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Research Article

# PURIFICATION AND CHARACTERIZATION OF TRYPSIN INHIBITOR PROTEIN FROM SEEDS OF *MOMORDICA DIOICA*

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**ABSTRACT**

**Objective:** To purify the trypsin inhibitor protein from the seeds of *Momordica dioica* and characterize the protein for its stability and effect on trypsin activity.

**Methods:** The total protein was extracted from the seeds of *M. dioica,* and the purification of the protein was performed by ion exchange chromatography and ultrafiltration technique. The antitrypsin activity assay of the purified inhibitor protein was carried out using N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as the chromogenic substrate at various pH and temperature ranges to determine the stability of the protein. The inhibitory effect of purified protein on trypsin activity was characterized by enzyme kinetic study. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was performed under the non-reducing condition to determine the presence of inhibitor protein in various fractions of elutes and its molecular mass.

**Results:** The purified protein from *M. dioica* seeds showed almost 96.17±0.034% trypsin activity inhibition and 0.96±0.00 U trypsin inhibitory activity (TIA) at 30°C and pH 8. This trypsin inhibitory protein from *M. dioica* (MdTi) was found to be stable over a temperature range of 30-100°C and the pH range of 3-10 retaining the antitrypsin activity. The molecular mass of MdTi was found to be~12 kDa. From the enzyme kinetics, it was found that Km value remains unaffected with decrease in the Vmax of trypsin in presence of inhibitor.

**Conclusion:** *M. dioica* seeds were found to posses serine protease inhibitor protein. The protein was purified and characterized as thermostable as well as pH tolerant trypsin inhibitor with high TIA. It can be well explored for its use in the agriculture industry for pest management as well as the therapeutic applications.

**Keywords:** Serine proteases, Trypsin inhibitor, *Momordica dioica*, Trypsin inhibitory protein from *Momordica dioica*, Non-competitive inhibition.

## INTRODUCTION

Proteases are enzymes that cleave proteins by the catalysis of peptide bond hydrolysis. They play an important role in all kinds of organisms in the regulation of protein synthesis and turnover [1-3]. The whole genome analyses suggest that proteases are widespread in plants, animals, and microorganisms and comprise approximately 2% of encoded proteins [4,5]. Activities of these powerful enzymes are well regulated in different organisms including animals and plants. Their corresponding protease inhibitors are also abundant in nature [6,7]. They are generally inactivated either by proteolytic degradation or by interaction with protease inhibitors [8]. Most of the protease inhibitors interact with its target protease at the catalytic domain, forming a stable protease inhibitor complex that leads to protease inactivation, and may also act as pseudo-substrates showing affinity to the enzymes catalytic sites [9-11]. These inhibitors have been reported as pharmacologically important molecules contributing in regulating diverse molecular processes such as inflammation, blood coagulation, platelet aggregation, and anti carcinogenesis fibrinolysis, intracellular protein breakdown, cell cycle, transcription, cell invasion, and apoptosis [12-15]. The agricultural importance of protease inhibitors has been reported as one of the molecules involved in protection against the plant predators [16]. Most serine protease inhibitors have been isolated and characterized from the seeds of Leguminosae, Cucurbitaceae, Solanceae, and Gramineae families [17,18]. Among the 80 species of the genus *Momordica* of Cucurbitaceae family, *Momordica cochinchinensis, Momordica repens,* and *Momordica charantia* have been reported for the presence of serine protease inhibitors in seed extracts [19-23], whereas *Momordica dioica* is not yet been investigated for the same. The species *M. dioica* has been investigated for the presence of various pharmacologically important properties in different plant parts by various researchers. Compounds such as lectins, triterpenes, vitamin C, iodine, alkaloid, flavanoids, and glycosides, were reported to be present in the fruit of *M. dioica* [24-27]. The fruit and fruit pulp of *M. dioica* were reported with analgesic, antipsychotic, anti-inflammatory, antidiabetic, antilipidemic, and antimicrobial activities [28-31]. The antiseptic, antioxidant, and hepatoprotective properties were reported in the root extracts of *M. dioica* [32]. Considering the medicinal potential of *M. dioica*, this study was performed to detect and characterize the serine protease inhibitor from seeds. Trypsin is one of the representative enzymes of family serine proteases which can be assayed using synthetic substrates for the screening of trypsin inhibitors [18]. The trypsin inhibitor was also analyzed for its stability at different temperatures and pH.

