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Molluscicidal and Anti-cholinesterase Activity of *Alstonia scholaris* Plant Against Freshwater Snail *Lymnaea acuminata*

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Abstract: *Alstonia scholaris* (Family-Apocynaceae) is a common medicinal plant of India. The aqueous as well as partially purified extracts of stem bark and leaf of *Alstonia scholaris* has molluscicidal as well as *in vivo* and *in vitro* anti-cholinesterase (AChE) activity against the snail *Lymnaea acuminata*. The aqueous stem-bark extract shows strong molluscicidal activity in comparison to leaf at all exposure periods in time as well as dose dependent manner (LC_{50} value decreases from 665.82 mg L^{-1} (24 h) to 138.32 mg L^{-1} (96 h) and 940.45 mg L^{-1} (24 h) to 485.06 mg L^{-1} (96 h) for stem bark and leaf extracts, respectively). Same trend was observed in case of partially purified extracts. The anti-AChE activity of *A. scholaris* was also time as well as dose dependent. At LC_{90} (24 h) of aqueous and partially purified stem bark and leaf extracts of this plant parts did not cause any mortality among fish in a mix population of snails and fish, which support the view that plant product are safer in use as molluscicides for non-target organism.

Key words: *Lymnaea acuminata*, AChE inhibition, molluscicides, *Alstonia scholaris*

Introduction

The freshwater snail *Lymnaea acuminata* is vectors of *Fasciola hepatica* and *Fasciola gigantica* and very common in the Northern part of India, which cause fascioliasis in cattle population of this region (Hyman, 1970). Snail control is a neglected aspect for the control of fascioliasis. However, it is an essential to eliminate this vector for the controlling this important disease in this region. Heavy use of synthetic pesticides has created another serious problem for the aquatic animals (Mian and Mulla, 1992). Now a day, several countries accepted natural pesticide due to its ideal properties such as less expensive, easily available and safe for users. These plant pesticides are effective in action without environmental hazards than synthetic pesticides.

A large number of plant families, which possess natural toxic compounds like such as saponins, tannins, alkaloids, alkenyl phenol, di and tri-terpenoids are reported by several workers (Alard *et al.,* 1991; Hostettmann and Lea, 1987; Singh and Singh, 1993; Singh *et al.,* 1996; Singh *et al.,* 2000). *Alstonia scholaris* (Chitwan) is a common medicinal plant of India belongs to family Apocynaceae. Banerji and Banerji (1977) reported that a large number of alkaloids (pseudo-akuammigine, betulin, ursolic acid and β-sitosterol), steroids and triterpenoids are present in *A. scholaris* and responsible for toxicological action. While another side, different parts of this plant were used for several purposes like traditional medicine, insecticides and others (Chopra *et al.,* 1958).

The aim of this paper to evaluate the molluscicidal and anti-cholinesterase activity of aqueous and partially purified stem-bark and leaf extracts against snail *Lymnaea acuminata* because mechanism by which these compounds causes snail death is not known. An attempt has been also made to know the effect of sub-lethal doses of these extracts on non-target fish *Channa punctatus*, which share the habitat of target organism.

Materials and Methods

Plant: *Alstonia scholaris* was collected around Gorakhpur from their natural habitat in winter season. That plant was identified by Prof. S.K. Singh, (Taxonomist) Botany Department, D.D.U. Gorakhpur University, Gorakhpur (U.P.) India where voucher No. 1792 is deposited.

Snail: Adult freshwater harmful snail *Lymnaea acuminata* (2.6±0.3 cm in shell height and 1.2±0.1 in shell width) were collected from local ponds and other water lodging areas of Gorakhpur district of Uttar Pradesh and stored at least for 72 h in 20 L de-chlorinated tap water for acclimatization under laboratory condition before experiment. Experimental conditions of water determined by the method of APHA/WPCF (1998). Atmospheric temperature was ranging from 29.8-31.9°C and 25.8-27.2°C, respectively, pH of water was 7.4-7.9, while dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were ranging from 6.9-7.7, 4.5-6.6 and 105.8-109.7 mg L^{-1}, respectively for whole experiments.

Preparation of aqueous extracts: The fresh leaves and stem bark were mined in 5.0 mL of distilled water, homogenised for 5 min and centrifuged at 1000 g for 10 min. The supernatant was used as a water extract for the molluscicidal and anti-cholinesterase activity.

