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Research Article

STUDIES ON PHYTOCHEMICAL EVALUATION OF *Tamarindus indica* EXTRACTS AS ANTI-SNAKE VENOM AGENTS

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ABSTRACT

Many medicinal plants have been recommended for the treatment of snakebites. Aqueous and alcoholic extracts of dried seed powder of *Tamarindus indica* were tested for their antioxidant and inhibitory activity of toxic enzymes like PLA2 and proteinases of *Naja naja* venom. Compared with alcoholic extracts the methanolic extract, displayed a significant inhibitory effect on the tested enzyme activities of venom. The present finding suggests that the methanolic extracts of *T. Indica* seed possess compounds, which inhibit the activity of Phospholipase A2 and Proteinases of cobra venom. It may be used as an alternative treatment to serum therapy and as a rich source of potential inhibitors of toxins involved in several pathological conditions of humans and animal diseases.

KEYWORDS: Snake venom; *Tamarindus indica*; antioxidants, PLA2

INTRODUCTION

Snake bite is a public health problem in many countries; Snake venom has been the cause of innumerable deaths worldwide. Accurate records to determine the exact epidemiology or even mortality of snake bite cases are generally unavailable¹. The global disparity in the epidemiological data reflects variations in health reporting accuracy as well as the diversity of economic and ecological conditions⁴. It is estimated that annual snake bites mortality in Indian subcontinent is more than 25000³⁶. Envenoming by *Naja nigricollis* induced clinical complications such as necrosis, haemorrhage, complement depletion³⁷; respiratory arrest or paralysis^{4,28}. The venom of *Naja nigricollis* consist of phospholipase A2¹³; and cardiotoxin³. In some cases envenoming by *Naja nigricollis* can induce corneal ulceration and anterior uveitis^{8,39}.

In India, the rural areas are most affected by snake envenomation and medicinal plant extracts are widely used as a remedy for treating snakebite^{26,30}. Medicinal plant extracts, rich source of natural inhibitors and pharmacologically active compounds, have been shown to antagonize the activity of some venoms and toxins^{10,18,35}. Anecdotal evidence abounds to indicate that plant remedies used are effective, and there appears to be a high rate of survival among snakebite patients with advanced clinical stages of venom toxicity.

Several chemical constituents like alkaloids, flavanoids, and phenolics are found to be present in varying proportions in plants. They have also been previously reported for anti snake venom activity. All these classes of chemical compounds are capable of interacting with macromolecular targets (enzymes or receptors) and can effectively inhibit the toxic effect of snake venoms in vitro than in vivo⁴³. The bioactive

products are generally secondary metabolites, such as alkaloids, flavanoids and tannins⁴²

Many Indian medicinal plants are recommended for the treatment of snakebite⁵, but so far no systematic analysis has been done. *Tamarindus indica* has long been used as a medicinal plant by traditional healers; the validity of the claims made for this plant has not been tested scientifically. Tamarind seeds are reported to contain phenolic antioxidants, procyanidins, epicatechin, taxifolin and eriodictyol³¹ and exhibit antimicrobial, antidiabetic, antioxidant and insecticidal and wound healing activities³². It has been noted that several anti snake venom activities are known to contain phenolic compounds.

The present study is to analyze the anti-snake venom activities of *Tamarindus indica* L. which is native to India and to assess how significant this folk tradition medicine is (i.e., *Tamarindus indica*) to neutralize snake venom activity of a typical and highly poisonous snake such as *Naja naja*.

MATERIALS AND METHODS

Extraction of Plant material

The fruits of *Tamarindus indica* were collected and seeds were separated. Seeds of *T. indica* were dried and made into a coarse powder. 4.0 g powder was placed in a beaker and soaked in 200 ml of distilled water with continuous stirring for 3 h at room temperature. The extract was filtered through muslin cloth and the filtrate was concentrated at 40°C. The dried residue was suspended in normal saline at a concentration of 0.1% and kept at 4°C. Alcoholic (methanolic and ethanolic) extractions were done separately with fresh powder following the method described by Alkofahi *et al.*, (1997)¹.

