In vitro screening of proteinase inhibitors in methanol and water extract of *Tridax procumbens*

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Abstract

Plant proteinase inhibitors accumulate in leaves and flowers in response to pathogen attack and various physiological stresses. These inhibitors have been exploited extremely in human health care system for preparation of drugs to control various disorders. In this study we studied the presence of proteinase inhibitors (trypsin, chymotrapsin, *Helicoverpa armigera* gut proteinase inhibitor) in leaves and flowers of *Tridax procumbens*. Water and methanol were used for isolation of protease inhibitors from leaves and flowers. The presence of inhibitor activity in aqueous and methanol extracts against trypsin, chymotrypsin and *H. armigera* gut proteinase was detected by using dot blot assay on X-ray film. Methanol extracts of leaves and flowers were found to be containing moderately prominent activity than aqueous extract. Both leaves and flowers extracts were observed more active against chymotrypsin as compared to trypsin and *H. armigera* gut proteinase. Both extracts were found to be unable of inhibition of all proteinase when incubation time extends from 15 to 30 min. Therefore it is necessary to characterise *T. procumbens* proteinase inhibitor for further applications in various field.

Key words: *Tridax procumbens*, Proteinase inhibitors, Trypsin, Chymotrypsin, Dot-blot assay, *H. armigera* gut proteinase.

1. Introduction

Tridax procumbens is a worldwide plant found in various tropical, subtropical regions and belonging to the daisy family[1]. It has been reported as a weed in many crops and traditionally used as medicine to cure liver diseases, diarrhoea, bronchial catarrh, and dysentery in Africa, South and Southeast Asia[2, 3, 4]. This plant contains strong allelopathic, larvicidal anti-cyclooxygenase, anti-inflammatory and antioxidant activities in ethyl acetate extract[5, 6]. Methanol and ethanol extracts of this plant exhibit anti-fungal[7], hepatoprotective activities[8], anti-hyperglycemic[9], and anti-leshmanial activities[10]. The leaves and flowers extracts are used to prevent haemorrhage from cut skin. This plant also possesses various pharmacological activities such as anti-diabetic, anti-inflammatory, insecticidal, antiseptic, analgesic and anti-parasitic. Generally, leaves extract of this plant is used to prevent hair loss[11]. Ethyl acetate extract of whole plant has antimicrobial activity against *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia* and *Bacillus cereus*[12].

Plant derived proteinase inhibitors are involved in defence mechanism against fungi, insects, pests, and herbivores. Medicinally, the proteinase inhibitors are used for treatment of many disorders such as osteoporosis, cardiovascular diseases, inflammatory diseases and neurological diseases[13]. They are signalling molecules, involved in many biological activities such as apoptosis, inflammation, hormone processing and blood clotting[13]. The proteinase inhibitor activity in *T. Procumbens* has not been studied yet. Therefore, in this study, we detected protease inhibitor activity in leaves and flowers of *T. Procumbens* by using dot blot assay on X-ray film.

2. Materials and Methods

2.1 Chemicals and reagents

Tris, HCL, NaOH, Glycine, Methanol and X-ray film were purchased from RANKEM. All chemicals and reagents used in this study were of analytical grade.

2.2 Collection of sample

Leaves and flowers of *T. Procumbens* were procured from campus of Ahmednagar College, Ahmednagar Maharashtra India. Fresh leaves and flowers were sorted out, washed thoroughly with distilled water and kept at 37^0C in an oven until completely dried. The dried samples were pulverized into fine powder by using grinder and mixer and preserved at 4^0C in refrigerator.

2.3 Preparation of extracts

For the preparation of extracts, distilled water and methanol were used. Fine powders were soaked and mixed properly in both solvents (1:10 w/v) and kept for stirring slowly by magnetic stirrer for 6 hrs. Thereafter, suspensions were filtered through whatman filter paper and the filtrates were preserved at 4^0C in refrigerator.

2.4 Extraction of HGPs (*Helicoverpa armigera*gut proteinase)

Fourth instar larvae of *H. armigera* were collected from the fields where pigeon are grown in agricultural area of Ahmednagar. The mid gut was excised from dissected larvae and preserved at -20 ^{0}C. Thawed mid gut was homogenized with 0.1 M glycine NaOH buffer (1:100 w/v) pH 10 for 20 min. The obtained suspension was centrifuged at 10,000 rpm for 15 at 4 ^{0}C and supernatant was used as source of proteases.

