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ORIGINAL ARTICLE

Screening different plants and fungal isolates for protease inhibitory activity and evaluation of the cytotoxicity of the most promising sample

Nischala Nadig[1], Monika Dhanapala[1], Prerana Venkatachalam[1] and Varalakshmi Kilingar Nadumane*[1]
[1]Department of Biotechnology, School of Sciences, Jain (Deemed-to-be- University), #18/3, 9th Main,3rd Block, Jayanagar, Bengaluru, India
* Corresponding author's Email: kn.varalakshmi@jainuniversity.ac.in

ABSTRACT
Protease inhibitors have shown to be an interesting group of molecules from the viewpoint of the modulation of physiological and pathological functions. There have been many reports about their therapeutic potentials including anti-cancer properties. In the current study, which was aimed at finding a best protease inhibitor (PI) from natural sources to treat cancer, a comparative analysis of PI activity among various plant sources and fungal isolates was carried out against the proteases, trypsin and chymotrypsin. PI from the best source was purified by Ammonium sulphate precipitation followed by dialysis, Ion exchange chromatography and finally Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed to characterize the PI. MTT[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was performed to assess the cytotoxicity of the extracted protease inhibitor on HeLa (human cervical carcinoma) and HepG2 (liver carcinoma) cell lines. Horse gram seeds (3rd day sprouted) were found to be the best source of a protease inhibitor, which also had highest cytotoxic activity towards in-vitro cancer cell lines. Further studies are required to establish the anticancer potential of the protease inhibitor from horse gram.
Key words: Protease inhibitors; Horse gram; Trypsin; purification fold; CMC column; Cytotoxic activity.

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INTRODUCTION

Protease inhibitors (PIs) constitute a group of proteins widely distributed among all organisms and their main function being their ability to form strong PIs complex, inhibiting the proteolytic activity. Protease inhibitors that areused in anticancer therapy have been isolated from plants, bacteria orhave been prepared synthetically. PIs play a very important role in regulation of various cellular physiological and biological processes, including cell cycle, cell death, differentiation and immune response[1][2]. Inhibitors of these proteases can suppress several stages of carcinogenesis including initiation, promotion and progression, although their mechanism of action is not yet fully clear.

Cancer is one of the most dreadful diseases with a death toll of several millions every year, around the world. Each year, of the tens of millions of people diagnosed with cancer, more than half of the patients die from it[**3**]. Though cancer therapy is in advance, side effects due to the non-specific cytotoxicity of drugs and resistance to drugs pose great problems in cancer management. Therefore, the development of novel anticancer agents, which in addition to being efficacious in curing/eradicating cancer cells, are at the same time not prone to resistance from target cells, has become a very important scientific pursuit[4].There are several small molecule protease inhibitors from microbes that have shown broad inhibitory specificity towards proteases of different catalytic classes[5].Protease inhibitors exhibit anti-tumor and anti-proliferative activities [6]. Hence, due to the need to develop an efficient cancer therapy, the current study was aimed at isolating protease inhibitors (PI) from plant and fungal sources,

purification, characterization of the potent PI and screening for its anticancer activity on *in-vitro* cancer cell lines.

MATERIAL AND METHODS

Different plant samples used

A variety of plants and plant parts were collected from different parts of Bangalore. The plant samples included different types of fruits, leaves, seeds, tubers and flowers. Some of the fruit samples used were turnip, coconut, kokum, Ricinus, banana, pomegranate seeds and mango pulp. Several seeds like mango seed, flax seeds, ajwain, jeera, methi and seeds from leguminous plants such as brown bean, chickpea, green peas and horsegram were also collected.

Microbes used in the study

Apart from the plant samples, fungal colonies were collected from air exposure method on CzapekDox Agar (CDA) plates, from which a few fungal colonies were subcultured to separate CDA plates to make pure cultures [7].