Partial purification of leaf and stem bark through organic solvents: The fresh stem bark and leaves were dried at 40°C over night and pulvorished in a mortar and pestle. For partial purification, dried powder of stem bark and leaf was sequentially extracted in Soxhlet apparatus, using 200 mL of five different organic solvents, viz. chloroform → carbon tetrachloride → acetone → diethyl ether → ethyl alcohol. The extracted material was dried through vacuum pump. The extracted dried powder was stored in airtight desiccator and used for further studies.

Toxicity experiments were performed by the method of Singh and Agarwal (1988). Ten experimental animals were kept in glass aquaria containing 3L de-chlorinated tap water. Control groups were kept in de-chlorinated tap water without any treatment. Each set of experiments, were replicated six times. Mortality was recorded after every 24 h up to 96 h. The LC values, UCL, LCL, 't' ratio, 'g' ratio and heterogeneity factor were calculated by the probit log method of Russell *et al.* (1977).

Table 1: Doses used for *in vivo* biochemical studies (Singh, 2000)

	Doses (mg L^{-1})			
Name of extract	40% of LC_{50} (24 h)	80% of LC_{50} (24 h)	40% of LC_{50} (96 h)	80% of LC_{50} (96 h)
Leaf	376.01	752.02	194.02	388.04
Stem bark	266.32	532.64	55.32	110.64

Table 1 shows doses used for the measurement of acetylcholinesterase activity in *in vivo* conditions in the nervous tissue of snails, after exposure to 24 - 96 h of 40 and 80% LC_{50} of the stem bark and leaf of *Alstonia scholaris*. Controls were kept in water alone.

The acetylcholinesterase activity was measured in the nervous tissue by the method of Ellman *et al.* (1961), as modified by Singh and Agarwal, 1982. 50 mg of nervous tissue dissected from buccal mass was homogenised in 1.0 mL of 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 enzyme containing supernatant (0.05 mL) was pipetted to a cuvette. To this was added $5x10^{-4}$M of freshly prepared acetylcholine iodide solution in distilled water as substrate, 1.45 mL of buffer (pH 8.0) and 0.05 mL of chromogenic agents, 5:5 dithio-bis-nitrobenzoate (DTNB). The change in optical density at 412 nm caused by the enzymatic reaction, was monitored for 3 min at 25°C.

In vitro enzyme activity was determined by the method of Singh and Agarwal (1982). This involves pre-incubation of the enzyme with the inhibitor. The stem bark and leaf of *A. scholaris* was suitably diluted in buffer and pipetted in the cuvettes; controls contained only buffer. Enzyme containing supernatant was then pre-incubated for 15 min at 25°C with the inhibitor following which cholinesterase activity was determined in the usual manner (Ellman *et al.*, 1961).

Protein estimation was done by the method of Lowry *et al.* (1951). Enzyme activity has been expressed as μ mol sulphhydril group (SH) hydrolysed min^{-1} mg^{-1} protein. Each experiment was replicated at least six times and data were expressed as mean±SE. Two-way analysis of variance and Student's `t' test were applied for locating significant differences (Sokal and Rohlf, 1973).

Results

The LC_{50} values of stem bark and leaf of *Alstonia scholaris* for periods ranging from 24 h or 96 h of snail *Lymnaea acuminata* is shown in (Table 2). The toxicity was time as well as dose dependent, as there was a significant negative correlation between LC_{50} values and exposure periods. Thus, the LC_{50} of aqueous extracts of stem bark of *Alstonia scholaris* for *Lymnaea acuminata* decreased from 665.82 mg L^{-1} (24 h) to 138.32 mg L^{-1} (96 h) and leaf extracts is 940.45 mg L^{-1} (24 h) to 485.06 mg L^{-1} (96 h), respectively (Table 2). Same trend was also observed in case of partially purified extracts of stem bark and leaf of *Alstonia scholaris* against *Lymnaea acuminata* respectively, (Table 3).

The slope values given in the (Table 2 and 3), were steep and heterogeneity factor was less than 1.0 indicates that the result found to be within the 95% confidence limits to LC_{50} values. The regression test ('t' ratio) was greater than 1.96 and the potency estimation test ('g' value) was less than 0.5 at all probability levels.

