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HPLC characterization of molluscicidal component of *Tamarindus indica* and its mode of action on nervous tissue of *Lymnaea acuminata*

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ABSTRACT

Background: Fasciolosis is a water-borne disease with gastropods snail (*Lymnaea acuminata*) act as key-link is still burden for mankind especially in developing countries. Snail control is one of the important tools to trim down the frequency of fasciolosis.
Objective: To evaluate the toxic effect and inhibitory potential of plant *Tamarindus indica* and their active constituent on the key enzyme of nervous tissue of snail *L. acuminata*.
Method: The present study deals with the chromatographic isolation and identification of molluscicidal component from *Tamarindus indica* bark and its effects on enzymes activities of vector snail *L. acuminata*.
Result: The toxicity study reveals that among all organic extract ethanol extract of *T. indica* bark (96 h LC_{50}:127.4 mgL^{-1}) was more effective than other organic extracts. The 96 h LC_{50} of column purified fraction of *T. indica* bark was 13.78 mgL^{-1} respectively. Saponin was isolated, characterized and identified as active molluscicidal component in the bark of *T. indica* by column chromatography, TLC and HPLC chromatographic methods. The *in vivo* and *in vitro* treatment of column purified fraction and saponin has significant inhibition in enzyme AChE, ACP and ALP activities. The study of inhibition kinetics indicates that inhibition of AChE and ALP is competitive, while ACP is uncompetitive in both the treatments.
Conclusion: Thus inhibition of these enzyme activities by *T. indica* bark column purified fraction and saponin in the snail *L. acuminata* may be the cause of its molluscicidal activity which leads snail death.

1. Introduction

The world wide liver fluke disease, fasciolosis is measured as one of the most important parasitic disease of domestic ruminants [1,2]. The causative agents of fasciolosis are flukes (Flat helminthes) *Fasciola hepatica* and *Fasciola gigantica* belonging to the genus *Fasciola* [3]. Occurrence of only *F. gigantica* species is reported in eastern Uttar Pradesh, India [4,5]. Fasciolosis causes significant economic losses to global agriculture estimated over 200 million US$ annually to the agriculture budget [6,7]. Human fasciolosis is also endemic in different parts of the world and now placed under neglected tropical disease [8]. About 2.4 to 18 million people are infected with *Fasciola* and 180 million people are at the risk of infection [9]. The snail *Lymnaea acuminata* act as carrier host of the liver fluke *F. gigantica* [5]. One of the possible approaches to control the fasciolosis is to disrupt the life cycle of *Fasciola* by killing the carrier snails *L. acuminata* [10]. The use of molluscicides either synthetic or plant derived has been and still is the most important method for controlling hosts population [11,12]. Synthetic molluscicides are toxic and hazardous to human being and ecosystem and cause serious environmental hazards [13]. Awareness in this field started the use of plant molluscicides which are ecologically safe, culturally and economically more acceptable [14,15]. The phytochemicals tannins, alkaloids, phenols, flavonoids, sesquiterpenes and glycosides find in different plant groups are isolated as potent molluscicides [16,17]. Pharmacological properties of plants *Tamarindus indica* (Family-Leguminosae) has been extensively explored by different workers [18,19]. In the present study, the molluscicidal activity of the bark powder of *T. indica* against the target snail *L. acuminata* was evaluated. The active molluscicidal component, responsible for snail death was identified, characterized and their mode of action on certain enzyme on acetylcholinesterase (AChE), acid

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phosphatase (ACP) and alkaline phosphatase (ALP) activity in the nervous tissue of *L. acuminata* were studied.

2. Materials and methods

2.1. Test animals

Adult *L. acuminata* (2.35 ± 0.30 cm in length) were collected locally from fresh water ponds and pools of Gorakhpur, Uttar Pradesh, India. Snails were acclimatized for 72 h in laboratory condition in dechlorinated tap water. Ten experimental snails were kept in glass aquaria containing 3 L of dechlorinated tap water at 24 ± 1 °C. The pH, dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.5–7.3 mgL^{-1}, 6.2–6.5 mgL^{-1} and 102–106 mg/L, respectively. The dead snails were removed after each observation to avoid any spoilage of the aquarium water.

2.2. Plants

Bark of *T. indica* (imli) was collected from Botanical garden of D. D. U. Gorakhpur University campus, Gorakhpur India and identified by retired Prof. S. K. Singh, plant taxonomist, Department of Botany D. D. U. Gorakhpur University Gorakhpur India.

2.3. Experimental design

2.3.1. Crude plant extract

The freshly collected stem barks of *T. indica* were kept in incubator at 45 °C for 72 h. The dried parts of were pulverized separately in electric grinder to obtained crude powders. The crude powder was then sieved with the help of fine meshed cotton cloth to obtain fine powder, this powder was then used for different toxicity experiments.

2.3.2. Organic solvent extract

Five gram of crude powder of bark of *T. indica* were extracted separately with 100 mL each chloroform (99%), ether (98%), acetone (99%), carbon tetra chloride (95.5%) and alcohol (95%) at the room temperature for 24 h. Each extracts was subsequently evaporated under vacuum at room temp. The residues thus obtained were used for determination of molluscicidal activity. The bark powder of *T. indica* stem yielded 83 mg of chloroform extract, 105 mg of ether extract, 97 mg of acetone extract, 120 mg of carbon tetra chloride extract and 170 mg of alcoholic extract.

2.4. Column chromatography

50 mL ethanol extract of stem bark *T. indica* extract was subjected to silica gel (60–120 mesh) Qualigens glass, Precious Electrochemical Industry, Pvt. Ltd. Mumbai, India Chromatography through 95 × 45 cm column. Elutent's of 5.0 mL will be collected from each column preparation of *T. indica*. Ethanol was evaporated under vacuum at 24 °C and the residues were used for the determination of molluscicidal activity.