Proteases chosen

Trypsin and chymotrypsin were used as proteolytic enzymes to screen for the protease inhibitory activity of the chosen natural sources. Trypsin: 0.25% trypsin with 0.02% EDTA in Dulbecco's phosphate buffer saline (HIMEDIA:TCL007); Chymotrypsin: 0.1mg/ml in phosphate buffer (HIMEDIA:RM801-1G)

Extraction of protease inhibitor (PI)

Plant sources: 100 g of seeds of leguminous plants were washed with water and soaked in water for 24 h at room temperature. After 24 h, they were divided into 4 parts and one of the parts was drained of water and stored at -4°C. The rest were incubated for 24h, 48h, and 72h which corresponds to day 1, 2, and 3 sprouted horse grams respectively and were stored at 4°C.25 g of each of the soaked, sprouted seeds (day 1, 2, 3) were weighed and kept separately. They were homogenized with 0.1M phosphate buffer of pH 7 at room temperature and centrifuged at 10000 g for 30 minutes at 4°C[8].Other plant parts like fruits, leaves and tubers (25g) were directly homogenized with 0.1M phosphate buffer and centrifuged as above. The resultant supernatant was used for protease inhibitory activity assays.

Fungal sources: Fungal isolates were cultured in 100 mL of Czapek Dox Broth (CDB) in 250 mL conical flasks at room temperature (26±2°C) for 5 days. The culture was then centrifuged at 10000 g for 10 min. The culture supernatant was used for analysing the protease inhibitory activity against trypsin and chymotrypsin.

Protease inhibitor (PI) activity assay

The protease inhibitor (PI) activity was assayed according to the procedure described by Kunitz with slight modifications [9]. Trypsin and chymotrypsin are the two proteases that were chosen for the experiment. Trypsin-EDTA solution (0.25%) and 0.1 mg/mL of chymotrypsin, prepared in 0.1 M phosphate buffer (pH 7.2) were used for the assays. 1 mL of trypsin/chymotrypsin was pre-incubated with 1mL of the culture supernatant of suitable dilutions at 37°C for 15 min. 2 mL of 1% casein, prepared in 0.1 M phosphate buffer of pH 7.2, was added and then incubated at 37°C for 30 min. The above reaction was terminated by the addition of 2.5 mL of 0.44 M trichloroacetic acid (TCA) solution. The reaction mixture was then centrifuged at 10,000 g for 15 min at 4°C and the optical density of the supernatant was measured at 280 nm using a spectrophotometer. Suitable blanks for substrate, enzyme and inhibitor were processed. TCA soluble peptide fractions of casein formed by the action of the two proteases in the presence and absence of the inhibitor were quantified in comparison with tyrosine as the standard. One unit of enzyme activity is defined as the amount of enzyme that liberates 1μmole/min/mL of tyrosine under specific assay conditions. One protease inhibitory activity unit (PIU) was defined as the amount of inhibitor that decreased the absorbance by one unit of TCA soluble casein hydrolysis product liberated by the enzyme action at 280 nm per minute under the assay conditions.The protease inhibitor activity was also expressed as inhibition percentage which was determined by comparison with a control experiment for comparative purposes and calculated as follows:

$$\text{Inhibition}(\%) = \frac{\text{Amount of tyrosine released (C)} - \text{Amount of tyrosine released (S)}}{\text{Amount of tyrosine released (C)}} \times 100$$

where (**C**) is tyrosine released without the inhibitor and (**S**) is tyrosine released with inhibitor

Protein estimation

Protein estimation was performed using Bovine Serum Albumin (BSA) as the standard [10]. The final protein concentration was expressed as mg/mL.

Purification of PI from the best source, horse gram (*Dolichs biflorus*)seeds

Ammonium sulphate precipitation

Ammonium sulphate precipitation of the sample was performed according to the method described by Englard and Seifter[11]. The fractionation using ammonium sulphate precipitation has the advantage of intermediate removal of unwanted proteins and simultaneously the protein of interest could be concentrated. Ammonium sulphate (Fisher scientific) required to precipitate the protease inhibitor was optimized by adding varying concentrations (30%, 60% and 90%) to the crude extract.The precipitate obtained after ammonium sulphate precipitation was further dialyzed against 0.01M phosphate buffer of pH 7.0, in order to remove ammonium sulphate from the precipitate. The precipitated PIs (which were proteins) were dissolved in minimal quantity of phosphate buffer of pH 7.2. The mixture was then lyophilized. 1 mg/mL stock concentration of the partially purified inhibitor was prepared and the protease inhibitory assay was performed.