Source of venom

The *Naja naja* snake venom was obtained from Hindustan Snake Park, Kolkata in lyophilised form. It was dissolved in Phosphate buffered saline and stored at 4°C for further use.

Total phenols estimation

The total phenols of all extracts were measured at 765 nm by Folin Ciocalteu reagent²⁰. The dilute methanolic extract (0.5 ml of 1:10 g ml⁻¹) or Tannic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1M). The mixture was allowed to stand for 15 min and the total phenols were determined by spectrophotometer at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg /ml

solutions of Tannic acid in methanol: water (50:50, v/v). Total phenol values were expressed in terms of Tannic acid equivalent (mg / l of dry mass), which is a common reference compound.

Total flavonoids estimation

Aluminum chloride colorimetric technique was used for flavonoids estimation⁷. Each extract (0.5 ml of 1:10 g ml⁻¹) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8ml of distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 415nm with a double beam UV/Visible spectrophotometer (SHIMADZU, Japan). The calibration curve was plotted by preparing the quercetin solutions at concentrations 12.5 to 100 g ml⁻¹ in methanol.

Phospholipase A2 assay

Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the method described by Gutierrez *et al.* (1988)²⁵ increasing doses of venom were added to wells in agarose gel contains 1.2% egg yolk and erythrocytes as a source of lecithin and 10mm CaCl₂. Slides were incubated at 37°C overnight and the diameters of the hemolytic haloes were measured.

Anti-proteolytic activity

Proteolytic activity of *Naja naja* venom was measured by modifying the methods of Kunitz (1947)¹⁵ and Tan *et al.* (1986)³⁴. Two ml of 1% casein in 0.25M sodium phosphate buffer pH 7.75 and 0.1 ml of venom (10–2000 µg) in physiological saline were incubated for 1 h at 37 °C. The undigested casein was precipitated and the reaction terminated by adding 2ml of 5% trichloroacetic acid. After centrifugation at 10,000 rpm for 10 min, the absorbance of the supernatant was measured at 280 nm. One unit of proteolytic activity was defined as an increase of 0.001 absorbance units at 280 nm/h. The initial proteolytic dose of *Naja* venom was obtained from the plot between proteolytic activity and the venom doses. Solutions of 5–25 mg/ml methanolic extract was evaluated for their anti-proteolytic potentials against *Naja* venom. Each 0.05 ml of the test solution was pre-incubated for 1 h at 37 °C with an equal amount of the venom solution (1 mg/ml) before the mixture was subjected to proteolytic activity evaluation.

RESULTS AND DISCUSSION

In recent time there is increasing awareness and interest in herbal medicines. Consequently, herbal medicines have received greater attention as an

alternative to clinical therapy leading to increasing demand². Phytochemical screening helps to reveal the chemical nature of the constituents of the plant extract and the one that predominates over the others. It may also be used to search for bioactive lead agents that could be used in the partial synthesis of some useful drugs⁴¹.

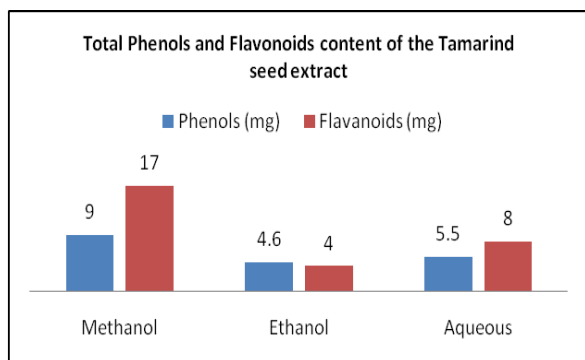
Total Phenols and Flavonoids content of the Tamarind seed extract