2.5 Dot-blot assay/spot test

The detection of proteinase inhibitor activity in aqueous and methanol extracts was performed by dot blot assay/spot test on X-ray film using the method of Pichare and. Kachole[14]. The principle of this method based on the digestion of gelatin by proteinase. The X-ray film is coated with gelatin, the drop of sample (containing proteinase) if loaded on film it hydrolyses the gelatin and forms clear transparent spot against dark background, hence proteinase present in sample. The solutions of trypsin and chymotrypsin were prepared in 0.1 M Tris-HCL buffers pH 7.5 (100µg/ml). The proteinase solutions such as *H. armigera* gut extract, trypsin and chymotrypsin were mixed with aqueous and methanol extracts of *T. Procumbens* with various proportions (3:1; 1:1; 1:3 v/v) and incubated at 37^0C for 10 min. The mixed samples were loaded on X-ray film and incubated at 37^0C for 15 and 30 min. At the same time, proteinase solutions were mixed with respective buffer solution and loaded on X-ray film as control. After incubation X-ray films were washed with warm distilled water and films were scanned by using digital scanner. The presence of proteinase inhibitor activity in extracts was assessed by comparing with control proteinase activity. The indigestion of X-ray film by mixed solution was indicated the presence of inhibitor activity in extract.

3. Results and Discussion

Secondary metabolites in plants have been gained attention for preparation of novel drugs against various diseases. Leaves of *T. procumbens* contain bioactive molecules such as alkaloids, flavonoids, hydroxylcinnamates, phytosterols, tannins, carotenoids, benzoic acid derivatives and lignans[15]. Figure 1 shows that detection of inhibitor activity in aqueous and methanol extracts of leaves and flowers against trypsin, chymotrypsin and *H. armigera* gut proteinase on X-ray film. Leaves aqueous, leaves methanol and flowers methanol extracts showed complete inhibition of trypsin when 3:1 proportion of extract/enzyme incubated for 15 min on X-ray film while flowers aqueous extract exhibited partial inhibition against trypsin as shown in Table 1. Leaves aqueous extract with 1:1 proportion of extract/enzyme showed partial inhibition against trypsin when incubated for 15 min on X-ray film while leaves methanol extract with same proportion of extract/enzyme showed completely inhibition against trypsin. Flowers methanol extract with 1:1 proportion of extract/enzyme showed partial inhibition against trypsin while flowers aqueous extract with same proportion did not exhibit inhibition. In earlier study it was reported that methanol extract of acerola bagasse flour containing major phenolic compounds like, gallic acid, syringic and *p*-coumaric acid, catechin, epigallocatechin gallate, epicatechin and quercetin inhibit digestive enzymes such as α-amylase, α-glucosidase, lipase and trypsin[16]. All mixed 1:3 proportions of extract/enzyme of leaves and flowers were unable to inhibit trypsin activity when 15 min incubation time applied. No inhibition of trypsin was found

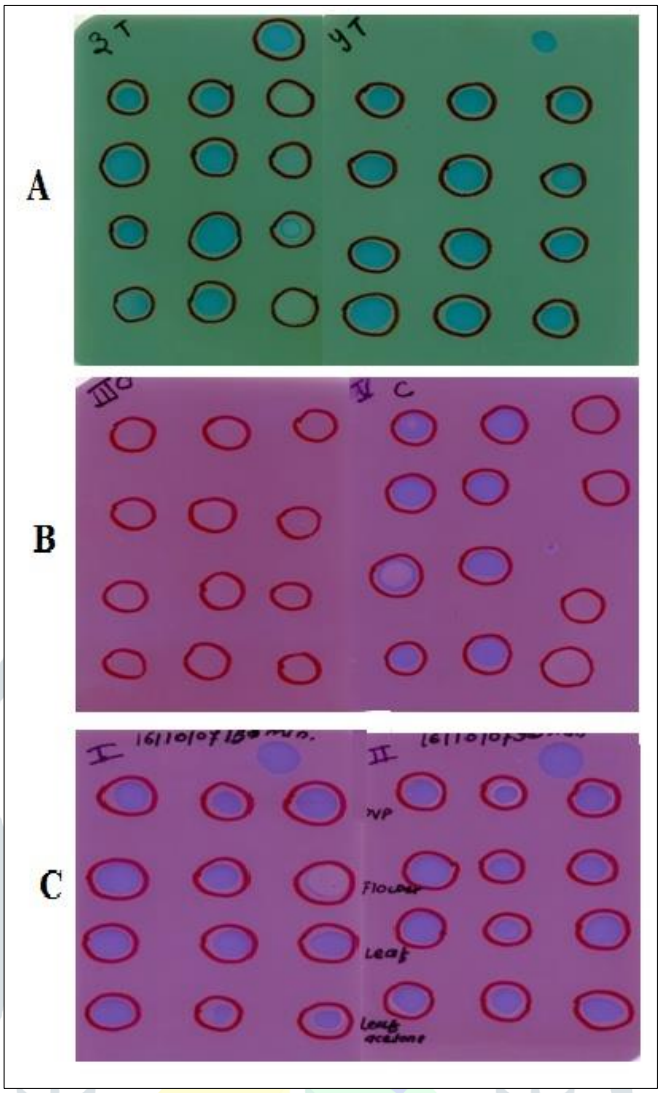
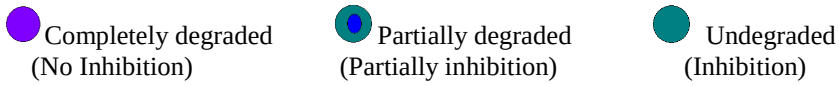