Ion exchange chromatography

The active PI fraction obtained after the dialysis of ammonium sulphate precipitation was further purified by Ion exchange chromatography, using CMC cellulose as the anion exchanger[12]. CMC - cellulose was first washed with a lower concentration of the adsorption buffer being used. 1mL of ammonium sulfate precipitated sample, at 5 mg/mL concentration was applied to the pre equilibrated CMC Cellulose column. The sample was eluted using 0.01M phosphate buffer with 0.5M sodium chloride. Fractions were collected with a flow rate of 0.15mL/ minute. The unbound proteins were washed out until the absorbance at 280nm reached near to zero. 3mL fractions were collected separately and the protein content of each fraction was determined by measuring the absorbance at 280nm. Active fractions from the column were pooled and lyophilized. 1mg/mL stock solution of the column purified active fraction was prepared and the protease inhibitory assay was performed as described earlier. The lyophilized fractions along with crude and dialyzed samples of soaked and day 3 sprouted horsegram seedswere assayed for PI activity, protein content and specific activity. The yieldand fold of purifications were calculated.

SDS-PAGE (SDS- polyacrylamide gel electrophoresis)

The sample extracts (soaked and day 3 sprouted HG), ammonium sulfate precipitate (soaked and day 3 sprouted HG) and column purified (day 3 sprouted HG) were subjected to SDS-PAGE for molecular weight determination as per the standard protocol [13]. A medium molecular weight marker of HIMEDIA was used as the molecular weight marker.

Cell lines

HeLa (human cervical carcinoma) and HepG2 (liver carcinoma) cell lines were procured from National Centre for Cell Science (NCCS), Pune. The cell lines were being maintained in MEM (Minimal Essential Media-HIMEDIA) supplemented with 10% FBS (Fetal Bovine Serum-HIMEDIA).

MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide])-assayfor Cytotoxicity

MTT assay was performed as per the standard protocol to assess the cytotoxicity of the extracted PIs on HeLa and HepG2 cancer cell lines[14]. The cell lines ($1x10^4$ cells/mL), after trypsinization were seeded onto 96-well microtiter plates. After 24 h, 10 µl of the inhibitor was added at varying concentrations (1, 10, 50 and 100 dilutions) for 48 h and afterwards 20 µl of MTT (5 mg/mL) was added to each well and the plates were incubated in a dark chamber for 3h. To dissolve the formazan crystals, 100 µl of DMSO was added and in an ELISA plate reader the absorbance was recorded at 540 nm. The percentage viability was calculated as follows:

$$\text{Viability (\%)} = \frac{\text{Optical density of the sample}}{\text{Optical density of the control}} \times 100$$

Statistical analysis

All experiments were replicated thrice,with 6 replicates being kept for each concentration and the results are expressed as mean ±SD. The results were analyzed statistically using one-way ANOVA (Prism 6 software) with $P<0.05$ significance level.Comparison of means was done by Duncan's multiple range test (DMRT) at a significance level of $P<0.05$.

RESULTS

PI activity of crude samples

The initial screening for protease inhibitory activity was performed for the chosen samples. The inhibitory activity of fruits and vegetables including turnip, coconut, banana, pomegranate, peas and raw mango pulp were calculated against chymotrypsin and the results are shown graphically (**Figure 1**). Turnip at 1:10 dilution showed the highest inhibitory activity among the lot with 5.7%. This was followed by raw mango pulp at 1:10 dilution giving 4.48% inhibition. The same sample at 1:50 dilution gave 2.8%

and 1:25 dilution with 2.62% inhibition. Banana showed the lowest inhibitory activity at 1:10 dilution with 0.08%. Pomegranate and green peas had no protease inhibitory activity at all the tested concentrations.

