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 their 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. Fibrolysis, 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, Solanaceae, 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, flavonoids, 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 anagelse, anti-psychotic, anti-inflammatory, antidiabetic, anti-lipidemic, 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 [16]. 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 ultrafiltration 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...
For calculating the % inhibition of the activity of trypsin by the inhibitor protein, following formula was used.

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

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].

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

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-Cl 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-Cl 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 10 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-Cl 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 spinning at 4000 g and 25°C for 10 minutes. Protein concentration 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-Cl buffer pH 8.0 containing 20 mM CaCl₂ and 0.001 M 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:

\[
\text{TIA (U/ml)} = \frac{[\text{Abs control} - \text{Abs sample}]}{[\text{Abs control} \times \text{Amt of trypsin (mg)}]}
\]

**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.

**RESULTS**

**TIA**

The crude extract of seed and MdTi were assayed for 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
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 3-11). 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±0.08% and 96.17±0.03% 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 (9.96±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. V_max 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 V_max 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.

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 erine 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 cysteiny1 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...
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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REFERENCES


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

<table>
<thead>
<tr>
<th>pH</th>
<th>TIA [U]±SD</th>
<th>% Inhibition±SD</th>
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<tbody>
<tr>
<td>3</td>
<td>0.950±0.000</td>
<td>95.38±0.162</td>
</tr>
<tr>
<td>4</td>
<td>0.950±0.000</td>
<td>95.63±0.040</td>
</tr>
<tr>
<td>5</td>
<td>0.957±0.005</td>
<td>96.09±0.137</td>
</tr>
<tr>
<td>6</td>
<td>0.952±0.005</td>
<td>94.99±0.083</td>
</tr>
<tr>
<td>7</td>
<td>0.955±0.005</td>
<td>96.00±0.147</td>
</tr>
<tr>
<td>8</td>
<td>0.960±0.000</td>
<td>96.17±0.034</td>
</tr>
<tr>
<td>9</td>
<td>0.950±0.000</td>
<td>95.55±0.076</td>
</tr>
<tr>
<td>10</td>
<td>0.950±0.000</td>
<td>95.41±0.059</td>
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</tbody>
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SD: Standard deviation, TIA: Trypsin inhibitory activity

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 4.75 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.


33. Lam SK, Ng TB. A dimeric high-molecular-weight chymotrypsin inhibitor with antitumor and HIV-1 reverse transcriptase inhibitory activities from seeds of Acacia confusa. Phytomedicine 2010;17(8-9):621-5.