## MATERIALS AND METHODS

### Chemicals and other laboratory materials

The chemicals and reagents used were of analytical grade. The DEAE Sepharose, CM Sepharose, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, trypsin, and substrate BAPNA were obtained from Sigma-Aldrich. Tris base, sodium chloride, glycine, glycerol, β-mercaptoethanol, sodium hydroxide, monobasic sodium phosphate, dibasic sodium phosphate, and sodium acetate were from HiMedia. Dimethyl sulfoxide (DMSO), bromophenol blue from SRL and calcium chloride, acetic acid, and methanol obtained from Fisher Scientific, 10 kDa cutoff ultra filtration membrane was obtained from Merck Scientific, and broad range molecular weight marker was obtained from Merck Genei.

### Collection of seed

The ripe fruits of *M. dioica* were collected from the nearby regions of Udaipur, Rajasthan, India. The seeds were extracted from the fruits

and washed with distilled water. The seeds were sun dried for about 2 weeks and stored at room temperature and used for extraction of trypsin inhibitor.

**Extraction of trypsin inhibitor from *M. dioica***

The extract preparation was performed by the method described by Huang *et al*. [20] with some modifications. The dried seeds were soaked in 50 mM Tris-HCL buffer (pH 8.0) for an hour and crushed in mortar pestle. Tris buffer was added in quantity sufficient to make slurry and it was incubated overnight at 4°C. The slurry was filtered with muslin cloth, and the filtrate was further centrifuged at 12000 rpm for 45 minutes at 10°C. The supernatant was re-spinned at 12000 rpm for 30 minutes at 10°C to remove any solid particle. The clear supernatant was used as a crude extract and preserved at 4°C for further study.

**Purification of trypsin inhibitor**

CM Sepharose and DEAE Sepharose column chromatography were performed to separate and purify the trypsin inhibitor from the extract using the modified method described by Lam and Ng [33]. The extract was applied to the cation exchange column of CM Sepharose, which was pre-equilibrated with 50 mM Tris-HCl Buffer (pH 8.0). The column was washed with the equilibration buffer, and the flow through was checked for the presence of protein by spectrophotometric analysis at 280 nm. The column was washed with the buffer till no protein was detected in the flow through. The fraction retained by the column was then eluted with Tris-HCL buffer at pH 8.0 using a linear gradient of NaCl (100 mM to 500 mM). The flow rate of 1 ml/min was maintained, and fractions of 1.0 ml each were collected. All the fractions were examined for protease inhibitory activity, protein content, and homogeneity by performing trypsin inhibitory assay, absorbance at 280 nm (spectrophotometric analysis), and SDS-polyacrylamide gel electrophoresis (PAGE), respectively.

The homogenous fractions of protein with the antitrypsin activity were eluted with 100 mM NaCl from cation exchange column. The pooled fractions were applied to the anion exchange chromatography column containing DEAE Sepharose which was pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The elution and analysis were performed as described above in the cation exchange chromatography [34].

The homogenous fractions from the anion exchange chromatography were pooled and concentrated with 10 kD ultrafiltration membrane [20]. The fractions were filtered through the 10 kD ultrafiltration membrane by spinning at 4000 g and 25°C for 10 minutes. Protein concentration in all the fractions collected at different steps of purification was determined by the Bradford's method using bovine serum albumin as a standard [35]. This purified and concentrated, active protein, i.e., trypsin inhibitor from *M. dioica* was labeled as MdTi and stored at 4°C.

**Trypsin inhibitory activity (TIA) assay**

The crude extract and purified MdTi were assayed for TIA and % inhibition using BAPNA as the chromogenic substrate. The assay was performed by incubating 200 µl of MdTi at a concentration of 1 mg/ml with 200 µl of trypsin (1 mg/ml in 50 mM Tris-HCl buffer pH 8.0 containing 20 mM $CaCl_2$ and 0.001 N HCl) at 27°C for 15 minutes. To this mixture 1.6 ml of 0.5 mM BAPNA in 2.5% DMSO was added as the enzyme substrate. Following a reaction time of 15 minutes, 200 µl of 10% acetic acid was added to the reaction mixture to stop the reaction. The enzymatic activity was analyzed on the basis of the liberation of *p*-nitroaniline which was measured at 410 nm [36,37]. The trypsin inhibitory activity was calculated as follows:

$$\text{Trypsin inhibitory activity (U)} = \frac{[\text{Abs control} - \text{Abs sample}]}{[\text{Abs control} \times \text{Amt of trypsin (mg)}]}$$