The treatment of snails with sub-lethal aqueous and partially purified dilution of the leaf and stem bark of *Alstonia scholaris* for 24 or 96 h of 40 and 80% of LC_{50} caused significant ($P<0.05$) inhibition of anti-AChE activity in the nervous tissue of snail *Lymnaea acuminata* of both the *In vivo* and *In vitro* treatment. Thus *In vivo* treatment of 24 h of exposure to 40% of LC_{50} of aqueous extracts of stem bark of *Alstonia scholaris* reduced the AChE activity of 90% of controls (Table 4). Similarly, 80% of 24 h, LC_{50} of aqueous stem bark extracts also significantly reduced AChE activity of control (Table 4). Analysis of variance demonstrated that the inhibition of AChE was both time and dose dependent ($P<0.05$). Stem bark is sequentially extracted with organic solvents also

Table 2: Toxicity (LC_{50}) of aqueous stem bark and leaf extracts of *A. scholaris* against freshwater harmful snail *L. acuminata* at different time intervals.

		Limits (mg L^{-1})					
Exposure periods	Effective dose (mg L^{-1})	UCL	LCL	Slope value	't' Ratio	'g' Factor	Hetero-Lgeneity
Stem bark							
24 h	LC_{50}= 665.82	709.20	454.84	2.79±0.60	4.62	0.17	0.29
48 h	LC_{50}= 427.07	609.72	354.47	2.30±0.51	4.55	0.18	0.17
72 h	LC_{50}= 295.71	406.11	227.82	1.58±0.46	3.41	0.33	0.12
96 h	LC_{50}= 138.32	180.36	72.67	2.01±0.49	4.09	0.23	0.21
Leaf							
24 h	LC_{50}= 940.45	1364.81	821.86	5.04± 1.30	3.86	0.25	0.16
48 h	LC_{50}= 882.69	950.45	750.80	2.57± 1.11	2.32	0.71	0.10
72 h	LC_{50}= 635.93	721.17	556.44	3.56± 1.08	3.28	0.35	0.11
96 h	LC_{50}= 485.06	540.55	373.63	4.74± 1.06	4.06	0.23	0.17

- Batches of ten snails were exposed to four different concentrations.
- Mortality was recorded every 24 h. Each set of experiment was replicated six times. Concentrations given are the final concentration (W/V) in the aquarium water.
- Regression coefficient showed that there was significant ($P<0.05$) negative regression between exposure periods and different values of LC_{50}.
- LCL - Lower confidence limits; UCL - Upper confidence limits.

Table 3: Toxicity (LC_{50}) of partially purified stem bark and leaf extracts of *A. scholaris* against freshwater water harmful snail *L. acuminata* at different time intervals

		Limits (mg L^{-1})					
Exposure periods	Effective dose (mg L^{-1})	UCL	LCL	Slope value	't' Ratio	'g' Factor	Hetero-Lgeneity
Stem bark							
24 h	LC_{50}= 334.18	401.22	254.80	2.15±0.63	4.14	0.18	0.19
48 h	LC_{50}= 214.23	303.76	214.37	2.35±0.61	3.52	0.17	0.13
72 h	LC_{50}= 145.27	206.32	129.85	1.67±0.56	3.67	0.39	0.15
96 h	LC_{50}= 70.65	90.33	62.69	1.95±0.50	3.34	0.26	0.24
Leaf							
24 h	LC_{50}= 460.13	512.34	354.40	2.67±0.89	4.45	0.22	0.25
48 h	LC_{50}= 413.24	502.12	295.56	2.56±0.69	4.59	0.19	0.19
72 h	LC_{50}= 315.45	423.15	259.22	2.63±0.54	3.94	0.32	0.20
96 h	LC_{50}= 202.24	302.34	132.45	2.59±0.52	3.65	0.21	0.27

• Serial extraction with organic solvents was carried out through $CHCl_3 \rightarrow CCl_4 \rightarrow CH_3COCH_3 \rightarrow C_2H_5OC_2H_5 \rightarrow C_2H_5OH$. •Details as given in table 2.