2.5. Pure compounds

Saponin (Sopogenin-10%) was purchased from sigma chemical Co. U.S.A.

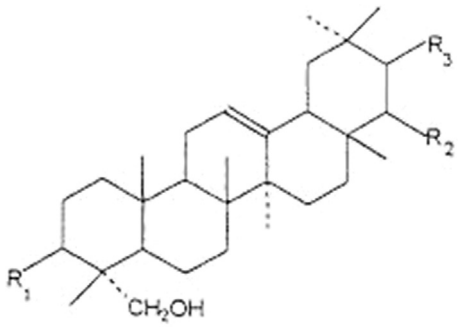


Fig. 1. Saponin.

2.6. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was performed by the method of Jaiswal and Singh [20] to identify active molluscicidal component present in the *T. indica* stem bark. TLC was performed on 20 × 20 cm precoated silica gel (Precious Electro Chemindus Industry Private Limited, Mumbai, India) using benzene/ethyl acetate (9:1, V:V) as the mobile phase. Co-migration of column purified fraction of plant along with its respective active component saponin was done for identification of molluscicidal components. TLC plate was developed by iodine.

2.7. High performance liquid chromatography (HPLC)

Identification of active component of present in *T. indica* bark was done by HPLC.

2.7.1. Sample preparation

The sample of *T. indica* bark was prepared by weighing 50 mg of column extract in 20 mL of acetonitrile. The samples were then properly vortexed to ensure proper dilution. The samples were properly vortexed to ensure dissolution. Prior to sample injection, the solutions were passed through a Millipore filter (ultra filter disc 3 K 43 mm 10 pk, Cole Parmer, Germany) to remove any undissolved particles.

2.7.2. Preparation of standard solution

Pure standard solution of saponin was prepared by weighing 10 mg, and then dissolve, it in 20 mL of acetonitrile. The mixtures were vortexed to ensure proper dissolution of pure compounds. The solutions, thus obtained, were passed through Millipore filter.

2.7.3. Instrumentation

The HPLC systems were equipped with two LC-10 AT VP pumps a cell CE 4201 UV variable detector and microliter ® # 702 (Hamilton- Bonaduz, Schweiz) syringe with a loop size of 20 μl. Reverse phase chromatography analysis was carried out in isocratic condition using a reverse phase Luna 5 μ C_{18} phenomenex column (250 mm × 4.6 mm) at 27 °C. Acetonitrile (HPLC grade) was used as mobile phase solvent under a pressure of 260–270 $kgf\ cm^{-2}$ and run time 15 min. The analysis was carried out at a flow rate of 1 mL/min with column effluent being monitored at 260 nm. Data acquisition was done with power stream™ software.

2.8. Toxicity experiment

2.8.1. Treatment protocol for concentration response relationship

Toxicity experiments were performed by the method of Singh and Agarwal [21]. Ten experimental snails were kept in glass aquarium containing 3 L of dechlorinated tap water. Snails were exposed continuously for 96 h to different concentration of plant products separately. Six aquaria were set up for each concentration. The control snails were kept in the equal volume of water under similar condition without any treatment. Mortality of snails was recorded at time interval of 24 h up to 96 h. The dead snails were removed immediately to avoid any contamination of aquarium water. The mortality of snail was established by showing discoloration of their shells, immobility, exposure of visceral mass, and release of mucus. The lower confidence values and upper confidence limits (LCL-UCL) slope value, t-ratio, g-value and heterogeneity factor were calculated by using Polo-Computer program software of Robertson et al. [22]. The regression coefficient between exposure time and different value of LC_{50} was determined by the method of Sokal and Rohlf [23].

2.9. Enzyme bioassay

2.9.1. In vivo treatment

Twenty experimental snails were kept in a glass aquarium containing 3 L of dechlorinated tap water. Six aquaria were set up for each concentration of 40% and 80% of 96 h LC_{50} of 24 h and 96 h exposure period of different column purified fraction of *T. indica* bark and its active component saponin (Table 1). Control aquarium contained only equal volume of dechlorinated tap water without treatment. After 24 h and 96 h of the treatment snail were washed with water the treatment snail were washed with water and nervous tissue was quickly taken out for the measurement of different enzyme activity such as AChE, ACP, and ALP. Nervous tissue was removed and place in ice cube. 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 in control group of test animal. In withdrawal experiments the snail were transferred from 24 h exposure of 80% of 96 h LC_{50} of different active components in to the freshwater then after 96 h of enzyme assays were estimated.

2.9.2. In vitro treatment

In *in vitro* treatments, column purified fraction and saponin of *T. indica* bark were (5, 7, 9 and 11 μg) were added to 10 mm path length cuvette. The ether was then allowed to evaporate. Each molluscicide was pre-incubated for 15 min at 25 °C with enzyme source and the enzyme activity was determined. The control treatment contains ether only.

Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were calculated by plotting Lineweaver–Burk plots for the hydrolysis of different concentration of substrate by the treated i.e.

Table 1
Toxicity of *Tamarindus indica* bark powder, different organic extract, column purified fraction and its active component against *Lymnaea acuminata* at different exposure period.

