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STUDIES ON A TRYPSIN INHIBITOR FROM THE SEEDS OF *MURRAYA KOENIGII*

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ABSTRACT

A novel proteinaceoustrypsin inhibitor from the seeds of *Murrayakoenigii* (Indian curry leaf tree) (MKTI)was isolated and purified to homogeneity by ion exchange chromatography followed by gel filtration chromatography on Sephadex G-75. The specific inhibition of the trypsin amongst the other serine proteases suggests that *Murrayakoenigii*trypsin inhibitor(MKTI) may belong to the Kunitz-type protease inhibitor family. The molecular mass of the novel cysteineless inhibitor was determined to be 21601.44 Daltons by MALDI-TOF. Kinetic studies revealed that *Murrayakoenigii*trypsin inhibitor is a competitive inhibitor having 1:1 stoichiometry of binding with trypsin. The trypsin inhibitor of the seeds of *Murrayakoenigii*, a non-glycoprotein, exists as a monomer under physiological conditions with 4.44% α helical content, 44.17% β sheet and 51.42% random coil structure as determined by circular dichroism studies.

KEYWORDS

MurrayaKoenigii, cysteinelesstrypsin inhibitor, MALDI-TOF, circular dichroism, fluorescence spectroscopy.

INTRODUCTION

Proteases play an important role in the biological regulation of many physiological processes and have been identified to be closely related to the pathophysiology of various diseased states[i, ii,iii,iv, v]. Moreover, their involvement in the life cycle of disease causing organisms has led to their becoming important targets for therapeutic intervention. Hence, there is a continued interest in the identification of new inhibitors to regulate their activity.

Proteinaceousproteinase inhibitors are important tools of nature and, like proteases, are ubiquitous proteins present in multiple forms in all-living organisms[vi, vii]. Plant proteinaceous inhibitors are stable proteins with selective specificity for serine proteinases, cysteineproteinases, aspartic proteinases and metalloproteinases respectively[viii]. They are generally present in plant storage tissues, viz. seeds, tubers, leaves and fruits in widely varying concentrations and thus play an important defensive role in the pathophysiology of the plants[ix, x]. The serine proteinase inhibitors, being the best known, extensively studied and well characterized inhibitors, are classified into 22 different families, based on their amino acid sequences, structural similarities, and mechanism of reaction with their respective enzymes[xi,xii]. Plant serine proteinase inhibitors are mainly grouped into Soybean (Kunitz), Bowman-Birk, Potato I and II, Squash as well as Barley, Ragi I and II, and Thaumatin families[xiii,xiv].

Murrayakoenigii Linn. (*Rutaceae*), commonly known as Indian Curry Leaf tree, is a small tree and its leaves are commonly used as a spice throughout India. Apart from this, it is well-known for its many medicinal properties since ancient time[xv,xvi]. In this paper, we report the purification and characterization of a proteinaceoustrypsin inhibitor from the seeds of *Murrayakoenigii*.

2. MATERIALS AND METHODS

2.1 Materials

Seeds of *Murrayakoenigii*were obtained locally. Trypsin, chymotrypsin, casein and Bovine Serum Albumin (BSA) were purchased from SRL (Sisco Research Laboratories, Mumbai, India). DEAE-Cellulose was purchased from Pharmacia Pvt. Ltd. Uppasala, Sweden. Sephadex G-75 was purchased from Amersham Pvt. Ltd. Molecular weight standards were obtained from Bio-Rad, Australia. All other chemicals used were purchased from SRL, India and were of analytical grade.

2.2 Protein estimation:

Protein estimation was carried out by the method of Lowry et al. using BSA as the standard [xvii]. Protein was also estimated by measuring the absorbance at 280 nm on a Shimadzu UV 1601 spectrophotometer using BSA as Standard. ($A^{1cm}_{1\%} = 6.45$)

2.3 Trypsin and Chymotrypsin Assay:

The activity of Trypsin was assayed by the method of Kakade et al[xviii] at 37^0C using Casein (2%) as substrate. Trypsin solution (2.0 mg/ml in 0.01M HCl) was prepared just before use. In this system, aliquots of trypsin (0.2-1.0 ml) were taken in a total volume of 2 ml adjusted with phosphate buffer (pH 7.4, 50 mM). The mixture was incubated at 37^0C for 10 minutes. Casein (2ml) was then added to the

solution and was incubated at 37^0C for 15 minutes. The enzyme action was arrested by adding 6 ml Trichloro acetic acid (TCA) (10% v/v). After 1 hour, TCA soluble peptides were collected by centrifugation, neutralized and estimated by reading absorbance at 280 nm or by Lowry's method. The activity of Chymotrypsin was assayed similarly by the method of Kakadeet al[18] at 37^0C using Casein (2%) as substrate. The trypsin inhibitory assay and the chymotrypsin inhibitory assay were carried out essentially as above but in the presence of an inhibitor under similar physiological conditions.

