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RESEARCH ARTICLE

Toxicological Safety Assessment of Molluscicides Against Non-target Aquatic Biota; *Colisa fasciatus*

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ABSTRACT

Plants *Terminalia arjuna* and *Tamarindus indica* are known to have a significant molluscicidal potential to control the population of vector snails. Since the molluscicides are considered an emerging pollutant and are frequently detected in surface water bodies and found to be a great threat for aquatic biota. Hence, the main objective of this research is to critically evaluate the ectotoxicological and chronic effect of plant-derived molluscicides on other aquatic biotas. With these references this study deals with the safety measurement of molluscicides arjnolic acid, saponin and procynadine from *T. arjuna* and *T. Indica* against the fish *Colisa fasciatus* which share the same habitat with snails. The result of toxicity experiment reveals that fishes showed no mortality against 24h LC_{90} (against *L. acuminata* and *I. exustus*) up to 96h exposure duration. The enzyme bioassays of these molluscicides on the nervous tissue of fish showed no significant effect on key enzymes Acetylcholinesterase, acid and alkaline phosphates activity in comparison to control group of fishes. These results indicated that the application of arjunolic acid, saponin, and procynadine derive from plant *T. arjuna* and *T. indica* at its maximum concentration (24h LC_{90} of *L. acuminata* and *I. exustus*) and exposure duration (96h) did not cause any mortality or treatment-related enzymes inactivity in fishes. The study conclusively proved the ecotoxicological and chronic safety of plant-derived molluscicides arjunolic acid, saponin, and procynadine on non-target animals in the aquatic environment.

Keywords: Molluscicides; Fish; Ectotoxicology; snails; Plants; Environmental safety; Snails

1 Introduction

The use of the molluscicides has been increased considerably to reduce the incident of fasciolosis, a food and waterborne disease caused by *fasciola* species commonly referred as liver flukes [1]. Among these molluscicides, plants derived molluscicides were commonly used because of their rapid biodegradability and non-persistent nature [2], [3], [4] [5]. These molluscicides, which frequently entered in the aquatic ecosystem through aquatic environment operations adversely, affect non-target animals such as fish [6], [7]. Medicinal plants are known to play an essential role in traditional and conventional medicine preparation [8]. The plant of *Terminalia arjuna* and *Tamarindus indica* are known to have high medicinal value in Indian traditional medicine [9], [10], [11], [12]. Since then the essays with this approach the two indigenous medicinal plants *Terminalia arjuna and Tamarindus indica* have successfully tested for their molluscicidal activity [2], [3], [12]. The molluscicidal component extracted from these two plant i.e arjunolic acid, saponin and procynadine significantly affect the brain tissue of targeted snails by affecting key enzymes AChE, ACP and ALP activities [12], [13]. The present study was carried out to explore the application safety of these molluscicidal drugs on non target aquatic biota such as, fish *Colisa fasciatus* and also study the effect of these molluscicidal drugs on the brain tissue of fishes, which share the same habitat with the fasciola vector snails i.e. *Lymnaea acuminata* and *Indoplanorbis exustus*.

2 Materials and Methods

2.1 Collection of Experimental Animals and Plant

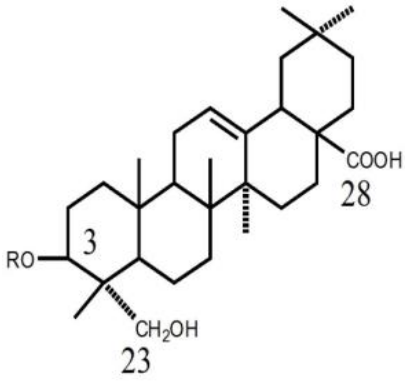
The Fish *Colisa fasciatus* (5.74±0.26 cm in length) were collected from Ramgarh lake of Gorakhpur District. The collected fish were stored in glass aquaria containing dechlorinated tap water for 72h in order to acclimatize these to laboratory conditions. Bark/seed of *Tamarindus indica* (imli) and *Terminalia arjuna* were 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.