Table 4: *In vivo* effect on acetylcholinesterase activity was found in the nervous tissue of snail *Lymnaea acuminata* after treatment with 40% and 80% of the LC_{50} of the aqueous and serially extracted stem bark and leaf extracts of *A. scholaris* for 24 h and 96 h

			In vivo AChE activity (µM 'SH' Hydrolysed/min/mg protein)		
Plant parts	Nature of extracts	Exposure periods	Control	40% of LC_{50} (+, *)	80% of LC_{50} (+, *)
Stem bark	A	24 h	0.071±0.0004 (100)	0.040±0.0004 (90)	0.024±0.0003 (71)
		96 h	0.071±0.0004 (100)	0.062±0.0003 (87)	0.049±0.0003 (69)
	B	24 h	0.071±0.0003 (100)	0.064±0.0004 (90)	0.051±0.0003 (71)
		96 h	0.071±0.0003 (100)	0.045±0.0004 (63)	0.030±0.0002 (42)
Leaf	A	24 h	0.071±0.0004 (100)	0.063±0.0004 (88)	0.053±0.0003 (74)
		96 h	0.071±0.0004 (100)	0.042±0.0003 (59)	0.026±0.0003 (36)
	B	24 h	0.072±0.0004 (100)	0.006±0.0004 (91)	0.053±0.0004 (73)
		96 h	0.072±0.0004 (100)	0.046±0.0006 (63)	0.032±0.0002 (44)

- Values are mean ±SE of six replicates. • Values in parenthesis indicate per cent enzyme activity with control taken as 100%.
- Serial extraction with organic solvents was carried out through $CHCl_3 \rightarrow CCl_4 \rightarrow CH_3COCH_3 \rightarrow C_2H_5OC_2H_5 \rightarrow C_2H_5OH$.
- +, *, Significant ($P<0.05$) when analysis of variance was applied to see whether AChE inhibition was time (+) and concentration (*) dependent.
- A – Water extract; B – Serially extracted with organic solvents.

Table 5: *In vitro* inhibition of the activity of acetylcholinesterase (µM 'SH' hydrolysed/min/mg protein) in the nervous tissue of snail *L. acuminata* after 15 min pre-incubation with the aqueous and serially extracted stem bark and leaf extracts of *A. scholaris*

	AChE activity (µM 'SH' hydrolysed/min/mg protein) Stem bark (w/v) in incubation mixture				
Nature of extracts	Control	190.25 mg L^{-1++}	210.00 mg L^{-1++}	225.75 mg L^{-1++}	240.15 mg L^{-1++}
A	0.064±0.0014 (100)	0.059±0.0013 (81)	0.053±0.0007 (76)	0.051±0.0012 (69)	0.049±0.0012 (63)
B	0.066±0.0022 (100)	0.061±0.0013 (83)	0.058±0.0007 (79)	0.052±0.0013 (71)	0.050±0.0013 (66)
Leaf (w/v) in incubation mixture					
	Control	250.00 mg L^{-1++}	265.25 mg L^{-1++}	280.75 mg L^{-1++}	300.15 mg L^{-1++}
A	0.063±0.0013 (100)	0.056±0.0007 (80)	0.054±0.0012 (78)	0.051±0.0006 (70)	0.046±0.0013 (62)
B	0.065±0.0014 (100)	0.058±0.0009 (82)	0.056±0.0013 (79)	0.052±0.0012 (72)	0.048±0.0012 (64)

- Values are mean ±SE of six replicates. • Values in parenthesis indicate per cent enzyme activity with control taken as 100%.
- ++, Significant ($P<0.05$) when analysis of variance was applied to see whether AChE inhibition was concentration dependent.
- A – Water extract; B – Serially extracted with organic solvents.

caused a similar inhibition of AChE activity (Table 4). Thus after 24 h of exposure to 40% of the LC_{50} for *Alstonia scholaris* leaf of aqueous extracts reduced the AChE activity to 88% of controls (Table 4), respectively. Similarly, 80% of 24 h, LC_{50} of leaf of aqueous extracts reduced AChE activity to 74% of controls (Table 4), respectively. And same trend was also observed in case of partially purified leaf extracts of *Alstonia scholaris* (Table 4).