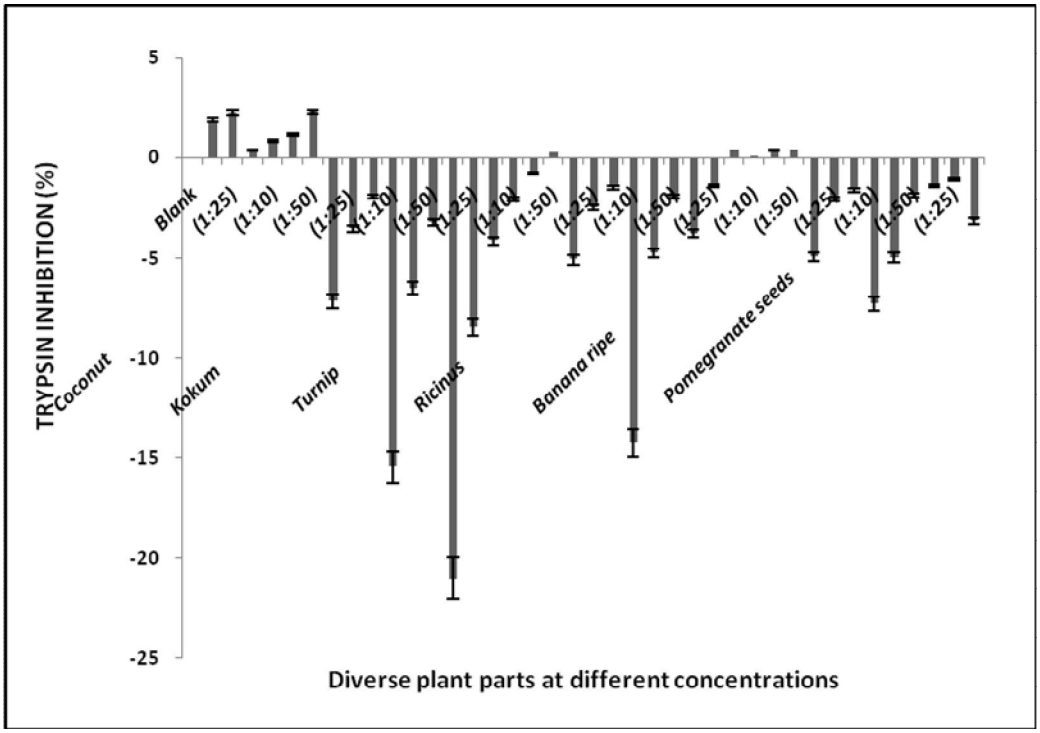

Figure 1: Protease inhibitory activity of different plant samples against trypsin

All the samples of green gram gave negative results for inhibition against trypsin, i.e., the samples augmented on protease activity rather than inhibiting it (**Figure 2).** Brown bean (soaked) showed an inhibition of 3.5% against trypsin at 1:10 and showed the highest inhibition of 23.4% at 1:25 dilution.

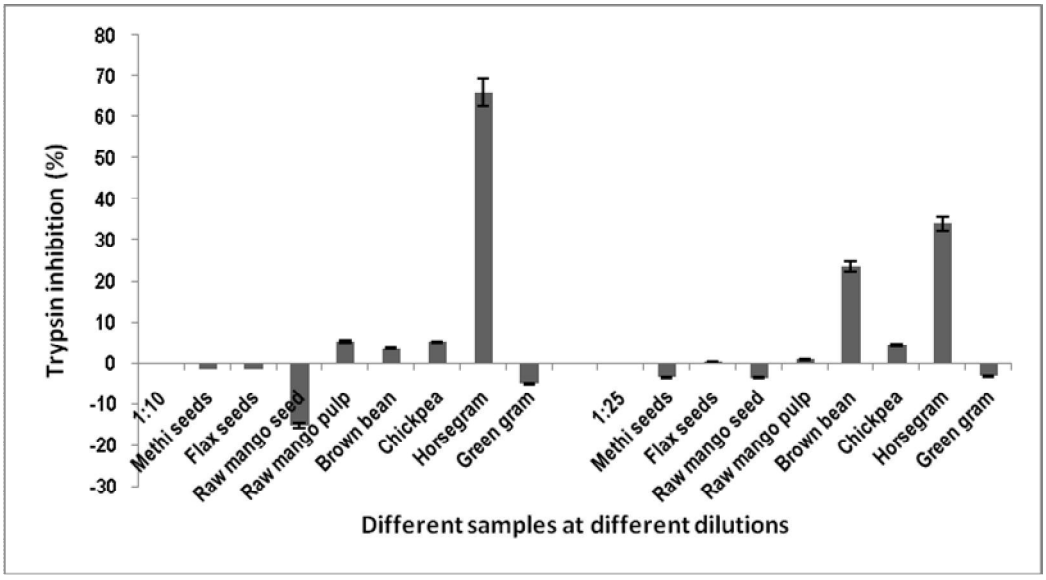

Figure 2: Protease inhibitory activity of pulses and other seeds against trypsin

Horse gram day 1 sprout at 1:10 dilution showed the maximum inhibition of 69.43% and the sample at 1:25 dilution showed inhibition on 33.9% against trypsin (**Figure 2**).