For calculating the % inhibition of the activity of trypsin by the inhibitor protein, following formula was used.

$$\% \text{ inhabition} = \frac{[\text{Abs control} - \text{Abs sample}]}{\text{Abs control}} \times 100$$

Where, Abs control is absorbance change in absence of sample, Abs sample is absorbance change in the presence of sample, and trypsin (mg) is the amount of trypsin in the assay mixture. One unit of TIA refers to the activity capable of inhibiting 1 mg trypsin [20].

**Determination of molecular weight of purified protein**

Discontinuous SDS-PAGE was performed for protein profiling according to the method described by Laemmli [38]. The electrophoresis was performed on a mini-vertical gel electrophoresis unit using a resolving gel of 15% acrylamide (pH 8.8) and a stacking gel of 5% acrylamide (pH 6.8). Protein sample preparation for electrophoresis was done by treating 30 µl of protein sample with 10 µl of the non-reducing sample loading buffer (0.5 M Tris-HCl, 2% SDS, tracking dye bromophenol blue, pH 6.8) at 100°C for 5 minutes. 20 µl of the treated sample was loaded into the gel. The electrophoresis was performed using Tris-glycine buffer containing 0.1% SDS as a running buffer at 100 V. Coomassie brilliant blue staining was performed for detection of the protein bands [38].

**Temperature stability**

The effect of temperature on the stability and antitrypsin activity of MdTi was determined by incubating MdTi samples at different temperatures ranging from 30°C to 100°C (interval of 10°C) for 1 hr and then cooling to 30°C [39]. Determination of inhibitory activity was performed using the TIA assay as described above.

**pH stability**

The pH stability of the purified MdTi was determined by pre-incubating the inhibitor at different pH for 1 hr at 30°C. The protease inhibitor activity was determined by performing the TIA assay as described above. The appropriate pH was obtained using different buffer solutions such as (a) 0.2 M glycine-HCl buffer (pH 3.0), (b) 0.2 M sodium acetate buffer (pH 4.0-5.0), (c) 0.2 M phosphate buffer (pH 6.0-7.0), and (d) 0.2 M Tris-HCl buffer (pH8.0-10.0) [40].

**Effect of inhibitor on enzyme kinetics**

The trypsin activity assay was performed at optimum reaction conditions. Initial reaction velocities ($V_0$) of trypsin were determined at increasing concentrations of substrate (BAPNA) ranging from 0.5 to 1.75 mM. Two sets of reactions were performed; one with and another without MdTi. The trypsin concentration used for the reaction was 0.1 mg/ml and that of the inhibitor was 1 mg/ml. The standard curve was plotted using the varying concentrations of the product *p*-nitroaniline (2.5-30 µM). The standard curve was used to convert the rates of trypsin activity from absorbance per minute to µM of product formed per minute. The reaction volume was then used to further convert the values from µM *p*-nitroaniline/min to µmol *p*-nitroaniline/min. The values thus obtained were plotted with BAPNA concentration on the X-axis and the rate of product formed per minute on the Y-axis. The Km and Vmax values were determined from the Lineweaver-Burk plot (double reciprocal plot) [41].

**RESULTS**

**TIA**

The crude extract of seed and MdTi were assayed for the TIA. The reaction mixture without MdTi showed a prominent color change from colorless to yellow due to the formation of chromogenic product *p*-nitroaniline. In the reaction mixtures with MdTi, no detectable color change was observed. The spectrophotometric analysis confirmed the presence of the trypsin inhibitor in the crude extract of seeds and MdTi. The crude extract showed 0.88±0.015 U TIA and 88.83±1.450% trypsin activity inhibition at pH 8 and 30°C. At same pH and temperature, MdTi showed TIA of 0.96±0.00 U, and the percent inhibition was detected to be 96.17±0.034 (Table 1).