2.4 Purification of Protein:

Proteins from mature seeds of *Murrayakoenigii*were extracted with physiological saline (0.145 M NaCl, 5ml/gm seed meal) for at least 6 hrs at 5^0C. The crude protein extract so obtained was centrifuged at 12000 rpm and then was subjected to fractional precipitation with ammonium sulphate. The protein fraction precipitating between 30% to 95% saturation of ammonium sulfate was collected by centrifugation, dissolved in minimum amount of distilled water and was dialyzed extensively against distilled water and finally against phosphate buffer (pH 7.4, 50 mM). The solution so obtained (fraction 'A') was clarified by centrifugation and was further used for ion-exchange chromatography.

2.4. (1) Ion-exchange Chromatography:

Trypsin inhibitor from fraction 'A' was purified by ion-exchange chromatography. Fraction 'A' was applied to a column of DEAE-Cellulose (1.5 X 30 cm) equilibrated with 50 mM phosphate buffer, pH 7.4 under cold conditions. The column was washed extensively with equilibration buffer at a flow rate of 25 ml/hr to remove unbound proteins. The absorbed proteins were eluted with a discontinuous salt gradient (NaCl at a concentration ranging from 0.1 M NaCl to 1 M NaCl in equilibration buffer). Fractions of 5 ml were collected at a flow rate of 25 ml/hr and were monitored for the protein content and for their inhibitory activity. Active fractions were further used for purification (Figure 1). Protein fractions showing trypsin inhibitory activity were pooled, concentrated and further purified by gel-filtration.

2.4 (2) Gel filtration Chromatography:

Above active fractions were subjected to gel filtration using Sephadex G-75 column (2.0 X 50 cm) equilibrated with physiological saline (0.145 M NaCl). Proteins were eluted with physiological saline at a flow rate of 24 ml/hr. The fractions of 3ml were collected and monitored for the protein content and for their trypsin inhibitory activity.

2.5 Molecular Weight Determination:

2.5. (1) By Mass Spectrometry method:

Molecular weight of purified MKTI was determined by MALDI-TOF. MALDI-TOF was performed by embedding purified MKTI in an excess of a specific wavelength-absorbing matrix (3, 2-dimethoxy-4 hydroxy-cinnamic acid).

2.5. (2) By SDS-PAGE:

SDS-PAGE was done essentially according to the method of Laemilli et al[xix]using standard molecular weight markers (Sigma Aldrich Pvt. Lyd). The relative mobilities of MKTI and of the markers were determined and the molecular weight (M.W.) of purified MKTI was calculated by plotting a graph of Log M.W. X 10^4 Vs relative mobilities.

2.6 Specificity of Inhibition:

Specificity of MKTI towards Chymotrypsin is studied essentially according to the method of Kakadeet al[18]. Specificity of Inhibition of MKTI towards Papain and Pepsin was also studied according to the modified method of Kakade et al[18] where the reaction was arrested by heating the reaction mixture at

70^0 C for 10 minutes and by raising the pH of the reaction mixture to pH 7.6 by 0.1 M Phosphate buffer respectively. Specificity of *Murrayakoenigii*trypsin inhibitor for its inhibitory activity against human salivary amylase was determined essentially by the modified method of Bernfeld[xx, xxi].

2.7 Determination of Carbohydrate content:

The total carbohydrate content was estimated by the method of Dubois et al[xxii] using glucose as standard. Phenol-sulphuric acid positive carbohydrates were expressed as percentage of glucose as determined from a standard calibration curve using 0.1 mg/ml glucose solution.

***2.8 Determination of total disulfide content*:**

The total disulfide content of *Murrayakoenigii*trypsin inhibitor was determined by reduction of the disulfide bonds according to the method of Cavallini et al[xxiii]. This sulfhydryl content was assayed by the method of Ellman[xxiv] using the 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) reagent. The total number of sulfydryl groups was calculated using the formula:

$$N = \frac{MW \times A \times V}{12000 \times M}$$

Where, N = total number of free –SH groups, MW = Molecular weight of the protein (inhibitor), A= absorbance at 412 nm, V = volume of the final reaction mixture, M = weight of the protein (Murrayakoenigiitrypsin inhibitor) in mg.