The PI activity for few medicinal plants such as tulasi, kokum, phyllanthus, turmeric and amla were calculated and graphically represented (**Figure 3**). It was found that turmeric at 1:50 dilution showed the highest trypsin inhibitory activity with 4.75% among the samples taken. No inhibition was seen for tulasi, kokum, phyllanthus and amla at all the tested concentrations.

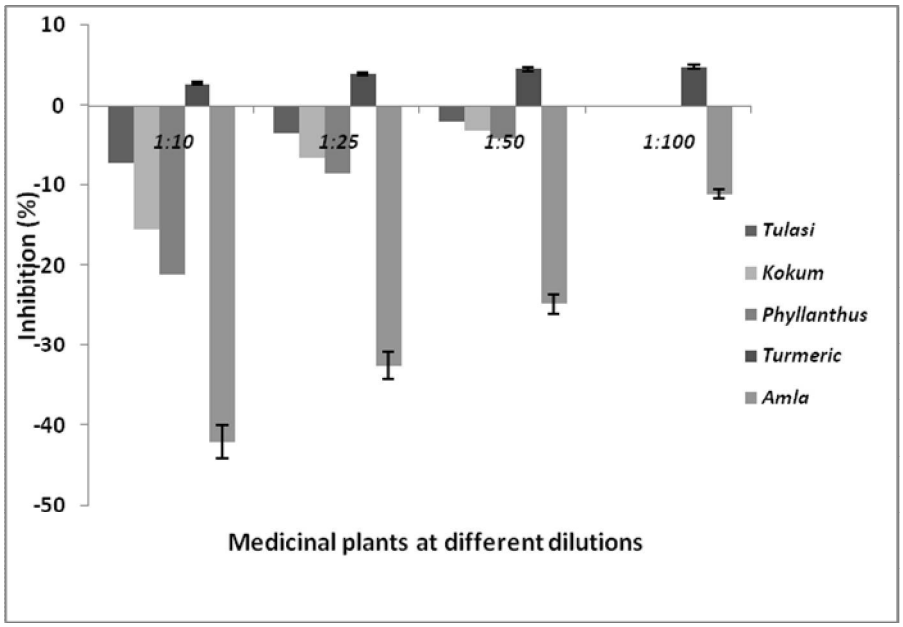

Figure 3: Protease inhibitory activity of different medicinal plants against trypsin

Among the fungi, the fungal isolate 'B' showed maximum inhibition against trypsin at 1:25 dilution with 5.41%, while the isolate 'F' showed the next highest inhibitory activity of 5.2% at 1:25 dilution (**Figure 4**).

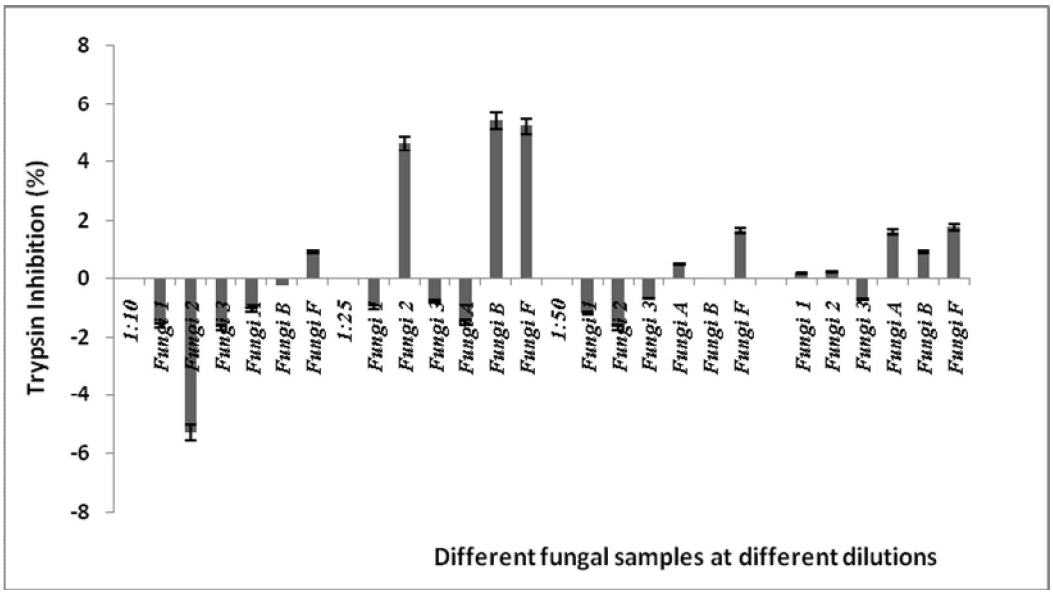

Figure 4: Protease inhibitory activity of different fungal isolates against trypsin