2.2 Preparation of Column Purified Fraction for Experimental Analysis

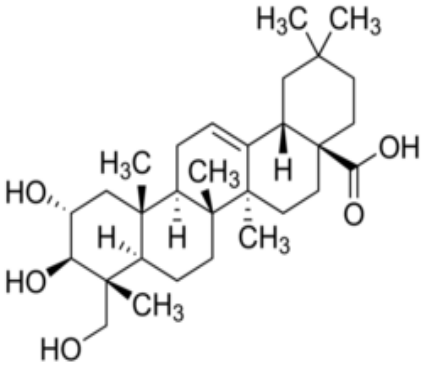
50 ml ethanol extract of each plant preparation i.e. bark, seed of *T. indica* and bark of *T. arjuna* were subjected to silica gel (60-120) mesh Qualigens glass, Precious Electro Chemindus Industry Privet Limited, Mumbai, India) Chromatography through 95×45 cm column. Five milliliters fractions of elutents were eluted with 95% ethanol for each column preparation. Ethanol was evaporated under vacuum and the remaining solids obtained from all 5 ml elutents were used for experimental study.

2.3 Pure Compounds

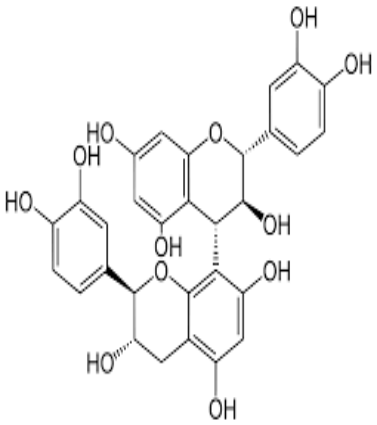
Saponin (Sopogenin-10%) Procynadine (cis, trans"-4,8"-Bi-(3,3',4',5,7-Pentahydroxyflavane), Arjunolic (2,3,23- Trihydrooxyolean-12-en, 28-oic acid) and purchased from Sigma Chemical Co., USA.



Saponin



Arjunolic Acid



Procynadine

2.4 Acute Lethal Fish Toxicity Test

Toxicity of column purified fractions and active (pure) components of *Tamarindus indica* and *Terminalia arjuna* were tested against the fish *Colisa fasciatus* to elucidate the toxic effect of molluscicides on other non target animal in aquatic environment. Ten fish were apportioned into each group and confined for an exposure of molluscicides at the interval of 96 h of dose of 24h LC_{90} (*L. acuminata* and *I. exustus*). Control group of fishes did not receive any treatment with all similar condition.

2.5 Enzyme Bioassay

Ten experimental fishes were kept in a glass aquarium containing 5 L of dechlorinated tap water. Six aquaria were set up for each concentration of 24h LC_{90} ((*L. acuminata* and *I. exustus*) for 96h exposure period of different column purified fractions and active components of viz- arjunolic, saponin and procynadine of *T. arjuna* bark and *T. Indica* bark and seed (Table.1). Control aquarium contained only equal volume of dechlorinated tap water without treatment. After termination of the treatment fishes were

Table 1: *Toxicity of 24h LC_{90} (L. acuminata and I. exustus) of column purified fractions and active component of T. arjuna bark and T. indica bark/seed against the fish Colisa fasciatus at different exposure period*.

Molluscicides	24h LC_{90} (mg/l) (*L. acuminata*) against fish *Colisa fasciatus*	24h LC_{90} (mg/l) (*I. exustus*) against fish *Colisa fasciatus*
T. arjuna bark (CPF)	20.41	161.56
Arjunolic acid	26.82	66.53
T. indica bark (CPF)	28.96	167.14
Saponin	28.17	150.45
T. indica seed (CPF)	4.68	82.38
Procynadine	5.13	62.68

washed with water and nervous tissue was quickly taken out and collected on ice trey for enzymatic analysis. The experiment was performed in treated as well as in control group of test animal.

2.5.1 Acetylcholinesterase

Acetylcholinesterase activity was measured according to the method of Ellman et al, [14]. Fifty milligrams of nervous tissue of *Colisa fasciatus* was taken around the buccal mass and homogenized in 1.0 ml of 0.1M phosphate buffer pH 8.0 for 5 minutes in an ice bath then centrifuged at 1000g for 30 minutes at 4^0C. The supernatant was used as an enzyme source. Enzyme using an incubation mixture consisting of 0.1mL of enzyme source, 2.9mL of 0.1M buffer pH 8.0, 0.1 ml of chromogenic agent DTNB (5,5-dithio-bis-2-nitrobenzoic acid), and 0.02mL of freshly prepared ATChI (acetylthiocholine iodide) solution in distilled water. The change in optical density at 412nm was recorded for 3 minutes after every 30 second interval at 25^0C. Enzyme activity has been expressed as µ mole "SH" hydrolyzed min/mg/protein.