***2.9 Effect of preincubation time on MKTI Activity*:**

MKTI (0.1 ml) was preincubated with trypsin (0.5 ml) at 37^0C for varying periods of time (0 to 60 minutes) in a total reaction mixture of 1.0 ml adjusted with phosphate buffer (50 mM, pH 7.4). The inhibitory activity was determined at the end of each incubation period using Kakadeet al[18] method as described above.

***2.10 Stoichiometric studies of binding of Murrayakoenigiitrypsin inhibitor to trypsin*:**

Stoichiometry of the interaction of MKTI with trypsin was studied by incubating varying concentrations of the MKTI with trypsin to determine the maximum extent of inhibition. A fixed amount of trypsin (2.0 mg/ml) was preincubated with varying concentrations of the inhibitor in phosphate buffer (pH 7.4, 50 mM) for 50 minutes in a total reaction mixture volume of 1.0 ml. Inhibitory activity was determined and

stoichiometry of binding was calculated by the trypsin-inhibitor ratio that corresponds to maximum inhibition.

Stoichiometry of the binding of the trypsin inhibitor with trypsin was also studied by the complex formation between MKTI and trypsin after preincubation on a Sephadex G-100 size exclusion chromatography column. Equal concentration of the solution of trypsin (500 µg/ml) was preincubated with the solution of MKTI (500 µg/ml) in phosphate buffer (pH 7.4, 50 mM) at 37°C for 50 minutes. This mixture was then applied to a Sephadex G-100 size exclusion chromatography column. The column was eluted with physiological saline and fractions of 3 ml were collected. The protein content of each fraction was determined by measuring absorbance at 280 nm. Trypsin and MKTI, in the amounts corresponding to that present in the mixture were also chromatographed separately on the same column.

2.11 pH Stability of MKTI:

MKTI was incubated with respective buffer solutions (acetate buffer: 50mM, pH 3.0-5.5, phosphate buffer: 50mM, pH 6.0- 8.0, and glycine-NaOH buffer: 50mM, pH 9.0 -10.0) in

equal volumes for 4 hours. At the end of incubation period, pH was adjusted to 7.4 and the resulting mixture (0.1 ml) was preincubated with trypsin (0.5 ml) at 37^0C in a total reaction mixture of 1.0 ml adjusted with phosphate buffer (50 mM, pH 7.4) for 50 minutes. Inhibitory activity was determined by the method of Kakadeet al[18].

2.12 Influence of pH on trypsin inhibitory activity:

MKTI was preincubated with trypsin in respective buffer solutions (50 mM, pH 2.0-10.0) in a total reaction mixture of 1.0 ml for 50 minutes at 37^0C. The residual trypsin activity at respective pH values were determined by the method of Kakadeet al[18]. Corresponding controls without MKTI were run simultaneously at all the respective pH values.

2.13 Heat stability of MKTI:

MKTI in phosphate buffer (50 mM, pH 7.4) was incubated at varying temperatures (10^0C – 100^0C) each for 15 minutes. After 15 minutes preincubated with trypsin at 37^0C in a total reaction volume of 1.0 ml adjusted with phosphate buffer (50 mM, pH 7.4) for 50 minutes at 37^0C. Inhibitory activity was determined by the method of Kakadeet al[18] as above.

2.14 Influence of temperature on trypsin inhibitory activity:

MKTI was preincubated with trypsin in a total reaction mixture of 1.0 ml adjusted with phosphate buffer (50 mM, pH 7.4) for 50 minutes at varying temperatures (10^0C – 100^0C). The residual trypsin activity at respective temperatures was determined by the method of Kakadeet al[18]. Corresponding controls without MKTI were run simultaneously at all the respective temperatures.

2.15 Kinetic Studies of MKTI:

The rate of trypsin activity, in the absence and in the presence of MKTI, was determined (Kakade et al[18]) at different substrate (casein 2%) concentrations in a total reaction volume of 2.0 ml adjusted with phosphate buffer (50 mM, pH 7.4) at 37^0C. Double reciprocal plots, according to the method of Lineweaver and Burke, were drawn for the uninhibited and partially inhibited trypsin[xxv]. Equilibrium dissociation constant (K_i) for the velocity of the reaction in the presence of the inhibitor was calculated from the graph of 1/S Vs 1/V. The nature of Lineweaver-Burke plot was used to determine the nature of inhibition.

2.16 IC_{50} Studies of MKTI:

Trypsin inhibitory activity (Kakade et al[18]) of MKTI at different concentrations of MKTI was carried out, to determine the inhibitory concentration of MKTI for 50% inhibition, in a total reaction volume of 1 ml adjusted with phosphate buffer (50 mM, pH 7.4) at 37^0C.