**Purification of trypsin inhibitor from *M. dioica* seed extract**

The crude protein extract of the seeds showing the positive inhibitory activity was processed for fractionation and purification. The initial flow through of the washing with equilibration buffer did not show any inhibitory activity. While screening all the eluted fractions obtained with the gradient elution buffer with NaCl (100-500 mM), fraction number

15-25 eluted with 100 mM NaCl appeared to be positive for the presence of protein (Fig. 1a) and TIA. The protein profile of this fraction showed the presence of multiple protein bands (Fig. 2). The processing of the obtained fractions through anion exchange chromatography using the DEAE Sepharose column removed the protein impurities. Among the collected fractions of anion exchange chromatography, fraction number 1-11 (Fig. 1b) showed positive TIA. The concentration of the protein in the fractions obtained from the DEAE column was comparatively less due to repeated washing. These proteins in different elutes were pooled together and concentrated with the 10 kDa molecular weight cutoff membrane. MdTi was obtained in the fraction retained by the membrane, and hence the molecular weight of MdTi was determined to be more than 10kDa. In SDS-PAGE analysis, this fraction showed a single band confirming the purity of the inhibitor protein MdTi (Fig. 2). The approximate molecular weight of MdTi was found to be 12 kDa.

**Effect of temperature and pH on the activity of MdTi**

The inhibitory activity of the purified protein was evaluated at different temperature treatments from 30 to 100°C. The minimum and maximum TIA were found to be 0.93 U at 90°C and 0.96 U at 30°C, respectively (Table 1). A similar pattern was obtained with the % inhibition at different temperatures. Therefore, 30°C was considered as the optimum temperature for the antitrypsin activity of MdTi and used for further study of stability of MdTi at acidic and alkaline pH range (pH 3-10). It was found that the protein was stable at acidic as well as alkaline pH showing minimum variation in the TIA and % inhibition. The minimum and maximum % inhibition of trypsin activity were found to be 94.99±0.083% and 96.17±0.034% at pH 6 and 8, respectively (Table 2). The TIA was also found to be minimum (0.95±0.00 U) at pH 3, 4, 9, and 10 and maximum at pH 8 (0.96±0.00 U) (Table 2).

**Kinetic study**

The initial velocity of trypsin without MdTi and with MdTi was calculated and used for determining the effect of the inhibitor on enzyme kinetics. From the Lineweaver-Burk plot, Vmax was calculated to be 333 µmol/min without inhibitor (y-intercept value 0.003, Fig. 3) and 10.41 µmol/min with inhibitor (y-intercept value 0.096, Fig. 3). The Michaelis constant Km of trypsin for BAPNA was found to be 1.587 mM with and without inhibitor. The values for Vmax decreased in the presence of inhibitor with no change in Km value. The graphical pattern obtained in Lineweaver-Burk plot (Fig. 3) showed that the mode of inhibition was non-competitive in nature.

**Table 1: Effect of different temperature treatments at pH 8 on TIA and percent inhibition by trypsin inhibitory protein from *Momordica dioica* seeds**

| Sample | Temperature | TIA[U]±SD | % Inhibition±SD |
|---|---|---|---|
| Crude extract | 30°C | 0.880±0.015 | 88.83±1.450 |
| MdTi | 30°C | 0.960±0.000 | 96.17±0.034 |
| | 40°C | 0.950±0.000 | 95.43±0.253 |
| | 50°C | 0.947±0.005 | 95.03±0.092 |
| | 60°C | 0.940±0.000 | 94.41±0.122 |
| | 70°C | 0.947±0.005 | 95.03±0.292 |
| | 80°C | 0.940±0.000 | 94.27±0.240 |
| | 90°C | 0.930±0.000 | 93.65±0.160 |
| | 100°C | 0.940±0.000 | 94.85±0.096 |

SD: Standard deviation, TIA: Trypsin inhibitory activity

## DISCUSSION

Plant seeds are a rich source of proteins and are considered important for their functional ingredients in the food system [42]. Different plant parts such as leaves, tubers, and seeds contain natural protease inhibitors that serve as defense and regulatory proteins [1]. During seed germination, the inhibitor is considered to serve as the substrates for proteolysis to provide amino acids for seed growth [43]. Due to the importance of protease inhibitors in medicine and agriculture, various studies have been performed for identification, purification, and characterization of inhibitors from different plant sources [1,44,45]. Considering the possibility of the potent trypsin inhibitor to be a protein, the seed proteins of *M. dioica* were isolated, purified, and characterized for its protease inhibitory activity. In this study, the seeds of the *M. dioica* plant were found to possess the trypsin inhibitory protein [MdTi] in abundance as determined from the content of purified protein obtained per gram of seeds (approximately 2 mg/g). In 50 mM Tris buffer at pH 8.0, MdTi acted as the cation which was retained on the CM-cation exchanger. Similar characteristic was reported in the other inhibitors from *M. cochinchinensis* [20], *Cucumis melo*, [46], and *M. charantia* Linn. [23,47].