2.5.2 Acid Phosphatase

Assays of acid and alkaline phosphatase activities were carried out by the method of Bergmeyer, [15]. Tissue homogenate (2% W/V) was prepared in ice cold 0.9% NaCl and centrifuged at 5000g for 15 minutes at 4ºC. Supernatant was used as an enzyme source. Standard curves were drawn with p-nitrophenol.

0.2ml of enzyme source was added to 1.0 ml of acid buffer substrate (0.41g citric acid, 1.125g sodium citrate, and 165 mg 4-nitrophenyl phosphatase sodium salt to 100ml of double distilled water) pre-incubated at 37ºC for 10 minutes. The incubation mixture was mixed thoroughly and incubated for 30 minutes at 37ºC. 4.0 ml of 0.1 NaOH was then added to the incubation mixture. The yellow colour developed due to the formation of p-nitrophenol was determined by spectrophotometer at 405 nm, standard curve was drawn with different concentration of 4- nitro phenol. The ACP activity has been expressed as µ mole substrate hydrolyzed/30 min/mg protein.

2.5.3 Alkaline Phosphatase

To determine the hydrolytic activity of alkaline phosphatases, 0.1ml of enzyme source was added to 1.0 ml of pre-incubated (10 min) alkaline buffer substrate (375 mg glycine, 10 mg $MgCl_2.6H_20$, 165 mg 4-nitrophenol phosphate sodium salt in 42 ml of 0.1N NaOH and a mixture was made up to 100 ml with double distilled water). The incubation mixture was mixed thoroughly and incubated for 30 minutes at 37ºC. 10ml of 0.02 N NaOH was then added to the incubation mixture. Reaction was stopped by the addition of an excess of NaOH solution. The yellow colour developed due to the formation of p-nitro phenyl phosphate. The activity of alkaline phosphatase was determined calorimetrically at 405 nm; it has been expressed as µ mole subtracts hydrolyzed/30 min/mg protein.

Table 2: *In vivo effect of 24h exposure of 80% 24h LC_{90} (against L. acuminata) column purified fraction and active component of T. arjuna and T. indica bark/seed on AChE, ACP and ALP activities in the nervous tissue of Fish Colisa fasciatus.*

Treatments	Concentration (mg/l) 80% of 24h LC_{90}	AChE µmole 'SH' hydrolyzed /min/mg protein	ACP µmole substrate hydrolyzed /30min/mg protein	ALP µmole substrate hydrolyzed /30min/mg protein
Control	—	0.836±0.04 (100)	56.95±0.84 (100)	49.10±0.83 (100)
***T. arjuna* bark (CPF)**	16.32 mg/l	0.804± 0.26 (96.17)	54.85±0.96 (96.31)	47.17±0.92 (96.99)
Arjunolic acid	21.45 mg/l	0.815±0.07 (97.48)	55.11±0.85 (96.76)	47.19±0.85 (96.10)
***T. indica* bark (CPF)**	23.16 mg/l	0.818±0.01 (97.84)	55.29±0.85 (98.82)	46.43±0.87 (94.56)
Saponin	22.53 mg/l	0.821±0.05 (98.20)	55.71±0.91 (97.82)	46.83±0.89 (95.37)
***T. indica* seed (CPF)**	3.74 mg/l	0.798±0.08 (95.45)	53.87±0.95 (94.59)	48.58±0.95 (98.94)
Procynadine	4.10 mg/l	0.809±0.01 (96.77)	55.09±0.95 (96.73)	48.04±0.95 (98.57)

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. Non-significant ($p>0.05$) when student's t-test was used for locating difference between treated and control group of snails. Abbreviations- *T. arjuna- Terminalia arjuna, T. indica –Tamarindus indica*, CPF-column purified fraction

2.5.4 Protein

Quantitative assessment of protein was made according to procedure of Lowry *et al* [16].