Exposure period	Treatment	LC_{50} (mg/L)	LCL	UCL	Slope value	t-ratio	g- value	Heterogeneity
24 h	*T. indica* bark powder	1517.26	1316.26	1869.91	4.70 ± 0.94	4.97	0.15	0.18
	Ethanol extract	201.31	185.29	238.83	6.59 ± 1.38	4.74	0.17	0.21
	Acetone extract	229.45	211.91	272.17	711 ± 1.54	4.61	0.18	0.20
	Ether extract	299.80	210.86	280.71	6.43 ± 1.50	4.27	0.21	0.22
	Chloroform extract	239.80	212.47	353.12	4.81 ± 1.43	3.35	0.34	0.56
	Carbon tetra chloride extract	221.81	207.76	251.18	8.00 ± 1.56	5.10	0.14	0.26
	Column purified	19.57	18.24	22.5	7.53 ± 1.57	4.77	0.16	0.28
	Saponin	17.65	16.24	21.00	6.31 ± 1.35	4.65	0.17	0.25
48 h	*T. indica* bark powder	1242.03	1139.85	1403.39	4.47 ± 0.86	5.15	0.14	0.27
	Ethanol extract	177.23	163.83	204.53	4.95 ± 1.17	4.21	0.21	0.25
	Acetone extract	200.07	184.61	217.42	7.39 ± 1.43	5.16	0.14	0.17
	Ether extract	214.04	196.95	262.71	5.18 ± 1.4	3.70	0.28	0.14
	Chloroform extract	197.73	185.65	219.53	6.04 ± 1.39	4.31	0.20	0.17
	Carbon tetra chloride extract	201.39	189.63	222.98	6.55 ± 1.41	4.64	0.17	0.19
	Column purified	16.88	15.95	18.26	6.67 ± 1.40	4.76	0.16	0.14
	Saponin	15.30	14.35	16.86	6.04 ± 1.23	4.87	0.16	0.11
72 h	*T. indica* bark powder	1004.20	896.75	1105.19	4.17 ± 0.85	4.89	0.16	0.16
	Ethanol extract	153.28	141.53	164.94	5.14 ± 1.48	4.71	0.17	0.21
	Acetone extract	177.89	166.79	188.71	6.71 ± 1.39	4.81	0.14	0.16
	Ether extract	176.18	163.80	187.57	6.24 ± 1.38	4.50	0.18	0.16
	Chloroform extract	174.14	163.61	183.49	7.34 ± 1.14	5.19	0.14	0.18
	Carbon tetra chloride extract	179.91	169.57	190.70	6.98 ± 1.39	4.99	0.15	0.20
	Column purified	14.86	13.55	15.82	6.00 ± 1.37	4.36	0.20	0.24
	Saponin	12.48	11.22	13.36	5.66 ± 1.21	4.67	0.17	0.13
96 h	*T. indica* bark powder	853.75	742.43	929.24	5.35 ± 0.96	5.56	0.12	0.16
	Ethanol extract	127.40	116.12	135.34	8.00 ± 1.28	6.24	0.09	0.45
	Acetone extract	155.83	145.47	162.97	10.31 ± 1.67	6.16	0.10	0.29
	Ether extract	150.51	135.73	159.46	8.61 ± 1.61	5.34	0.13	0.28
	Chloroform extract	160.04	150.54	166.98	10.12 ± 1.60	2.58	0.09	0.27
	Carbon tetra chloride extract	160.69	152.30	169.05	11.14 ± 1.66	6.67	0.08	0.30
	Column purified	13.78	12.84	14.44	9.45 ± 1.55	6.14	0.10	0.35
	Saponin	11.50	10.54	12.16	8.51 ± 1.39	6.11	0.10	0.31

Mortality was determined at every 24 h up to 96 h. Each set of experiment was replicated six times; Concentration given is the final concentration (W/V) in aquarium water. Significant negative ($P < 0.05$) was observed between exposure time and LC_{50} of treatments.
Ts, testing significance of the regression coefficient – *T. indica* bark powder, 9.31^{++}; Ethanol extract, 8.85^{+}; acetone, 8.14^{+}; ether extract, 6.50^{+}; chloroform, 14.58^{+}; Carbon tetra chloride, 15.02^{+}; column purified, 6.28^{++}; saponin, 7.37^{++}.
+, linear regression between x and y.
++, non-linear regression between log x and log y.
Abbreviation: *T. indica* seed powder = *Tamarindus indica* bark powder; LCL = lower confidence limit. UCL = upper confidence limits.

T. indica bark column purified and active component saponin (9.0 μg) and untreated enzyme [11].

2.9.3. Acetylcholinesterase

Acetylcholinesterase activity was measured according to the method of Ellman et al. [24] as modified by Singh et al. [25]. 50 mg of nervous tissue of *L. acuminata* was taken around the buccal mass and homogenized in 1.0 mL of 0.1 M 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 0.1 M 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 s interval at 25 °C. Enzyme activity has been expressed as μ mole "SH" hydrolyzed min/mg/protein.

For the estimation of kinetics constant (K_m) and maximum velocity (V_{max}) of AChE, *in vitro* experiment of enzyme was carried out at different concentration (3.0×10^{-4}, 5.0×10^{-4}, 7.0×10^{-4}, 1.0×10^{-3} M) of substrate acetylthiocholine iodide (ATChI).

2.9.4. Phosphatases

Acid and alkaline phosphatase activity in the nervous tissue of *L. acuminata* was measured by the method of Bergmeyer [26] as modified by Singh and Agarwal [27]. Tissue homogenate (2% W/V) was prepared in ice cold 0.9% NaCl and centrifuged at 5000 g for 15 min at 4 °C. The supernatant was used as enzyme source 0.2 mL of enzyme source was added to 1.0 mL of acid buffer subtract (0.41 g citric acid, 1.125 g sodium citrate, and 165 mg 4-nitrophenyl phosphatase sodium salt to 100 mL of double distilled water) for acid phosphatase measurement and pre-incubated at 37 °C for 10 min 0.1 mL of enzyme source was added to 1.0 mL of alkaline buffer substrate (375 mg glycine, 10 mg $mgcl_2.6H_20$, 165 mg 4-nitrophenol phosphate sodium salt in 42 mL of 0.1 N NaOH and a mixture was made up to 100 ml with double distilled water) for alkaline phosphatase measurement. The incubation mixture was mixed thoroughly and incubated for 30 min at 37 °C. 4.0 mL of 0.1 NaOH was then added to the incubation mixture. The yellow color developed due to the formation of 4- nitro phenol was due to the formation of 4-nitrophenol, was determined by spectrophotometer at 420 nm standard curve were drawn with different concentration of 4-nitro phenol. The ACP and ALP activities have been expressed as μ mole subtracts hydrolyzed 30 min/mg protein.