Figure 1: X-ray film represent the detection of inhibitor activity in aqueous and methanol extracts of leaves and flowers against trypsin (A), chymotrypsin (B) and *H. armigera* gut proteases (C).

when all proportion of extract/enzyme incubated for 30 min on X-ray film. Both leaves and flowers extracts were unable to inhibit trypsin activity if incubation time extended up to 30 min as shown in Table 1. It seems that weak inhibitors of trypsin may exist in leaves and flowers of *T. procumbens*. It has been investigated that some phenolics such as cinnamic acid, caffeic acid, eugenol, gallic acid, ferulic acid, vanillin and catechol isolated from plants (Cinnamomum zeylanicum, Impatiens bicolor, Melia azedarach, Allium cepa) exhibit trypsin inhibitors[17]. Leaves and flowers extracts exhibited prominent activity against chymotrypsin as compared to trypsin and *H. armigera* gut proteinase as shown in Table 1. This indicates that leaves and flowers extract of *T. Procumbens* containing compounds have good efficiency of chymotrypsin inhibition. All proportions of extract/enzyme showed inhibition against chymotrypsin when incubated for 15 min on X-ray film. All leaves and flowers extracts with 3:1 proportion of extract/enzyme showed inhibition against chymotrypsin when incubated up to 30 min on X-ray film. Aqueous extracts of

Table 1: Inhibition of trypsin, chymotrypsin and *H. armigera* gut proteases by aqueous and methanol extracts of leaves and flowers. Various proportions of proteases and inhibitor solutions were mixed and incubated at 37 ^{0}C for 10 min and inhibitor activity was checked by dot blot assay on X-ray film (details as described in Materials and methods).



Enzyme	Time (min)	TP extract: Enzyme (Mixed)	Leaves Aqueous extract	Leaves Methanol extract	Flowers Aqueous extract	Flowers Methanol extract
Trypsin	15	1:3				
		1:1				
		3:1				
	30	1:3				
		1:1				
		3:1				
Chymotrypsin	15	1:3				
		1:1				
		3:1				
	30	1:3				
		1:1				
		3:1				
H. armigera gut proteases	15	1:3				
		1:1				
		3:1				
	30	1:3				
		1:1				
		3:1				

leaves and flowers with 1:1 proportion of extract/enzyme exhibited partial inhibition against chymotrypsin when incubated up to 30 min as shown in Table 1. The weak inhibition was found in case of *H. armigera* gut proteinase. The reasons of weak inhibition may be due to *T. procumbens* is unable to inhibit a group of various proteinase such as trypsin, chymotrypsin, cysteine protease, carboxypeptidase-A and aminopeptidase-N available in *H. armigera* gut proteinase[18]. Leaves aqueous extract with 1:1 and 3:1 proportion of extract/enzyme showed partial inhibition against *H. armigera* gut proteinase when incubated for 15 min. Flowers aqueous extract with 3:1 proportion of extract/enzyme exhibited complete inhibition against *H. armigera* gut proteinase when incubated for 15 min. No inhibition of *H. armigera* gut proteinase was found when all proportion of extract/enzyme incubated for 30 min on X-ray film. It indicates that inhibitor activity showing compounds in *T. Procumbens* leaves and flowers ineffective when incubated at longer time with *H. armigera* gut proteinase. Methanol extracts of leaves and flowers were quite more active than aqueous extracts. From inhibition study it quite clear that non-proteinaceous protease inhibitors are available in *T. procumbens* leaves and flowers. Plant derived compounds such as terpenes, flavones, polyphenols, saponins, alkaloids, tannins, amino acids, di- and tripeptides act as protease inhibitors[19].

4. Conclusion

From the result of this study it was concluded that leaves and flowers of *T. procumbens* are sources of trypsin, chymotrypsin and *H. armigera* gut proteinase inhibitors. Methanol extracts of leaves and flowers showed prominent inhibitor activity. It indicates the presence of inhibitors in *T. procumbens* are secondary metabolites which inhibit proteinase. The inhibitors of *T. procumbens* could be utilized for formulation of novel drugs in human health care system for prevention of various disorders.

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