008 (55)	0.30±0.008 $^{+}$(91)
	HP	0.14±0.004(100)	0.10±0.004^{+}(71)	0.08±0.004 (57)	0.13±0.004^{+}(93)
	OT	0.11±0.006(100)	0.07±0.008^{+}(63)	0.06±0.006 (54)	0.10±0.004^{+}(91)
SDH	NT	28.78±0.004(100)	35.06±0.07(127)	39.65±0.089 (137)	29.5±0.008 $^{+}$(102)
	HP	11.41±0.006(100)	23.94±0.028(122)	25.8±0.060 (132)	19.95±0.004^{+}(103)
	OT	16.36±0.006(100)	21.40±0.13(130)	22.36±0.005^{+}(138)	17.91±0.006^{+}(109)
Cytochrome oxidase	NT	15.64±0.006(100)	10.43±0.016 (67)	9.26±0.008^{+}(59)	15.04±0.010 (96)
	HP	9.76±0.006 (100)	6.28±0.06^{+} (65)	4.74±0.004^{+} (49)	9.39±0.004^{+} (96)

Parameter	Tissue	Control	40% of LC_{50}(24h)	80% of LC_{50}(24h)	7th day of withdrawal
	OT	12.73±0.014(100)	7.60±0.042 (60)	7.10±0.032 (56)	12.44±0.004^{+}(97)
AChE	NT	1.27±0.012 (100)	0.91±0.008^{+}(71)	0.69±0.012 (55)	1.18±0.008^{+}(93)
	HP	0.41±0.006(100)	0.30±0.014(73)	0.22±0.006^{+}(54)	0.39±0.007 $^{+}$ (96)
	OT	2.46±0.008(100)	2.07±0.033(84)	1.47±0.006^{+}(59)	2.36±0.004^{+}(95)

- $^{+}$*, Significant (P<0.05) Student's 't' test was applied between treated groups and withdrawal groups.*
- *Values are mean ± SE of six replicas.*
- *Values in parenthesis are percent change with control taken as 100%.*

4. DISCUSSION

The reduction of the protein fraction in the various tissues of the snails may have resulted from the breakdown of those proteins and the potential metabolic use of the degraded products. According to Mommensen and Walsh [34] proteins, which are the primary source of the nitrogenous metabolism, are primarily engaged in the architecture of the cell and also serve as a source of energy during extended periods of stress. The increment in the free amino acids level resulted in breakdown of protein for energy requirement and impaired incorporation of amino acids in protein synthesis. Inhibition of DNA synthesis might affect protein as well as amino acid levels by decreasing the level of RNA in the protein synthesis machinery [35] However, in any tissue total depletion of glycogen will not occur, because it would result in the disruption of enzyme systems associated with the carbohydrate metabolism [36] since the enzyme systems are associated with glycolysis and TCA cycle from a constitutive enzyme system.[37]Carbohydrates are the primary and immediate source of the metabolism.[38] Suggesting that, in stress conditions, carbohydrate reserves deplete to meet energy demand, thus depletion of glycogen may be due to direct utilization for energy generation, a demand caused by active moiety-induced hypoxia. The glycogenolysis seems to be the result of increased secretion of catecholamine due to stress. Higher energy demands during exposure result in a fall in pyruvate a level, which raises the probability of a switch to anaerobic dependency due to a striking decrease in oxygen consumption. The level of tissue lactic acid is known to act as an index of anaerobiosis which might be beneficial to the animal to tolerate hypoxic conditions. [39] The increase in lactate also suggests a shift towards anaerobiosis because of hypoxia leading to respiratory distress.[40] Lactic dehydrogenase catalyzes the interconversions of lactic acid and pyruvic acid during anaerobic conditions. Inhibition of lactic dehydrogenase and cytochrome oxidase activity shows that aqueous extracts of stem bark of *E. tirucalli* significantly inhibits the aerobic as well as anaerobic metabolism in exposed animals [41]. Succinic dehydrogenase is one of the active regulatory enzymes of the TCA cycle, while inhibition of cytochrome oxidase activity supports that Euphorbiales show a profound impact on the oxidative metabolism.

Withdrawal experiments were performed to see whether biochemical alteration caused by aqueous extracts of stem bark and leaf of *P. niruri* would return to normal, if the treatment ends. In the various body tissues of the freshwater snail *L. acuminata*, there was a nearly complete recovery of the total protein, total free amino acid, lactate, nucleic acid (DNA and RNA), and pyruvate level. There was also a partial recovery of the glycogen level.

5. CONCLUSION

The results showed that aqueous stem bark and leaf extracts of P.niruri induced toxicity to *Lymnaea acuminata* snails , and molluscicidal activity progressively increased with the extract concentration and exposure time. It is believed that the aqueous extracts of stem bark and leaf of *P. niruri* may be used as a potent source of molluscicides; being less expensive, easily available, easily soluble in water, and more safe for the non-target animals than synthetic molluscicides.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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