In this study, the molecular mass of protein MdTi as determined from the SDS-PAGE analysis was found to be 12 kDa (approximate). Mostly, the molecular mass of serine protease inhibitors varies from 4 to 85 kDa, with majority in the range of 8-20 kDa. Squash-type trypsin inhibitors are the smallest serine proteases known that have been purified from members of *Cucurbitaceae* family, of genus *Cucurbita*, *Cucumis,* and *Momordica* [20]. Their polypeptide chains are usually comprised of 27-34 amino acid residues, with a high relative content of cysteinyl residues, six in the case of squash inhibitors [48]. The molecular weight of the trypsin inhibitor from *Erythrina velutina* seeds was reported to be 20 kDa by SDS-PAGE and 19,210.48 Da by mass spectrometry [49]. Compared to these results, our protein was found to be a middle range molecular weight inhibitor protein.

In this study, the TIA in the crude extract was found to be 0.88 ± 0.015 U and that in purified protein (MdTi) was 0.96 U, which can be considered to be comparatively high activity. The similar TIA reported in a protein from *M. charantia* Linn was 0.85 unit/mg [23] and 1.58 units/mg in *M. cochinchinensis* [20]. The protease inhibitor proteins from soybean were reported to be stable at higher temperatures (0-100°C) and a wide range of pH (3-11) [34]. The protease inhibitors from *Cucurbita ficifolia* have been reported with the maximum TIA at alkaline pH [50]. In this study, MdTi has also been characterized as thermostable at a temperature

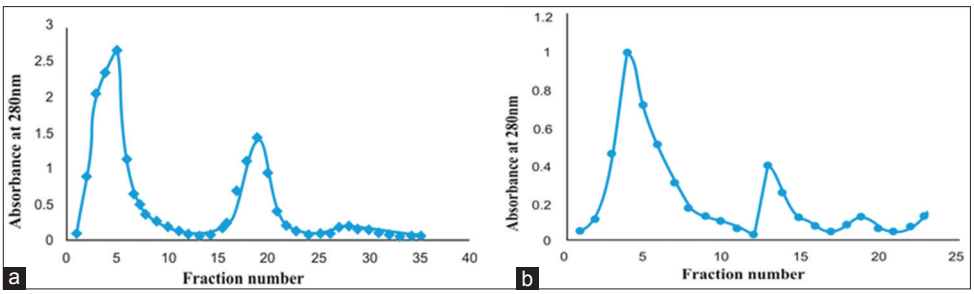


**Fig. 1: Purification of trypsin inhibitor protein from seed extract by ion exchange chromatography. (a) Elution profile of CM Sepharose cation exchange column chromatography obtained with elution buffer of concentration gradient 100-500 mM NaCl. Fraction no. 15-25 were the bound protein fractions containing trypsin inhibitory protein from *M. dioica* (MdTi) eluted with 100 mM NaCl, (b) elution profile of DEAE Sepharose anion exchange column chromatography eluted with pre-equilibrated. Fraction no. 1-11 were the fractions of the unbound protein containing MdTi**