2.17 Circular dichroism studies:

Circular Dichroism (CD) spectrum of *Murrayakoenigii*trypsin inhibitor (100 µg/ml dissolved in phosphate buffer 50 mM, pH 7.4) was obtained under native conditions. The spectrum was recorded in a 1 cm path length cell, on a JASCO J- 715 spectropolarimeter using a scan speed of 100 nm/min, a bandwidth of 1.0 nm with a resolution of 1.0 nm in UV and near UV region. 5 scans were accumulated. Scans were accumulated at room temperature at a response time of 1 second with a sensitivity of 5 mdeg. Results were reported as mean residue ellipticity with units of degree.cm^2.$dmol^{-1}$. CD analysis was carried out by online K2d[xxvi] analysis software.

2.18 Fluorescence studies:

Fluorescence studies of *Murrayakoenigii*trypsin inhibitor were carried out on a RF-5301PC Shimadzu spectrofluorimeter. MKTI was left overnight at room temperature in 0.1 N HCl, 0.1 N NaOH and in the presence of denaturating agents such as 6 M Urea and 5 M Guanidine

hydrochloride respectively. The samples were excited at 280 nm and the emission spectra were recorded between 300 nm and 500 nm. Base line corrections were carried out using 50 mM phosphate buffer, pH 7.4 without protein sample in all cases. *Murrayakoenigii*trypsin inhibitor at a final protein concentration of 100 μg/ml was used for these studies.

3. RESULTS AND DISCUSSION

Murrayakoenigii is a well-known herbal plant that is widely consumed in India as a spice in food preparations. It is also well known for its medicinal properties. Although the presence of proteinaceousproteinase inhibitors in plants and predominantly in the seeds is well known, their function is still the subject of much interest due to their role as defense proteins in pathophysiology of various diseases. Present investigation reports the presence of a potent proteinaceousproteinase inhibitor from the seeds of *Murrayakoenigii* (MKTI) with a specific activity against serine proteinase - trypsin.

3.1 Purification of Protein (MKTI):

The seeds of *Murrayakoenigii* exhibited higher trypsin inhibitory activity than the other parts of the plant. Preliminary results show that MKTI exhibited optimum inhibitory activity in alkaline pH range. So, ion-exchange chromatography on DEAE-Cellulose was employed as one of the purification steps. The trypsin inhibitor (MKTI) from the seeds of *Murrayakoenigii* was purified to homogeneity by salt precipitation, ion-exchange chromatography on DEAE-Cellulose column at pH 7.4 (Figure 1) and by gel filtration chromatography (G-75) with a total of 75.61 folds purification (Table 1). Purified MKTI appeared as a single band in SDS-PAGE analysis under both reducing and non-reducing conditions (Figure 3).

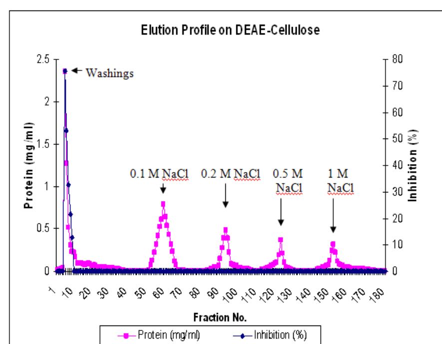

Figure (1)

Elution profile of Murrayakoenigiitrypsin inhibitor on DEAE-Cellulose.

Table (1)

Summary of purification of the trypsin inhibitor from 100 grams of seeds of Murrayakoenigii

Stage of purification	Volume (ml)	Inhibitory activity (Units/ml)	Proteins (mg/ml)	Specific activity (Units/mg)	Fold purification
Saline Extraction	920	12.25	33.26	0.36	1
Fraction-A	50	10.31	10.33	0.9980	2.77
DEAE-Cellulose chromatography	5	8.383	1.52	5.5151	15.31
Gel filtration chromatography (Sephadex G-75)	3	6.533	0.24	27.2208	75.61