PI activity of different pulses(soaked, day 1, day 2and day 3 sprouts)

All the samples of green gram gave negative results for inhibition against trypsin, i.e., the samples augmented on protease activity rather than inhibiting it (**Figure 5a).** Brown bean (soaked) showed the highest inhibition of 11.15% against trypsin at 1:10 and showed the lowest inhibition of 0.08% at 1:100 dilution (**Figure 5b**). Chickpea sprout at day 2 showed maximum inhibition of 23.37% at 1:10 dilution and chickpea sprout at day 1 showed the least inhibition of 0.16% at 1:25 dilution against trypsin (**Figure 5c**). Horse gram day 1 sprout at 1:10 dilution showed the maximum inhibition of 69.43% and the sample at 1:100 dilution showed minimum inhibition on 2.51% with trypsin (**Figure 5d**). The PI activity of soaked, day 1, day 2 and day 3 sprouts of Horsegram did not vary significantly among each other, but highest activity was observed for 1:10 dilution of all these samples.

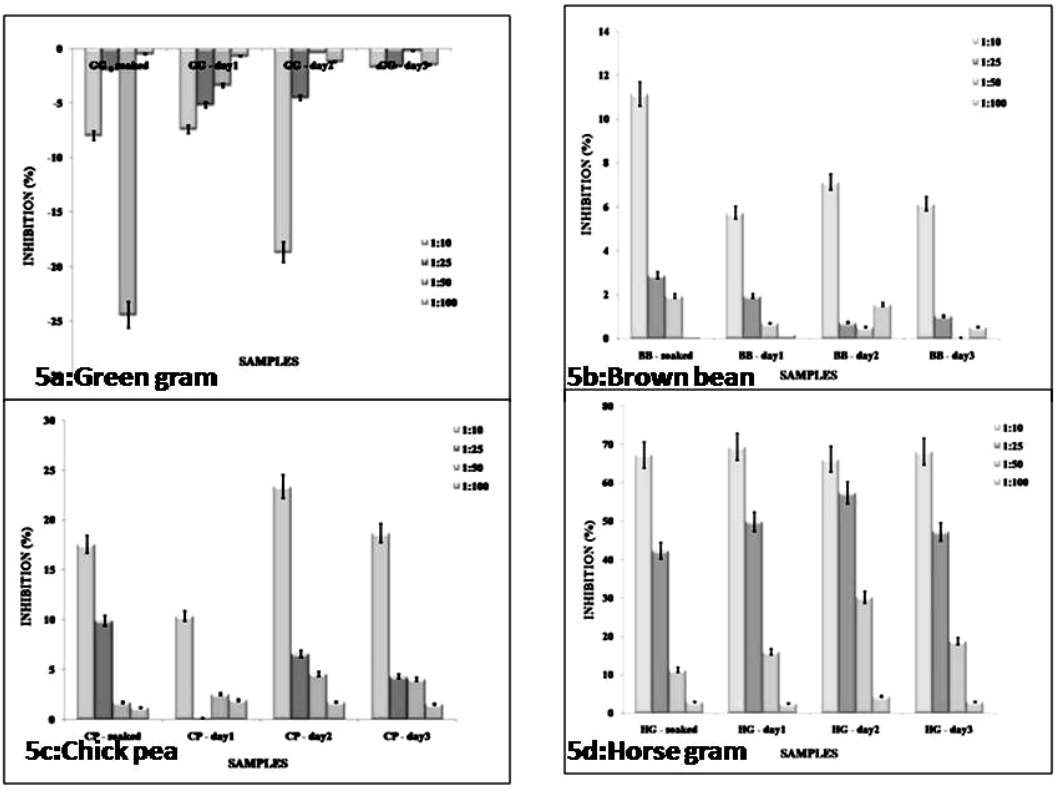

Figure 5: PI activity of different pulses along with their day1, day 2 and day 3 sprouts against trypsin.