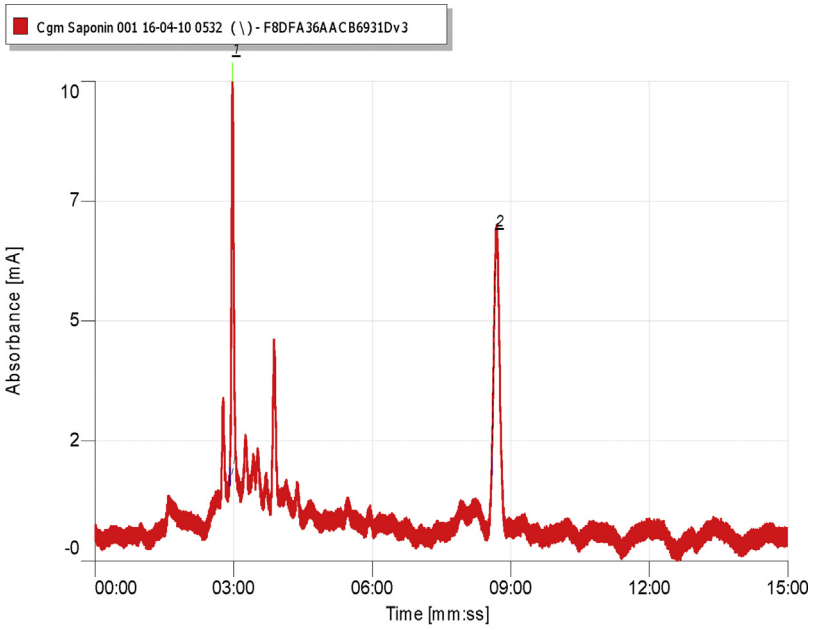
For estimation of kinetic constant (K_m) and maximum velocity (V_{max}) of acid and alkaline phosphatase, *in vitro* inhibition of the enzyme was carried out at different concentration (1.25×10^{-5}, 1.8×10^{-5}, 3.0×10^{-5}, and 5.4×10^{-5} M) of subtract p-nitrophenyl phosphate.

2.9.5. Protein

Protein was estimated in the enzyme source supernatant by the method of Lowry et al. [28].

2.10. Statistical analysis

Each experimental was replicated at least six times and results were expressed as mean ± SE of Six replicates. Student's test was applied between control and treated groups to locate significant ($p < 0.05$) variations [23].



Peak	Ret. Time(nm:ss)	Start Time(mm:ss)	End Time(mm:ss)	Area (mAS)	Hight (mA)
Saponin	02:58.3	02:58.4	03:2.3	27.4	8.3
***	08:39.9	08:35.3	08:40.1	0.5	0.2

Fig. 2. High performance liquid chromatography of pure component Saponin.

3. Results

3.1. Molluscicidal activity

The toxicity of crude and different organic solvent extracts of bark of *T. indica* against *L. acuminata* was time and concentration dependent. The 24 h LC_{50} and 96 h LC_{50} of crude bark of *T. indica* 1517.26 mgL^{-1}, 853.75 mgL^{-1}, against *L. acuminata*, respectively (Table 1). There was a significant negative correlation between the LC_{50} and exposure time of corresponding treatments. The maximum toxicity among all organic extract was noticed in ethanol extract (96 h LC_{50} 127.4 mgL^{-1}) (Table 1). Molluscicidal activity of column extract of bark was noted in 15th to 25th 5 mL fraction eluted from silica gel. The 96 h LC_{50} of column purified fraction of *T. indica* bark was 13.78 mgL^{-1} (Table 1). Active component saponin (96 h LC_{50} 11.50 mgL^{-1}) was highly toxic against *L. acuminata* (Table 1). In control group of snails no mortality were observed even up to 96 h of experimental time.

The slope values given in Table 1 were steep and separate estimation of LC based on each of six replicate were found to be within 95% confidence limit of LC_{50}. The t-ratio is higher than 1.96 and heterogeneity factor was less than 1.0 .The g-value was less than 0.5 at all probability level i.e. 90, 95, 99. There was negative regression ($p < 0.05$) between exposure time and LC_{50} treatment (Table 1). The Thin Layer Chromatography analysis demonstrates that the R*f* value of column purified fraction *T. indica* bark (0.68) were equivalent to R*f* value saponin (0.68).

The identification of active component was done by comparing the retention time *(Rt)* and chromatographic peaks of *T. indica* bark column purified sample with its respective active component saponin (Figs. 2 and 3). The HPLC fingerprint profile of the samples of *T. indica* bark showed major peaks at the retention time of 2.84 min respectively, whereas, the pure standard solution saponin showed major peaks at the retention time of 2.58 min respectively, indicate the presence of saponin on column purified sample.