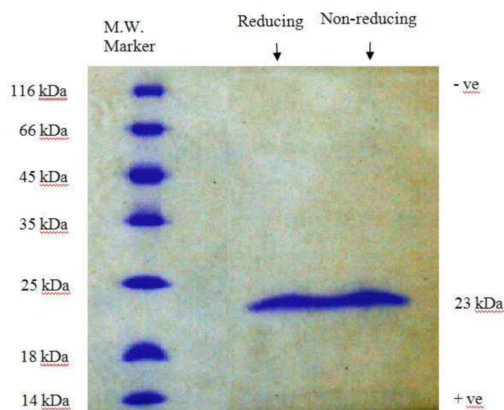

Figure (3)
SDS PAGE analysis of Murrayakoenigiitrypsin inhibitor

3.2 Molecular weight of MKTI:

Molecular weights of plant proteinase inhibitors generally vary from 4 to 85 kDa. However, majority of them are found in the range of 8 to 20 kDa. In the present investigation, the molecular weight of the purified MKTI was determined by MALDI-TOF, SDS-PAGE and gel filtration. The Molecular weight determined by MALDI-TOF (21601.44 Daltons) (Figure 2), by SDS-PAGE (23000 $\pm$ 1040 Daltons) (Figure 3) and by gel filtration (19950 $\pm$ 1023 Daltons) suggests that MKTI exists as a monomeric subunit under both reducing and non-reducing conditions

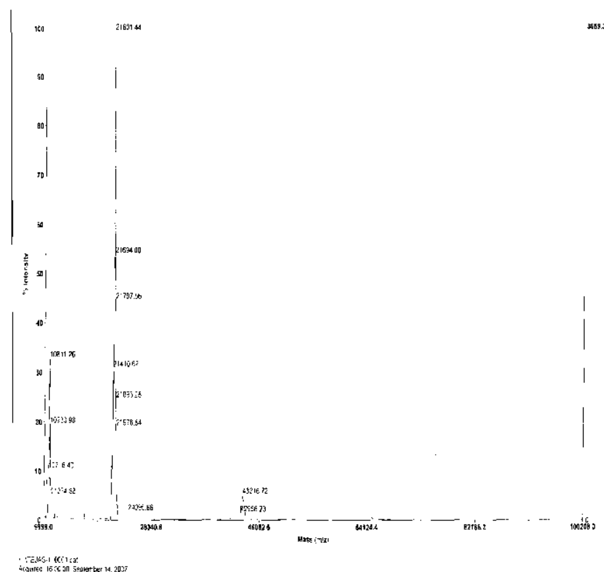

Figure (2)
Molecular weight of Murrayakoenigiitrypsin inhibitor by MALDI-TOF.

3.3 Specificity of Inhibition:

Proteinaceoustrypsin inhibitors derived from various plants have been found to be single headed, double headed, multi-headed and bifunctional[xxvii, xxviii, xxix, xxx, xxxi]. However, MKTI exhibited inhibitory activity specifically against trypsin and did not show any inhibitory activity against other serine proteinases like

chymotrypsin suggesting that it may belong to the Kunitz-type protease inhibitor family. MKTI did not also show any inhibitory activity against the proteinases of other classes like pepsin, Papain etc. which suggests that it is not a double headed inhibitor. MKTI also did not show any inhibition of human salivary amylase suggesting that it is not a bi-functional inhibitor.

Although the archetypical member of the Kunitz inhibitor family, Soybean trypsin inhibitor is not a glycoprotein, the presence of glycosylated inhibitors has also been commonly reported[xxxii, xxxiii, xxxiv, xxxv]. In the present study, the absence of any carbohydrate content (as observed by phenol-sulphuric assay) suggests that MKTI is not a glycoprotein.

3.4 Total disulfide content:

Plant proteinase inhibitors usually have a high content of cysteine residues that form disulfide bridges. One of the distinct features of the plant Kunitz (STI) family is the presence of four cysteine residues connected by two highly conserved disulfide bridges. However, Oliva et al[xxxvi] and Gowda et al[xxxvii] have reported the presence of a Kunitztrypsin inhibitor having a single disulfide bridge from the seeds of *Swartziapickellii* and *Entadascandens* respectively. Recently, Oliva et al[xxxviii] reported, for the first time, the presence of a novel cysteinless plant Kunitz-type proteinase inhibitor (18kDa) from the seeds of *Bauhinia bauhinioides*. Stability of such proteinaceous inhibitors has been attributed to other strong protein interactions. *Murrayakoenigii*trypsin inhibitor isolated in our laboratory was also found to be a cysteine-less proteinaceous inhibitor.

3.5 Effect of preincubation time on MKTI activity:

Preincubation studies of MKTI with trypsin at 37^0C for varying periods of time (0 to 60 minutes) suggested that a preincubation period of 50 minutes is essential for optimal trypsin inhibitory activity (Figure 4). The need for preincubation clearly shows that there is a definite complex being formed between the trypsin inhibitor and trypsin.