Partial purification of the best PI (HG seeds) by ammonium sulfate precipitation

Among all the tested samples, the extract of the horse gram (HG) seeds demonstrated the highest protease inhibitory activity (67-70%), followed by chickpea (day 2 sprout) with 23% inhibition of trypsin. Hence, soaked horse gram and day 3 sprout from the horse gram samples were taken for further purification studies.Ammonium sulfate precipitation was carried out with soaked horse gram and horse gram day 3 sprout samples at 0-30%, 30-60% and 60-80% precipitation.The protein was precipitated at30-60% with highest PI activity in day 3 sprout of horse gram with a value of 82.12% at 1:10 dilution (**Figure 6a &6b)**

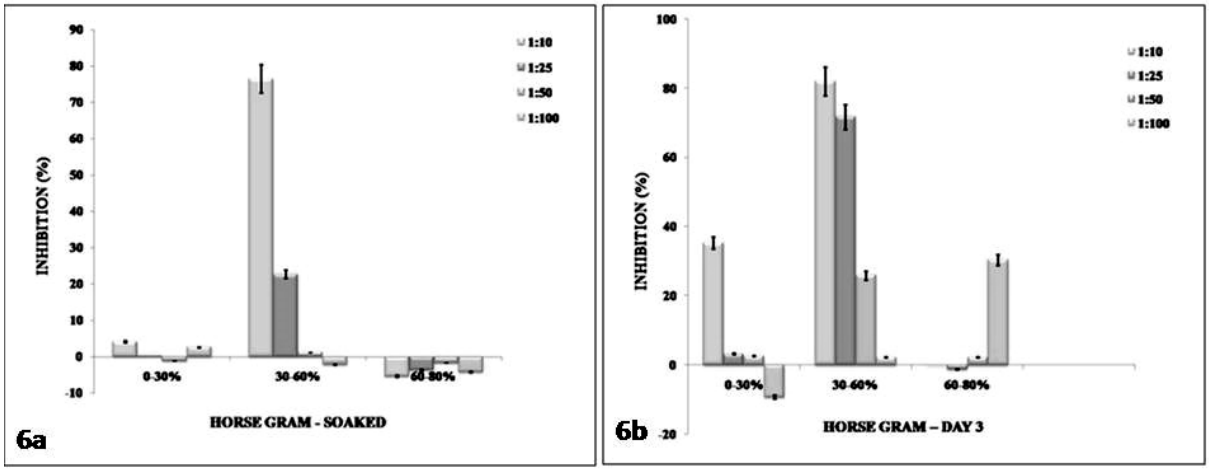

Figure 6: Protease inhibitory activity for ammonium sulphate precipitate from HG.**6a:** HG-soaked sample. **6b:** HG -day 3 sprouted sample.

Purification by CMC column chromatography

When ammonium sulfate precipitated sample from day 3 sprout of horse gram was subjected to CMC column purification, the eluted fractions were collected in different vials (3mL each). All the fractions

were checked for protease inhibitory activity and it was found that fraction 5 gave the highest inhibition of trypsin activity at 65.4% (**Figure7a**), followed by fraction 6 (54%). When checked against chymotrypsin, again, fraction 5 demonstrated the highest inhibition with 86.7% (**Figure 7b**).

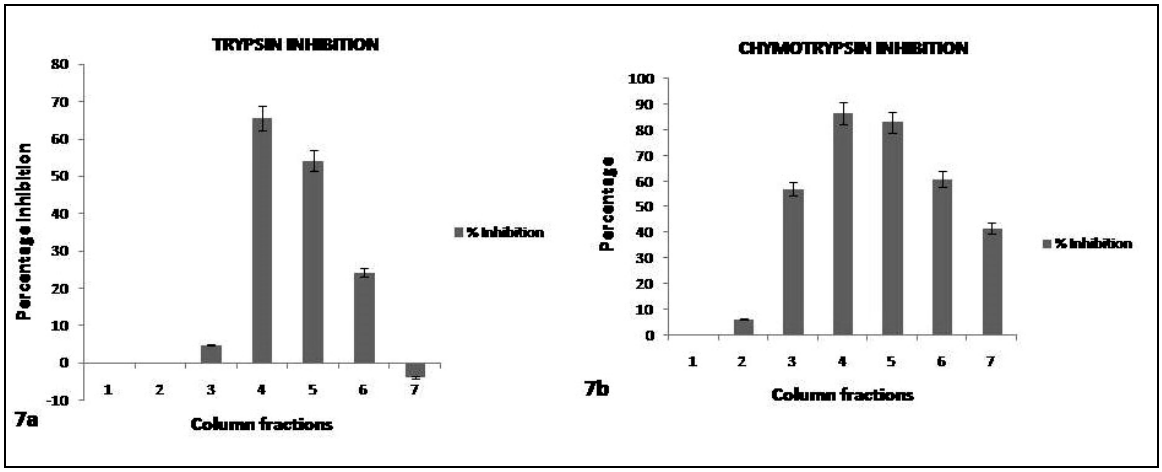

Figure 7: Protease inhibitory activity of the column purified PI sample from HG seeds. **7a.**PI against trypsin. **7b.** PI against chymotrypsin