3.2. In vivo and in vitro inhibition of enzyme activity

In vivo sublethal treatment of 40% and 80% of 96 h LC_{50} of active component of column purified bark of *T. indica* and its active component saponin caused significant inhibition in AChE, ACP and ALP activity in the nervous tissue of *L. acuminata*. In the control group of snail, acetylcholinesterase, acid phosphates and alkaline phosphates activity in the nervous of *L. acuminata* were 0.668 μmole "SH" hydrolyzed/minute/mg proteins, 20.22 and 18.11 μmole subtract hydrolyzed/30 min/mg protein respectively. Maximum inhibition in AChE (44.07% of control), ACP (37.93% of control) and ALP (29.21% of control) activity were observed in snail exposed to 80% of 96 h LC_{50} for 96 h exposure period of active component Saponin (Table 2).

In withdrawn experiments, significant recovery was observed in AChE, ALP and ACP activity in the nervous tissue of *L. acuminata*. Maximum recovery 76.5%, 85.29% and 90.96% of control in AChE, ACP, and ALP activity, respectively was noted in nervous tissue of snail withdrawn from 24 h treatment of 80% of 96 h LC_{50} of column purified fraction for next 96 h (Table 1).

In vitro pre-incubation of 5.0, 7.0, 9.0 and 11.00 μg of column purified fraction and active component (saponin) of *T. indica* bark caused significant concentration dependent inhibition in key enzyme activity. *In vitro* treatment with 11.00 μg saponin caused maximum inhibition in AChE (48.27% of control), ACP (41.03 of control) and ALP (34.52 of control) activities in the nervous tissue of *L. acuminata* (Table 3).

Lineweaver–Burk plot of column purified fraction and saponin shows inhibited and uninhibited enzyme activity at different substrate concentration. The plot shows that K_m and V_{max} of uninhibited AChE, ACP and ALP were 6.73×10^{-3} and 0.94, 1.42×10^{-5} and 28.57, 2.02×10^{-5} and 24.39 respectively. K_m of column purified fraction and saponin inhibited AChE were 12.71×10^{-3} and 10.65×10^{-3} respectively. V_{max} of column purified fraction and saponin inhibited AChE were 0.94 and 0.94 μmole 'SH' hydrolyzed/min/mg protein, respectively .K_m of column purified fraction and saponin inhibited ACP were 1.24×10^{-5} and 0.96×10^{-5} respectively. V_{max} of column purified fraction and saponin inhibited ACP were 22.67 and 21.88 μmole substrate hydrolyzed/30 min/mg protein, respectively. K_m of column purified fraction and saponin inhibited ALP were 3.23×10^{-5} and 2.97×10^{-5} respectively. V_{max} of column purified fraction and saponin inhibited ALP were 24.39 and 24.39 μmole substrate hydrolyzed/30 min/mg protein, respectively (Table 4).

4. Discussion

The results of the present study clearly demonstrate that the bark of *T. indica* is potent molluscicidal drug. Toxicity of crude powder reveals that the toxic substances present in the plant are soluble in water. Their toxic effects are time as well concentration

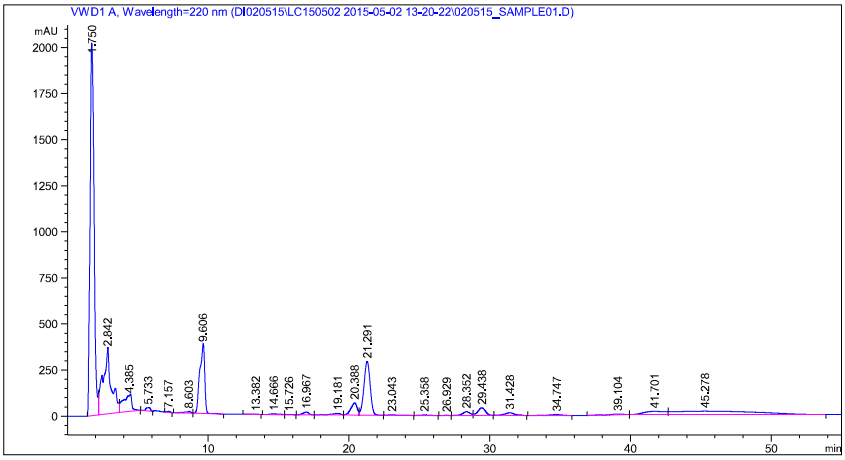


Fig. 3. High performance liquid chromatography of *T. indica* bark column purified fraction.

Table 2
In vivo effects of 24 h and 96 h exposure of subleathal concentration of column purified fraction of *T. indica* bark and its active component saponin on acetylcholinesterase (AChE), acid phosphatase (ACP) and alkaline phosphatase (ALP) activity on the nervous tissue of *Lymnaea acuminata*.

Enzyme	Treatment	Enzyme activity					
		Control	24 h Treatment		96 h Treatment		Withdrawal Control (0.653 ± 0.008) (100)
			40% of 96 h LC_{50} C.P. (5.51 mg/L) saponin (4.6 mg/L)	80% of 96 h LC_{50} C.P. (11.02 mg/L) saponin (9.2 mg/L)	40% of 96 h LC_{50} C.P. (5.51 mg/L) saponin (4.6 mg/L)	80% of 96 h LC_{50} C.P. (11.02 mg/L) saponin (9.2 mg/L)	
AchE	Column purified fraction	0.668 ± 0.004 (100)	0.578 ± 0.003 (86.52)	0.422 ± 0.001 (63.17)	0.468 ± 0.003 (70.05)	0.336 ± 0.002 (50.29)	0.594 ± 0.006 (90.96)
	Saponin	0.668 ± 0.004 (100)	0.587 ± 0.001 (87.87)	0.545 ± 0.004 (76.96)	0.448 ± 0.003 (67.06)	0.249 ± 0.008 (44.07)	0.546 ± 0.006 (83.60)
ACP	Column purified fraction	20.22 ± 0.05 (100)	16.52 ± 0.05 (81.70)	12.51 ± 0.06 (61.86)	8.89 ± 0.04 (43.96)	7.72 ± 0.05 (38.18)	14.99 ± 0.16 (76.5)
	Saponin	20.22 ± 0.05 (100)	13.84 ± 0.02 (68.64)	10.78 ± 0.03 (53.31)	8.33 ± 0.07 (41.19)	7.67 ± 0.02 (37.93)	13.63 ± 0.03 (69.57)
ALP	Column purified fraction	18.11 ± 0.07 (100)	14.29 ± 0.06 (78.95)	11.52 ± 0.06 (63.61)	8.47 ± 0.08 (44.78)	6.50 ± 0.07 (35.84)	15.16 ± 0.07 (85.29)
	Saponin	18.11 ± 0.07 (100)	15.72 ± 0.07 (86.80)	13.42 ± 0.02 (74.10)	6.32 ± 0.09 (34.89)	5.29 ± 0.03 (29.21)	15.83 ± 0.09 (81.68)