Table 1: Total Phenols and Flavonoids content of the Tamarind seed extract

S.NO	EXTRACTION SOLVENT	PHENOLS (mg/g)	FLAVONOIDS (mg/g)
1	Methanol	9	17
2	Ethanol	4.6	4
3	Aqueous	5.5	8

mg/g = milligram/gram

Fig 1: Total Phenols and Flavonoids content of the Tamarind seed extract



mg = milligram

The tamarind seed powder which was extracted by three different solvents had varied concentrations of Phenols and Flavonoids (Table 1; Fig.1). The concentration of both the components was higher which was extracted by Methanol followed by aqueous extraction. Both the components was least extracted through Ethanol. This indicates that Methanol was efficient in extracting Phenols and flavonoids from Tamarind seed powder. The total phenol content was expressed as tannic acid equivalents. The total flavonoids were measured by aluminum chloride colorimetric technique in term of

quercetin equivalent and the total phenols by Folin Ciocalteu reagent in terms of tannic acid equivalent. 1g of methanolic seed powder extract contains 9mg of phenols. 1g of methanolic seed powder extract contains 17 mg of flavonoids.

Over the years, the studies on medicinal plants revealing the mechanism of action and to justify their claims by traditional healers have been increased. As the study is on analyzing bioactive components and antioxidant properties of the *T.indica*, it has been acknowledged that flavonoids show significant antioxidant action on human health and fitness. The flavonoids act through scavenging or chelating process^{14,6}. The high potential of phenolics to scavenge free radicals may be due to many phenolic hydroxyl groups they possess²⁷. Free radicals possess the ability to reduce the oxidative damage associated with many disease including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS^{27,24,17,19}.

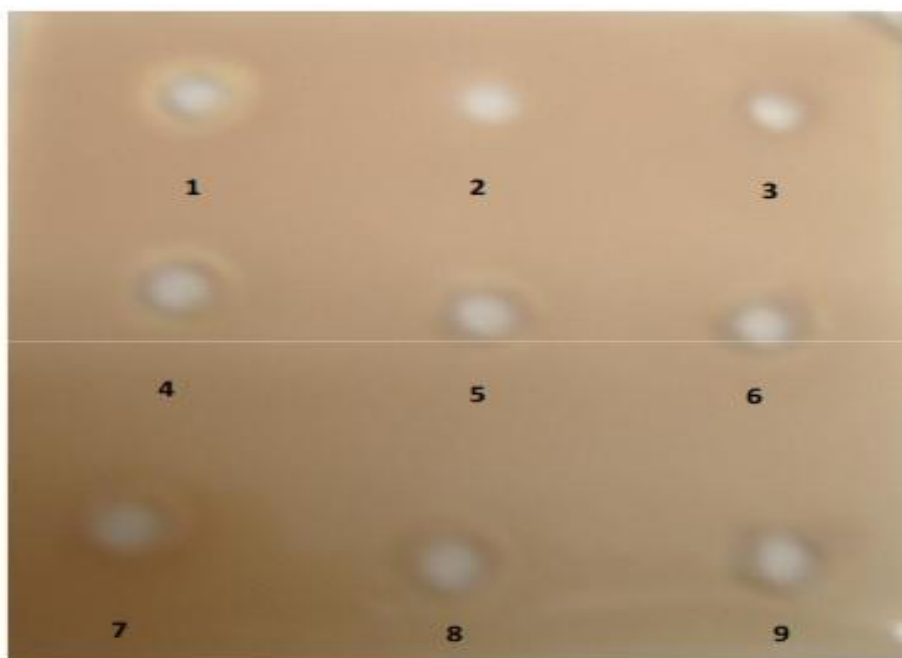
Phospholipase A2 assay

By increasing the concentration of venom in wells the zone formation was increased. The formation of a zone indicates that the venom having the enzyme PLA2 which degraded the phospholipid source. The zone formation was stopped when 25 μ l plant extract was added to the well having 3 μ l venom which indicates the 100% of inhibition of PLA2 activity by plant extract.

