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 mg L$^{-1}$) was more effective than other organic extracts. The 96 h LC$_{50}$ of column purified fraction of *T. indica* bark was 13.78 mg L$^{-1}$ 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.

**Keywords:** fasciolosis, Snail, Molluscicidal drugs, Saponin, Enzyme

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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 mg L⁻¹, 6.2–6.5 mg L⁻¹ and 102–106 mg L⁻¹, 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 molluscidical 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. Eluent’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 molluscidical activity.

2.5. Pure compounds

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

2.6. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was performed by the method of Jaiswal and Singh [20] to identify active molluscidical component present in the *T. indica* stem bark. TLC was performed on 20 × 20 cm precoated silica gel (Precious Electroindus 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 molluscidical 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 isotropic condition using a reverse phase Luna 5 µ C18 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 LC50 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 LC50 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 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 LC50 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.

| 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 | LC50 (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 | 7.11 ± 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 | 7.53 ± 1.57 | 4.77 | 0.16 | 0.25 |
|                  | Column purified | 19.57 | 18.24 | 22.5 | 6.31 ± 1.35 | 4.65 | 0.17 | 0.25 |
|                  | 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 ± 1.14 | 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 | 0.20 |
|                  | 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 | 851.75 | 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 LC50 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.
2.9.3. Acetylcholinesterase

Acetylcholinesterase activity was measured according to the method of Elman 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-dithiobi-2-nitrobenzoic acid), and 0.02 mL of freshly prepared ATChI (acyethylthiocholine 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 (Km) and maximum velocity (Vmax) 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 substrate (0.41 g citric acid, 1.125 g sodium citrate, and 165 mg 4-nitrophenyl phosphate 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 mgCl2.6H2O, 165 mg 4-nitrophenol phosphate sodium salt in 42 mL of 0.1 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 substrates hydrolyzed 30 min/mg protein.

For estimation of kinetic constant (Km) and maximum velocity (Vmax) 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 substrate 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].
3. Results

3.1. Molluscidal 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 LC50 and 96 h LC50 of crude bark of *T. indica* 1517.26 mg L⁻¹, 853.75 mg L⁻¹, against *L. acuminata*, respectively (Table 1). There was a significant negative correlation between the LC50 and exposure time of corresponding treatments. The maximum toxicity among all organic extract was noticed in ethanol extract (96 h LC50 127.4 mg L⁻¹) (Table 1). Molluscidal activity of column extract of bark was noted in 15th to 25th 5 mL fraction eluted from silica gel. The 96 h LC50 of column purified fraction of *T. indica* bark was 13.78 mg L⁻¹ (Table 1). Active component saponin (96 h LC50 11.50 mg L⁻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 LC50. 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 LC50 treatment (Table 1). The Thin Layer Chromatography analysis demonstrates that the Rf value of column purified fraction *T. indica* bark (0.68) were equivalent to RF 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 LC50 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 substrate 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 LC50 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 LC50 of column purified fraction for next 96 h (Table 1).

*In vitro* pre-incubation of 5.0, 7.0, 9.0 and 11.0 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 11.0 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 $6.73 \times 10^{-3}$ and $0.94, 1.42 \times 10^{-5}$ and $28.57, 2.02 \times 10^{-5}$ and 24.39 respectively. $K_m$ of column purified fraction and saponin inhibited AChE were $12.71 \times 10^{-3}$ and $10.65 \times 10^{-3}$ 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 $1.24 \times 10^{-5}$ and $0.96 \times 10^{-5}$ 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 $3.23 \times 10^{-5}$ and $2.97 \times 10^{-5}$ 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-dependent. 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-dependent.
Table 2
In vivo effects of 24 h and 96 h exposure of sublethal 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 | 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) | 0.338 ± 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) | 15.08 ± 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 (34.32) |

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 | 40% of 96 h LC50 | 80% of 96 h LC50 | 40% of 96 h LC50 | 80% of 96 h LC50 |
|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control | 6.73 × 10⁻³ | 9.4 | 1.42 × 10⁻³ | 28.57 | 2.02 × 10⁻⁵ | 24.39 |
| T. indica bark (C.P.) | 12.71 × 10⁻³ | 9.4 | 1.24 × 10⁻³ | 22.67 | 3.23 × 10⁻⁵ | 24.39 |
| Saponin | 10.65 × 10⁻³ | 9.4 | 0.96 × 10⁻³ | 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 myoneural 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 [36,37,38].

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, gonadal 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 secretory 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. indica against L. acuminata. Result of the kinetic study clearly indicate that inhibition of AChE by Column purified fraction and saponin is competitive, as Km value of inhibited and uninhibited enzyme were different, while Vmax value were same (Same intercept 1/Vmax 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 Vmax of control and inhibited enzyme were different. Inhibition of ALP by both of treatments was competitive because

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**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.
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 LC50 for next 96 h untreated water caused trend to recovery enzymes directly/or indirectly. Withdrawal of snail from 80% of 96 h LC50 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 mollusccidal 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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Conflicts of interest

None.

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