Values are mean ± SE of Six replicates. Values in parentheses indicate percent enzyme activity with control taken as 100%. Concentrations (W/V) have been expressed as final concentration in aquarium water. Acetylcholinesterase activity, μmol "SH" hydrolyzed/min/mg protein. Acid phosphatase activity, μmol substrate hydrolyzed/30 min/mg protein. Alkaline phosphatase activity, μmol substrate hydrolyzed/30 min/mg protein. C.P. (column purified fraction).* Significant ($P < 0.05$) when student's t-test was used for locating difference between treated and control group of snails.

Table 3
In vitro effects Column purified fraction of *T. indica* bark and its active component saponin on acetylcholinesterase (AChE), acid phosphatase (ACP) and alkaline phosphatase (ALP) activity on the nervous tissue of *Lymnaea acuminata*.

Enzyme	Treatment	Enzyme activity				
		Control	5.0 μg	7.0 μg	9.0 μg	11.0 μg
AChE	Column purified fraction	0.607 ± 0.002 (100)	0.503 ± 0.006 (82.86)	0.444 ± 0.001 (72.93)	0.347 ± 0.001 (57.16)	0.340 ± 0.001 (56.01)
	Saponin		0.442 ± 0.002 (72.81)	0.383 ± 0.0004 (63.07)	0.351 ± 0.0005 (57.82)	0.293 ± 0.0007 (48.27)
ACP	Column purified fraction	22.59 ± 0.01 (100)	16.61 ± 0.06 (73.52)	14.31 ± 0.03 (63.34)	12.56 ± 0.05 (55.59)	10.8 ± 0.04 (47.80)
	Saponin		16.84 ± 0.03 (74.54)	15.06 ± 0.03 (66.66)	11.38 ± 0.03 (50.37)	9.27 ± 0.03 (41.03)
ALP	Column purified fraction	22.40 ± 0.18 (100)	13.57 ± 0.07 (60.58)	11.14 ± 0.02 (49.75)	9.62 ± 0.04 (42.94)	8.30 ± 0.02 (37.05)
	Saponin		12.92 ± 0.04 (57.67)	9.89 ± 0.03 (44.15)	8.92 ± 0.04 (39.82)	7.51 ± 0.02 (34.52)

Values are mean ± SE of Six replicates. Values in parentheses indicate percent enzyme activity with control taken as 100%. Concentrations (W/V) have been expressed as final concentration in aquarium water. Acetylcholinesterase activity, μmol "SH" hydrolyzed/min/mg protein. Acid and alkaline phosphatase activity, μmol substrate hydrolyzed/30 min/mg protein. C.P. (column purified fraction).* Significant ($P < 0.05$) when student's t-test was used for locating difference between treated and control group of snails.

dependent, as evident from negative regression between exposure time and different LC_{50}. Time dependence toxic effect may be due to the more uptake of the active molluscicidal component inside the body of snail at higher exposure period or it may be possible that the active component could be changed into more toxic form in the aquarium water or in the snail body. Higher toxicity of ethanol extract among other organic solution indicates that active molluscicidal components present in plant extract are easily soluble in ethanol. Co-migration of column purified extract and pure compound on TLC plate demonstrate same R*f* value it indicate that probably molluscicidal activity of bark of *T. indica* is due to the presence of active component saponin. HPLC fingerprinting is the best way for chemical characterization [29]. High performance liquid chromatography studied indicates the presence of pure saponin (Rt value 2.58 min) in column purified extract of *T. indica* (Rt value 2.84 min). HPLC clearly indicate that saponin is active molluscicidal component in *T. indica* bark against *L. acuminata*. Saponins are potent molluscicides [30]. The plant of *T. indica* has known for greater pharmacological significance [19] and cytotoxic activity [31]. Hostettman and Lea (1987), noted that the bio product is potent molluscicides, if the LC_{50} is less than 100 ppm. In the present study 96 h LC_{50} of *T. indica* bark treatments are less than 100 ppm. Those plants, which have biotoxins, are of great interest due to their inherent combinations of chemical. It is harder for a snail to develop resistance to combinations of plant derived molluscicides than a single compound.

Among all organic extract ethanol extract (96 h LC_{50} 127.4 mgL^{-1}) was found to be highly toxic. The 96 h LC_{50} of column purified fraction of *T. indica* bark (13.78 mgL^{-1}) is lower than those of synthetic molluscicides *carbaryl* (14.4 mgL^{-1}), *phorate* (15.5 mgL^{-1}) [11] and column purified fraction of other plant derived molluscicide viz *Mimusops elengi* bark (16.27 mgL^{-1}), *Bauhinia variegata* leaf (19.72 mgL^{-1}) [32], *Moringa oleifera* leaf (22.52 mgL^{-1}) [15] (Table 1). It is evident from the steep slope value that the small increase in the concentration of different treatment

Table 4
Kinetics constant (K_m and V_{max}) of *in vitro* enzyme inhibition by *T. indica* bark column purified fraction and saponin (9.0 μg) in snail *Lymnaea acuminata*.