Acetylcholinesterase inhibition observed under the *In vitro* experiment was a direct result of the treatment with the stem bark and leaf of *Alstonia scholaris* or due to some unknown complex interactions with in the body of the snail *Lymnaea acuminata*. For that purpose nerve tissue extracts of untreated snails were pre-incubation for 15 min with aqueous as well as partially purified extracts. *In vitro* treatment with aqueous stem bark extracts of *Alstonia scholaris* caused significant ($P<0.05$) inhibition of AChE activity. Fifteen minutes pre-incubation with 190.25 mg L^{-1} aqueous stem bark extracts reduced the enzyme activity to 81% of controls while treatment with higher 240.15 mg L^{-1} was also observed in to reduced the enzyme activity to 63% of controls of *Alstonia scholaris* against nerve tissue of snail *Lymnaea acuminata* of controls respectively, (Table 5). Same trend was also observed in case of serially extracted stem bark of this plant against *Lymnaea acuminata*, respectively (Table 5). In case of aqueous extracts of leaf of this plant reduced the enzyme activity to 80% of controls while treatment with higher 300.15 mg L^{-1} reduced it to 62% of controls respectively, (Table 5). Same trend was also observed in case of serially extracted leaf of this plant against *Lymnaea acuminata*, respectively (Table 5).

Discussion

In beginning of experiments snail's behavioural changes appear within 5 to 10 min while primitive 30-40 min they become hyperactive and started crawling on each other like carbamate, organophosphates pesticides treated animals as reported by Singh and Agarwal (1984ab and 1990). Gill *et al.* (1991) reported that these behavioural anomalies are due to inhibition of cholinergic impulse by the hydrolysis of neurotransmitter acetylcholinesterase released during synaptic transmission. So the possibility cannot be ruled out in case plant extracts of *A. scholaris* caused behavioural changes.

As the poison enters in the snails body a muscular twitching and the snails become spirally twisted, which resulted ataxia, convulsion, paralysis and finally death of snails. The mortality data presented in aqueous and partially purified extracts of stem bark and leaf of *Alstonia scholaris* is lethal to snail *Lymnaea acuminata* (Table 2 and 3). Their mortality against snail is time as well as dose dependent. The increase in mortality with increase in exposure period could be due to several factors, which may be acting separately or jointly. For example, uptake of the active moiety of aqueous and partially purified extracts could be time dependent leading to a progressive increase in the titre of the active ingredient and its effects in the snail tissue or the active moiety of aqueous and partially purified extracts could be get converted into more toxic metabolites in the body of snail, resulting in a time dependent effect. Toxicity data also indicates that the aqueous and partially purified extracts of stem bark of *Alstonia scholaris* has the highest molluscicidal activity followed by leaf extracts of this plant against *Lymnaea acuminata* at all the exposure periods.

It is evident from the result that the stem bark and leaf of *Alstonia scholaris* has strong *In vivo* and *In vitro* inhibitory effects on acetylcholinesterase of *Lymnaea acuminata*. The inhibition of AChE activity was found to be both concentration and time dependent. The enzyme acetylcholinesterase occurs in the outer basal lamina of nerve synapses (Taylor, 1980), neuromuscular junction and in certain other tissues (Hall, 1973). It is responsible for the termination of cholinergic impulses by the hydrolysis of ACh, released during synaptic transmission; inhibition of AChE thus permits accumulation of ACh at the synapses which concentration rises several folds in comparison to the normal levels leading first to paralysis and then eventually to death, (Koelle, 1975; Singh and Singh, 2003). Results obtained from the present study indicate that the aqueous and partially purified both the extracts of this plant inhibit the acetylcholinesterase activity in *L. acuminata* in dose dependent manner. The dose dependent effect may be due to several factors. Singh and Agarwal (1982) reported a dose related response presumably due to conversion to more toxic metabolites in the body of freshwater snail *L. acuminata*.

Several studies on the mode of action and inhibition of acetylcholinesterase have been carried out for the last few decades. Indeed, inhibition of this enzyme is the focal target for most of the current synthetic pesticides. It has been established that the AChE enzyme unit consists of a negative subsite, which attracts the quaternary group of choline through both coulombic and hydrophobic forces and an esteratic subsite, where nucleophilic attack occurs on the acyl carbon of the substrate (Taylor, 1985).

The concentration dependent inhibition of AChE could be due to several factors such as rate of penetration of active moiety in the body of the animal, rate of inactivation, variability or increased competition with natural substrate at the active sites. It has been observed that carbamate and organophosphorus compounds (e.g. mexacarbate,

phorate etc.) also show a concentration related response probably due to conversion to more active metabolites in the body of the snails (Singh and Agarwal, 1982; 1983). It's clear from the above discussion that crude and partially purified extracts contains an anti-AChE moiety more potent than synthetic molluscicides. The author believes that these extracts may eventually be of great value of the control of snails and safe for other non-target organism.

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