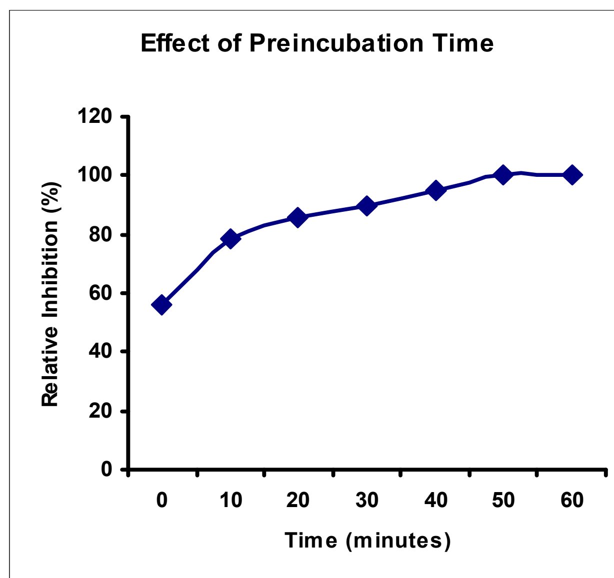

Figure (4)

Effect of preincubation time of Murrayakoenigiitrypsin inhibitor with trypsin.

3.6 Stoichiometry of binding of MKTI with trypsin:

Complex formation between the *Murrayakoenigii*trypsin inhibitor and the trypsin has been demonstrated by gel filtration on a sephadex G-100 column. A 1:1 stoichiometry of the binding of the *Murrayakoenigii*trypsin inhibitor with trypsin was observed. Comparison of the molecular weights of the individual components of the complex (M.W. of trypsin = 25118 Da, M.W. of *Murrayakoenigii*trypsin inhibitor = 19950 Da) and the complex itself (M.W. = 43151 Da) on Sephadex G 100 showed that the *Murrayakoenigii*trypsin inhibitor bound to trypsin in a 1:1 ratio.

3.7 pH and Heat stability of MKTI:

*Murrayakoenigii*trypsin inhibitor exhibited a broad range of pH stability ranging from pH 2.2 to pH 10.6 (Figure 5) with optimum inhibitory activity at pH 9.0 (Figure 6). The trypsin inhibitors from *Cassia obtusifolia*[xxxix], *Terminaliaarjuna*[xl]also exhibit similar pH stabilities. Studies on heat stability of *Murrayakoenigii*trypsin revealed that the inhibitory activity of inhibitor is stable up to 50^0C (Figure 7) with optimum inhibitory activity at 37^0C (Figure 8)

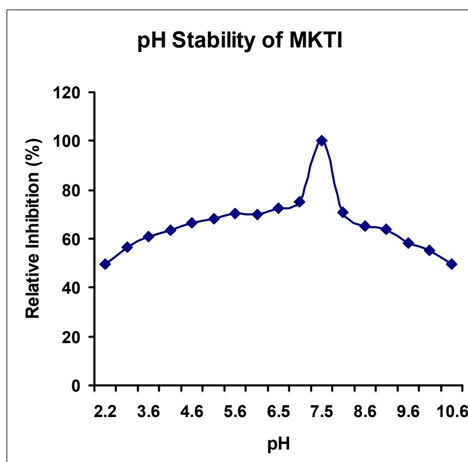

Figure (5)

pH stability of Murrayakoenigiitrypsin inhibitor

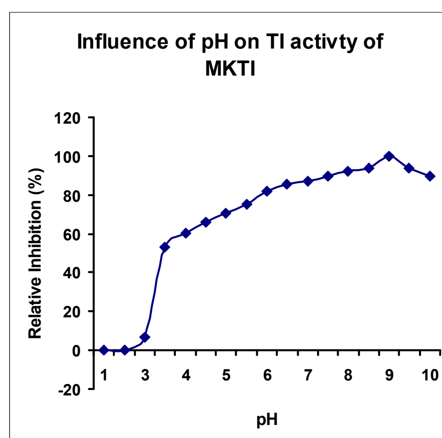

Figure (6)

Effect of pH on trypsin inhibitory activity of Murrayakoenigiitrypsin inhibitor.