Treatments	Acetylcholinesterase		Acid phosphatase		Alkaline phosphatase	
	K_m (M)	V_{max}	K_m (M)	V_{max}	K_m (M)	V_{max}
Control	6.73×10^{-3}	0.94	1.42×10^{-5}	28.57	2.02×10^{-5}	24.39
T. indica bark (C·P.)	12.71×10^{-3}	0.94	1.24×10^{-5}	22.67	3.23×10^{-5}	24.39
Saponin	10.65×10^{-3}	0.94	0.96×10^{-5}	21.88	2.97×10^{-5}	24.39

Michaelis–Menten constant K_m and V_{max} of different enzyme were calculated from Lineweaver–Burk plots (1/V versus 1/S).

causes marked mortality in the snails. A t-ratio value is greater than 1.96 indicate that regression is significant. The value of heterogeneity factor is less than 1.0 denotes that in the replicate tests of random sample the concentration response line would fall within 95% confidence limits and thus model fits the data adequately. The index of significance of potency estimation value indicate that the value of mean are within the limits of all probability levels (90, 95, 99) as it is less than 0.5.

It is clear from the result section that *in vivo* and *in vitro* exposure to sublethal concentration of column purified fraction and saponin caused a significant inhibition of AChE, ACP, and ALP activity in the nervous tissue of snail *L. acuminata*. In the above experiment it has been found that active component saponin causes more AChE, ACP and ALP inhibition then column purified fraction of *T. indica* bark. Saponins are natural glycosides which possess a wide range of pharmacological properties including cytotoxic activity [33] and causes hemolysis of red blood cells [34]. AChE plays a significant role in nerve conduction process at myoneutral junction of nerve ending of muscle tissue [35]. Inhibition of AChE activity result in accumulation of acetylcholine at the nervous synapses, such that the post synaptic membrane in a state of a permanent stimulation, resulting in producing paralysis, ataxia, general lack of coordination in neurotransmitter system and eventual death [36,11,37].

Acid phosphatase is a lysozyme enzyme [38] which plays an important role in catabolism pathological nacrosis, autolysis and phagocytosis [39]. ACP is involved in immune defense in oyster [40,41]. ACP and ALP are involved in metabolic functions such as permeability, growth and cell differentiation, protein synthesis, absorption and transport of nutrients, gonodal maturation and steroid genesis [42]. ALP play a important role in protein synthesis [43], it may be possible that inhibition in ALP may cause reduction in protein level, shell formation and other secretary activities [44]. It play an important role in transport of metabolite across the membrane [45] in gastropods were also inhibited by treatment with column purified and saponin of *T. indic*a against *L. acuminata*. Result of the kinetic study clearly indicate that inhibition of AChE by Column purified fraction and saponin is competitive, as K_m value of inhibited and uninhibited enzyme were different, while V_{max} value were same (Same intercept $1/V_{max}$ on the Y axis of Lineweaver–Burk plots) (Fig. 4a and b). Inhibition of ACP by column purified fraction and saponin were uncompetitive, as evident from Lineweaver–Burk plot that the slope of inhibited and uninhibited ACP were not changed, both were parallel to each other, whereas intercepts of inhibited and uninhibited ACP on the y axis were different (Fig. 5a and b). The Km and V_{max} of control and inhibited enzyme were different. Inhibition of ALP by both of treatments was competitive because

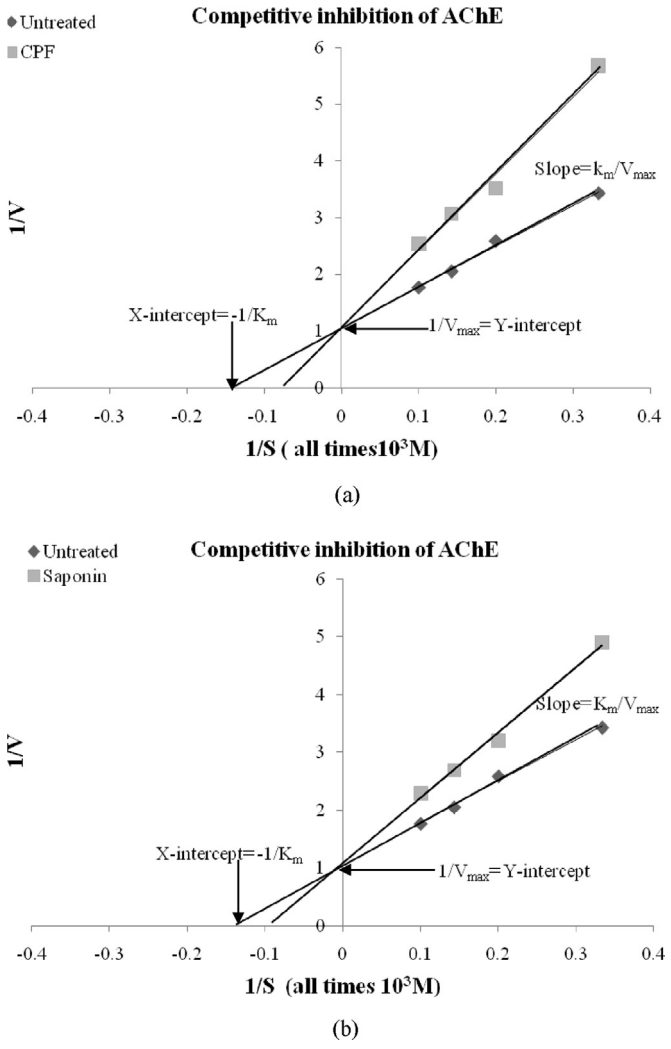


Fig. 4. Lineweaver–Burk plots showing the effects of column purified fraction (a) and active component saponin (b) of *T. indica* bark (9.0 μg) on the inhibition acetylcholinesterase (AChE) activity in the nervous of snail *L. acuminata*.