2.6 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 [17].

3 Results

3.1 Toxicity Experiment

The result of the toxicity experiments revels that there was no mortality in fish *Colisa fasciatus* exposed to 24h LC_{90} (against *L. acuminata* and *I. exustus*) of all column purified fractions and active molluscicidal components viz arjunolic acid, saponin and procynadine of *T. arjuna* and *T. indica* up to 96h of exposure period (Table 1). Even not any behavioural changes were noted after the exposure of molluscicides.

3.2 Biochemical measurement

In vivo effect of plant preparations on enzymatic activities in the nervous tissue of fish *Colisa fasciatus*

In control group of experiments acetylcholinesterase, acid and alkaline phosphatase activities activity in the nervous tissue of *Colisa fasciatus* were 0.836 µmole 'SH' hydrolyzed/min/mg proteins, 56.95 µmole substrate hydrolyzed/30min/mg protein and 49.10 µmole substrate hydrolyzed/30min/mg protein, respectively (Table 2). *In vivo* treatments, 24h exposure of 24h LC_{90} (against *L. acuminata*) of column purified fraction (96.17% of control) and arjunolic acid (97.48% of control), of *T. arjuna* bark, column purified fraction (97.84% of control) and saponin (98.20% of control) of *T. indica* bark, and column purified fraction (95.45% of control) and procynadine (96.77% of control) of *T. indica* seed caused no significant ($p>0.05$) inhibition in AChE activity (Table 2, Figure 1).

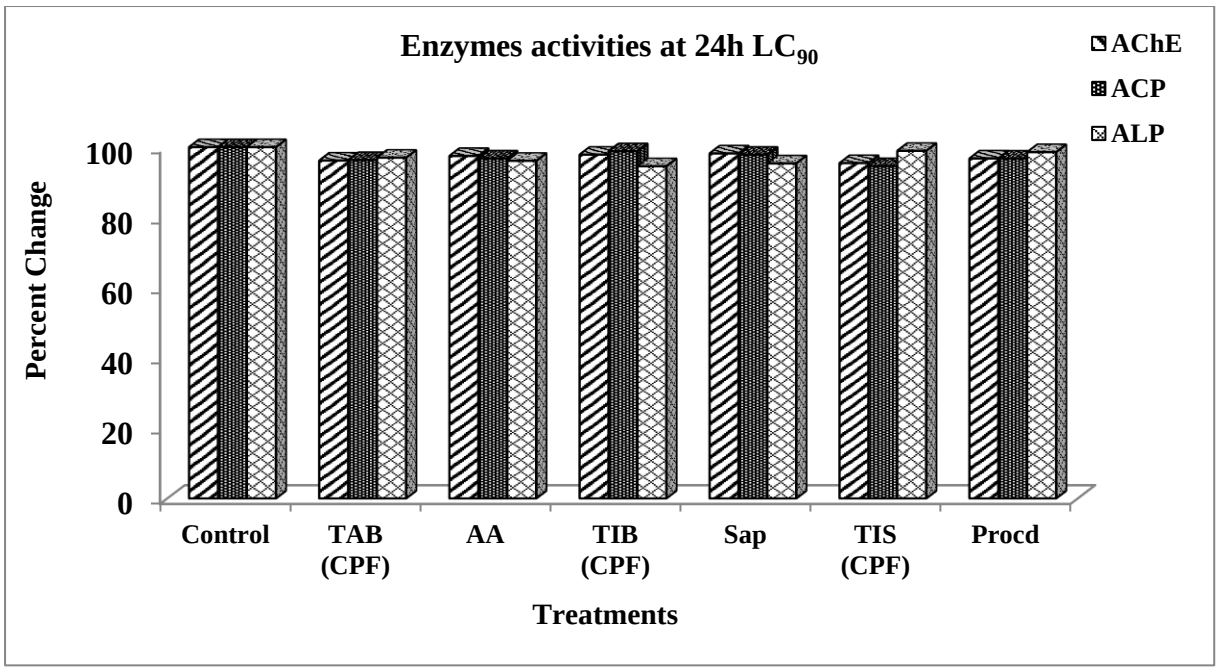


Figure 1: *Histogram showing in vivo effect of 24h exposure of 80% of 24h LC_{90} (against L. acuminata) of column purified fractions and active components (arjunolic acid of T. arjuna bark), (saponin of T. indica bark), (procynadine of T. indica seed) on the AChE, ACP and ALP activities in the nervous tissue of fish Colisa fasciatus. Abbreviations; TAB (CPF)- Terminalia arjuna bark (column purified fraction); AA- Arjunolic acid; TIB (CPF)-Tamarindus indica bark (column purified fraction); Sap- Saponin; TIS (CPF)- Tamarindus indica seed (column purified fraction); Procd, Procynadine.*