Table 2: Inhibition of phospholipase A2 enzyme by the plant extract of *Tamarindus indica*

S.no	Venom	seed extract	zone size (mm)	% of inhibition
1	3 μ l	5 μ l	9	18.18
2	3 μ l	10 μ l	7	36.36
3	3 μ l	15 μ l	3	72.72
4	3 μ l	20 μ l	1	90.90
5	3 μ l	25 μ l	0	100

Mm=millimetre

Fig 2: Inhibition of phospholipase A2 enzyme by the plant extract of *Tamarindus indica*

1. 3µl venom +5µl seed extract, 2. Buffer 3. Seed extract 4. 3µl venom +10µl seed extract 5. 3µl venom +15µl seed extract 6. 3µl venom +20µl seed extract 7. 3µl venom +25µl seed extract 8. 3µl venom +30µl seed extract 9. 3µl venom +35µl seed extract

Anti-proteolytic activity

Table: 3 Inhibition of venom proteinases by the plant extract of *Tamarindus indica*

S . N o	Sample o.1ml	1% casein MI	Incubation	5% TCA ml	C e n t r i f u g e	O. D at 280 nm	Units
1	Venom	2	1hr At 37°C	2		0.09	90
2	Venom	2		2		0.08	80
4	Venom+ plant extract	2		2		0.02	20
5	Venom+ plant extract	2		2		0.02	20

1unit=0.001 absorbance units at 280nm/hr

The medical and economical importance of plants has motivated several scientific and ethanopharmacological studies that have resulted in the discovery of many interesting properties. Several medicinal plants have been used widely in popular culture as neutralizers of the many effects induced by snake venoms (Soares *et al.*, 2004b, 2005). Phytochemical agents such as flavonoids inhibit snake venom phospholipase A2 activity was reported

earlier⁶. Flavonoids also effective in increasing survival time of rats injected with cobra venom (Gujral and Dhawan, 1956). Several substances have been isolated from plants and tested against the lethal action of the venoms (Mors *et al.*, 1989; Pereira *et al.*, 1994). As a result, a large number of plants have been found to be effective as antidotes against snake venoms in India (Chopra *et al.*, 1956; Usher, 1974; Kirtikar and Basu, 1975; Nadkarni, 1976; Lewis and Elvin-Lewis, 1977; Alam and Gomes, 2003).

In medicinal flora, the search for active principles capable of neutralizing the venom lesion effects constitutes an important field of scientific investigation. In the present study, the neutralizing effects of *T. indica* extract against some enzymatic and biological activities from snake venom was evaluated. This was very effective in inhibiting the activities induced by *Naja naja* venom. There was a significant inhibition of PLA2, Proteinase activity. The antivenom activity of several extracts may be due to the presence of enzyme inhibitors, chemical inactivators or immunomodulating principles (Soares *et al.*, 2004b). The efficacy of plant species as inhibitors of the toxic and pharmacological actions of snake venoms may be attributed to the presence of multiple factors.

Based on our results, *T.indica* seems to be a potent anti-snake venom mixture. Many vegetal extracts contain compounds capable of neutralizing the PLA2 and proteolytic activities of snake and bee venoms³³; Biondo *et al.*, 2003; Izidoro *et al.*, 2003; Maiorano *et al.*, 2005; Silva *et al.*, 2005; Oliveira *et al.*, 2005;⁴⁰).

CONCLUSION

In conclusion, it was demonstrated that the enzymatic and biological activities of venoms and some toxins were inhibited by *T.indica*. Although the mode of action of *T.indica* extract is still unknown, our studies support the hypothesis that the neutralizing properties occur probably due the association of its components with the venom proteins. Three extracts have been taken in the present study – Aqueous, Ethanolic and Methanolic extracts. Anti snake venom activities like PLA2 inhibition assay, Proteinase inhibition assay has been performed using methanolic extract as it was showed the maximum amount of phenolic compounds which posses the neutralisation of venom proteins. Further analytical methods like GC-MS analysis can help us to characterise the biochemical nature of the Phenolic compounds. The isolation and structural elucidation of these compounds will allow the understanding of the interaction with these proteins and consequently the inhibition mechanism.

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