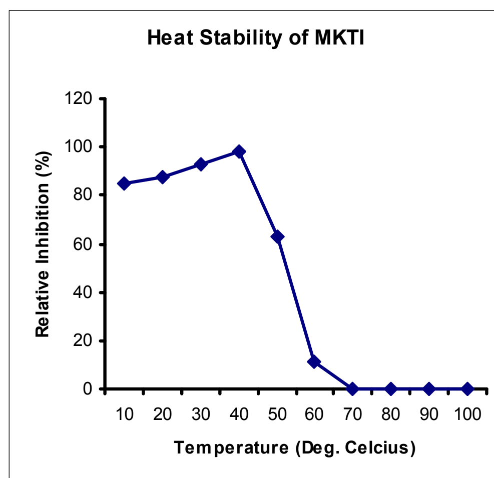

Figure (7)
Heat stability of Murrayakoenigiitrypsin inhibitor.

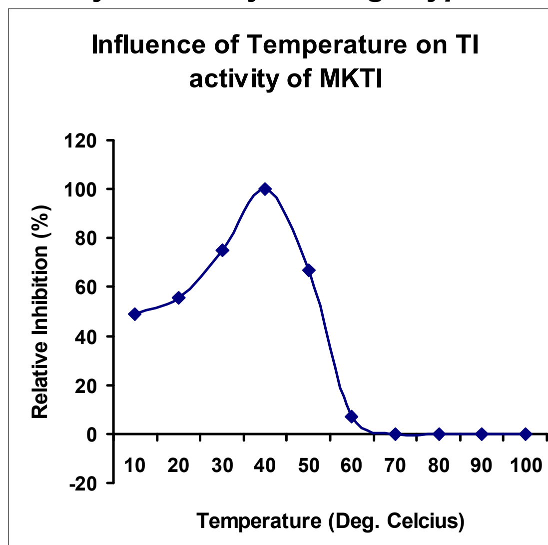

Figure (8)
Influence of temperature on trypsin inhibitory activity of Murrayakoenigiitrypsin inhibitor

***8 Kinetic Studies of MKTI*:**

Kinetic studies demonstrated that MKTI is a competitive inhibitor of trypsin. Velocity of the reaction in the absence and in the presence of MKTI was observed to be same i. e. $76.92X10^{-3}$ mg/ml/min. Dissociation constant (Km) in the absence of MKTI was found to be 4.54 mg/ml whereas dissociation constant (Ki) in the presence of MKTI was calculated to be 29.41 mg/ml (Figure 9).

IC_{50} studies of MKTI demonstrated that a concentration of 0.088 mg/ml of MKTI is essential for 50% inhibition of trypsin

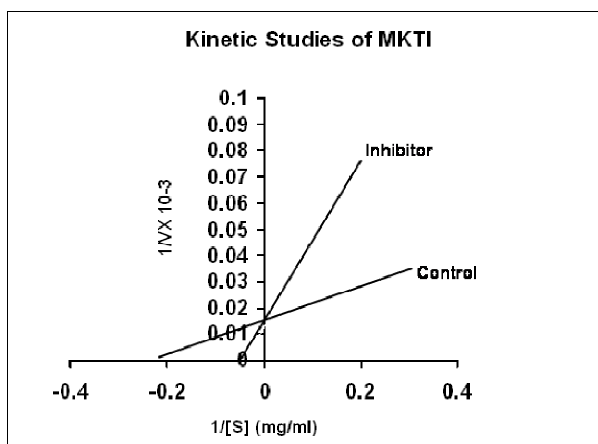

Figure (9)
Kinetic studies of Murrayakoenigiitrypsin inhibitor.

***3.9 Circular Dichroism studies*:**

Circular dichroism (CD) is being increasingly recognized as a valuable technique for examining secondary structures of proteins in solution[xli]. The X-ray and CD studies of Kunitz type proteinase inhibitors have shown that they are mostly β-sheet structures with almost little or none α-helical content. *Murraya koenigii*trypsin inhibitor revealed the presence of 4.44 % α helical content, 44.17% β sheet and 51.42% random coil structure (Figure 10). These studies further support the suggestion that the trypsin inhibitor from the seeds of *Murrayakoenigii* may belong to the classical Kunitz type proteinase inhibitors

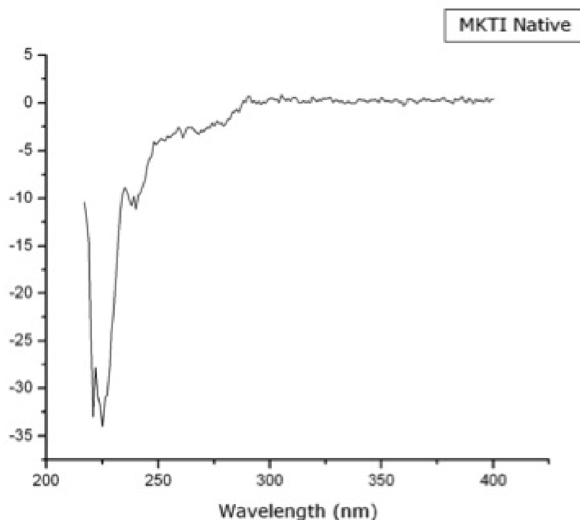

Figure (10)
Circular dichroism spectrum of Murrayakoenigiitrypsin inhibitor under native conditions.