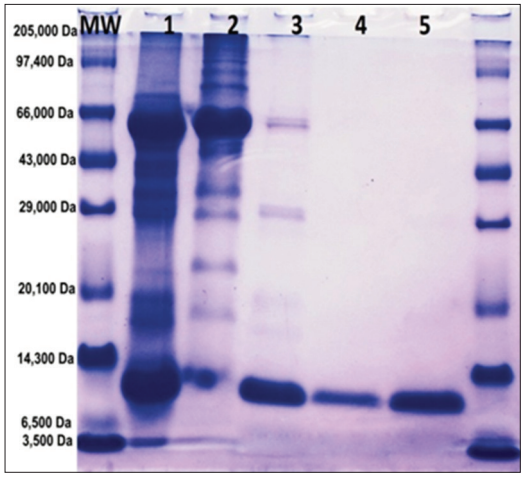


**Fig. 2: Protein profile of crude extract of seed and purified trypsin inhibitory protein from *M. dioica* (MdTi) obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Indents: M: Broad range protein molecular weight marker, (1): Crude seed extract, (2 and 3): Unbound and bound fractions from CM Sepharose chromatography, (4): Unbound fraction of MdTi from DEAE chromatography, (5): Purified MdTi concentrated with ultrafiltration membrane of 10 kD**

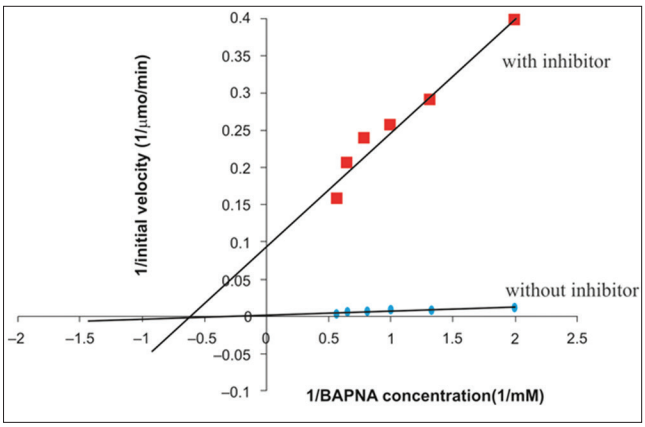


**Fig. 3: Lineweaver-Burk double reciprocal plot for the inhibition of trypsin by trypsin inhibitory protein from *M. dioica* (MdTi) using the concentrations of substrate BAPNA from 0.5 to 1.75 mM. Where Vmax and Km values for trypsin decreased in the presence of MdTi. Series A: Initial reaction velocity of trypsin without inhibitor at varying substrate concentrations of BAPNA. Series B: Initial reaction velocity of trypsin in the presence of MdTi**

as high as 100°C and found to be stable to retain the inhibitory activity in a wide range of acidic as well as alkaline pH (Tables 1 and 2).

Kinetic study of enzyme trypsin in the presence of MdTi showed that there was a significant decrease in Vmax compared to that without inhibitor, whereas no change was observed in the Km value. The characteristic of no alteration in the Km, with reduction in Vmax, is a typical trait for a non-competitive type of inhibition. Hence, the inhibitor was characterized as non-competitive type. In this type of inhibition, the binding of substrate and the inhibitor is independent as inhibitor does not bind to the catalytic site rather binds to different sites and results in the reduction of the turnover rate of the reaction. A pure non-competitive inhibitor has an equal affinity for free enzymes and enzyme substrate complexes [41,51].

From the results, it can be interpreted that the protein MdTi had a potential to be used as a serine protease inhibitor and explored further for its therapeutic applications.

**Table 2: Comparative study of TIA and percent inhibition by MdTi at different pH**

| pH | TIA [U]±SD | % Inhibition±SD |
|---|---|---|
| 3 | 0.950±0.000 | 95.38±0.162 |
| 4 | 0.950±0.000 | 95.63±0.040 |
| 5 | 0.957±0.005 | 96.09±0.137 |
| 6 | 0.952±0.005 | 94.99±0.083 |
| 7 | 0.955±0.005 | 96.00±0.147 |
| 8 | 0.960±0.000 | 96.17±0.034 |
| 9 | 0.950±0.000 | 95.55±0.076 |
| 10 | 0.950±0.000 | 95.41±0.059 |

SD: Standard deviation, TIA: Trypsin inhibitory activity

## CONCLUSIONS

The trypsin inhibitor MdTi isolated from the seeds of *M. dioica* is a medium size range protein which is highly stable toward temperature and pH change with no reduction in the TIA and has non-competitive mode of inhibition. To our knowledge, this is the first report of the purification and characterization of trypsin inhibitor proteins from the seeds of *M. dioica*. Ion exchange chromatography can be very well used for purification of MdTi protein. This potent protein can be investigated further for its inhibitory activities against the proteinases from various sources and characterized for its medicinal potential.

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