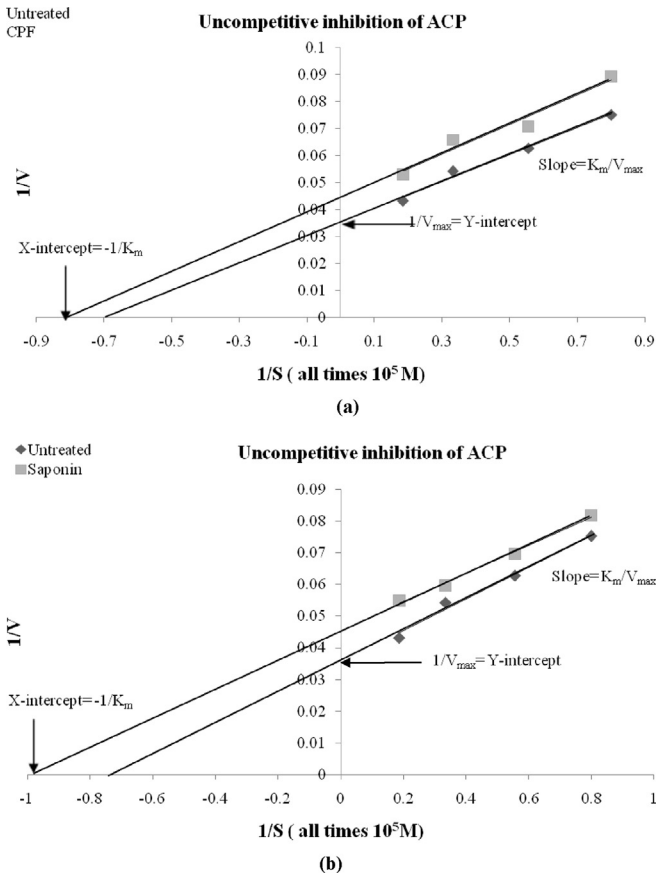


Fig. 5. Lineweaver–Burk plots showing the effects of column purified fraction and active component saponin of *T. indica* bark (9.0 μg) on the inhibition of acid phosphatase (ACP) activity in the nervous of snail *L. acuminata*.

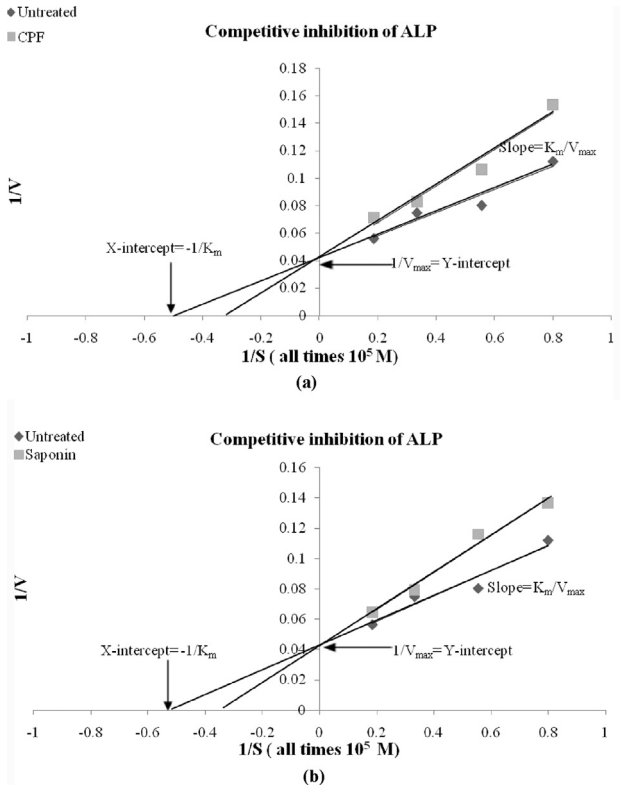


Fig. 6. Lineweaver–Burk plots showing the effects of column purified fraction (a) and active component saponin (b) of *T. indica* bark (9.0 μg) on the inhibition of alkaline phosphatase (ALP) activity in the nervous of snail *L. acuminata*.

the intercept of uninhibited and inhibited enzyme on the y intercept (1/V) axis is the same. Km value of inhibited and uninhibited enzyme was different while V_{max} values were same (Fig. 6a and b) It seems that alteration in enzyme activity in the nervous tissue of snails by the active component saponin present in *T. indica* bark may cause of snail death by inhibiting these enzymes directly/or indirectly. Withdrawal of snail from 80% of 96 h LC_{50} for next 96 h untreated water caused trend to recovery in enzymes activities indicates that treatment of column fraction of *T. indica* bark and its active component saponin caused reversible inhibition of these enzyme.

5. Conclusion

It can be concluded from present study that the *T. indica* bark have great potential with respect to synthetic molluscicides. Inhibition of enzymes (AChE, ACP and ALP) in the nervous tissue of *L. acuminata* by purified extracts (saponins) may be responsible for the molluscicidal activity of *T. indica*. Therefore, purified extracts can be used as potent molluscicides as they are easily available, eco-friendly and culturally more acceptable.

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None declared.

Conflicts of interest

None.

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