3.10 Fluorescence studies:

The emission maxima of *Murrayakoenigii*trypsin inhibitor under native conditions was observed at 318 nm (Figure 11) (Table 2). In acidic condition, there was a slight blue shift of 2 nm with an increase in the fluorescence intensity. This may be due to the tryptophan residues getting more buried in the hydrophobic environment and the protein attaining a more folded state conformation. However, under alkaline conditions, a minor blue shift of 2 nm with an appreciable decrease in the fluorescence intensity was observed. This suggests that, under alkaline conditions, although in a compact state, the protein structure is more open or loose than in the native state. In the presence of 6 M urea, *Murrayakoenigii*trypsin inhibitor exhibited a slight red shift of 2 nm to 320 nm, suggesting a slight unfolding of the native conformation of the inhibitor. These observations that MKTI does not undergo appreciable change in its conformation in the presence of 0.1 M HCl, 0.1 M NaOH and 6 M Urea also support the broad range pH stability as well as stability towards denaturating agent such as Urea. However, the treatment of *Murrayakoenigii*trypsin inhibitor with 5 M guanidine hydrochloride showed a marked red shift of 35 nm. This is well in agreement with the observations that proteins are completely unfolded in the presence of high concentrations of guanidine hydrochloride.

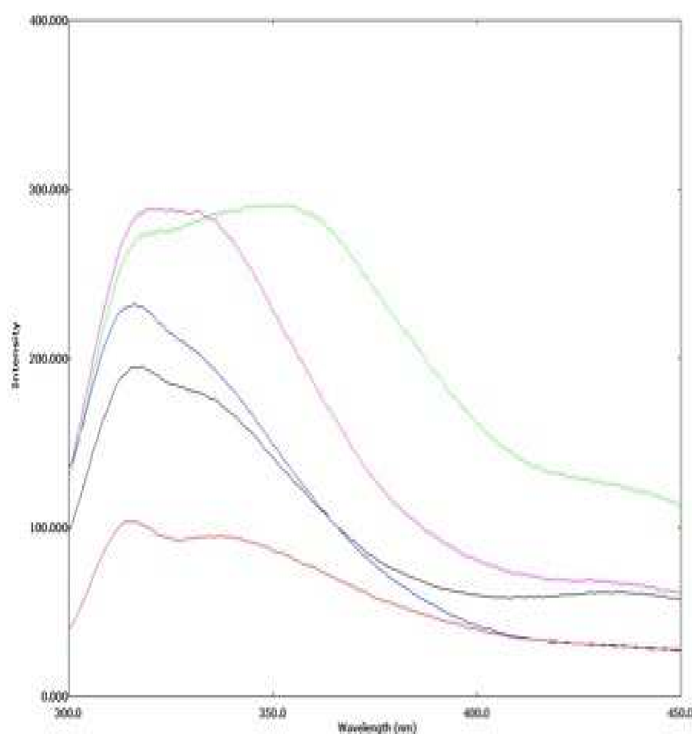

Figure (11)
***Fluorescence spectra of Murrayakoenigiitrypsin inhibitor under native conditions*.**

Black lined curve represents fluorescence emission spectra of native MKTI, Blue lined, Red lined, Green lined and Pink lined curves represent emission spectra of MKTI when treated with acid, alkali, guanidine hydrochloride and urea respectively.

Table (2)
Fluorescence emission maxima and corresponding intensities of Murrayakoenigiitrypsininhibitor.

No.	Treatment	Emission Wavelength (nm)	Intensity
1	Native	318.00	195.301
2	Acid (0.1 N)	316.00	231.929
3	Alkali (0.1 N)	316.00	103.775
4	Guanidine hydrochloride (5 M)	353.00	290.912
5	Urea (6 M)	320.00	288.630

CONCLUSION

We report the presence of a novel cysteinlessKunitz type trypsin inhibitor with a molecular mass of 21.60 kDa from the seeds of *Murrayakoenigii*. The broad pH stability and specificity of the competitive trypsininhibtor – MKTI can be further explored as a potent therapeutic intervention against the plausible patho-physiological conditions where serine proteinase inhibitors are known to be effective biomarker

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