Non-significant changes were also noticed in ACP inhibition by column purified fraction (96.31% of control) and arjunolic acid (96.76% of control), of *T. arjuna* bark, column purified fraction (98.82% of control) and saponin (97.82% of control) of *T. indica* bark, and column purified fraction (94.59% of control) and procynadine (96.73% of control) of *T. indica* seed (Figure 1). Inhibition of ALP activities in the nervous tissue of fish *Colisa fasciatus* by column purified fraction (96.99% of control) and arjunolic acid (96.10% of control), of *T. arjuna* bark, column purified fraction (94.56 of control) and saponin (95.37% of control) of *T. indica* bark, and column purified fraction (98.94 of control) and procynadine (98.57% of control) of *T. indica* seed was not significant ($p>0.05$) (Fig 1)

Student's t-test was applied between treated and control group of animals to determined significant ($p<0.05$) variations.

4 Discussion

The application of environmental toxicology studies on non mammalian vertebrates is rapidly expanding, and for aquatic system, fish have become indicators to evaluate the effect of noxious compound**.** Synthetic molluscicides was known for knock-down effect in controlling the population of harmful gastropod, however adverse effect of the synthetic molluscicides on fishes as well as to other non target organisms and great threat to ecosystem, have stimulated the interest in the search for plant derived molluscicides [18]. The plants are virtually inexhaustible natural resource of structurally diverse and biologically active substances [19], [20]. Plant molluscicides are now gaining great importance because they are economic, effective, and safe to non-target animal and culturally more acceptable [21], [22]. Two indigenous medicinal plants *Terminalia arjuna* (Family-Combretaceae) and *Tamarindus indica* (Family- Leguminosae) earlier tested for its molluscicidal activity against *L. acuminata* and *I. exustus*. The active component i.e. arjunolic acid, saponin and procynadine present in the plants was responsible for its toxicological action against snails [2], [3], [12], [13]. The result of the present study clearly

demonstrated that the column purified fractions and their active molluscicidal components of both the plants (24h LC_{90} against *L. acuminata* and *I. exustus*) does not show any toxic effect in fish *Colisa fasciatus* as no mortality was reported up to 96h exposure period, because the concentration of molluscicides used to kill the snails *L. acuminata* and *I. exustus* was too low to cause any mortality in fishes. *In vivo* exposure of 80% of 24h LC_{90} of column purified fractions and active constituents viz- arjunolic acid, saponin and procynadine of *T. arjuna* bark and *T. indica* bark and seed caused no significant ($p>0.05$) inhibition in AChE, ACP and ALP activities in the nervous tissue of fish *Colisa fasciatus*. The result of this experiment clearly indicates the tolerance limits and safety of these molluscicides against non targeted animals.

5 Conclusions

From present study it can be concluded that plant of *T. arjuna* and *T. indica* can be used as potent molluscicides since the concentration used to kill 90% of the snails were not toxic to the fish *Colisa fasciatus* which share the same habitat with the snails. These indigenous plant molluscicides might be a valuable, environmentally safe and sound source of among the plant derived molluscicides and may be helpful to combat fasciolosis in endemic areas by interrupting the parasitic life cycle via control of snail population without affecting the population of non targets animals.

6 Declarations

6.1 Study Limitations

The finding of the study has been seen under the light of potential limitations such as time cast, convenient sampling of experimental animal which may not be representative of actual population and a large number of environmental factors that interpret with the result of the study.

6.2 Acknowledgement

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6.3 Ethical Consideration

The authors declare that they have followed the protocols of their work centre on the publication of humans & animals related study.

6.4 Funding Source:

None

6.5 Competing Interests

The authors declare that they have no competing interest.

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