Fold purification

The fold purification of ammonium sulfate purified and CM cellulose purified protease inhibitor fractions (PI) of horse gram day 3 sprout samples were found to be 5.85 and 7.25 respectively against trypsin (**Table 1**), and 25.31 and 56.55 against chymotrypsin (**Table2**). Hence, the chymotrypsin inhibiting sample was found to show two-fold increase in purification.

SDS-PAGE

The result of SDS-PAGE (**Figure 8**) shows that the lanes 4 and 7 loaded with column purified PI show three clear bands of proteins, indicating that the PI could be trimeric or it still needs to be purified to homogeneity.

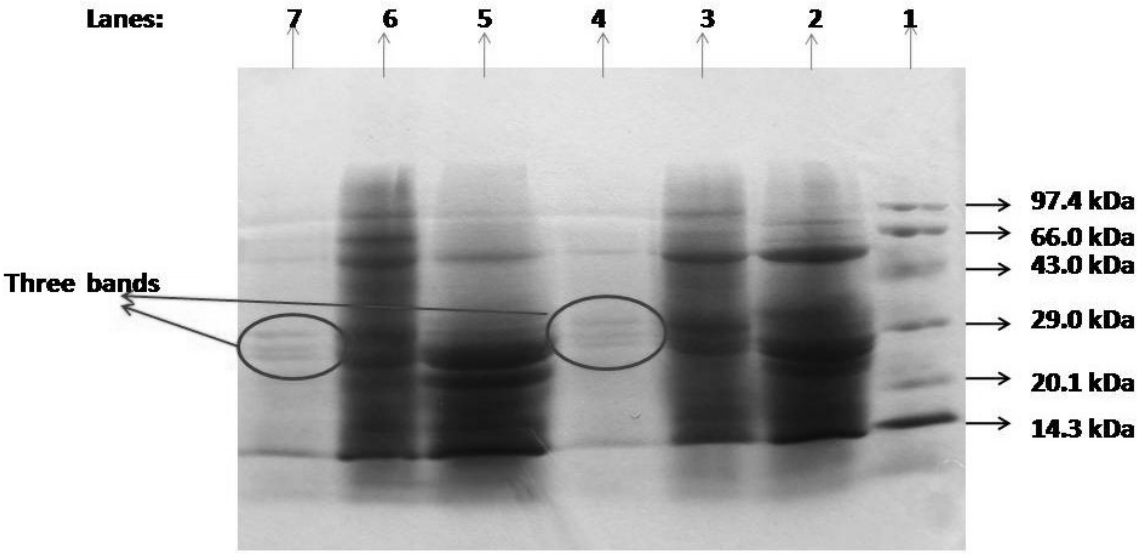

Figure 8: Molecular weight determination of samples using SDS-PAGE
Lanes 1: Marker; **2 &5:** crude PI samples, **3 & 6:** ammonium sulphate precipitated samples; **4 & 7:** column purified samples showing three bands.

Cytotoxicity assay

To analyze the ability of the protease inhibitor to control the growth of cancer cells, the percentage viability of HeLa cells was checked after treating with samples having protease inhibitory activity. Samples such as soaked horse gram extract day 3, sprouted horse gram extract, ammonium sulphate

precipitated sample, turmeric extractand chickpea extract.After 48 hours of incubation, the percent viability of treated HeLa cells decreased in a few tested samples. The highest decrease in the viability was seen in 1:10 dilution of turmeric with a viability percentage of 76.34%, followed by day 3 sprouted horse gram with 77.2% viability at 1:10 dilution. The samples have sown some degree of cytotoxicity as evident from the results (**Table 3**). When one-way ANOVA was performed using Graph pad prism version 6, the p value was found to be 0.0077 which was significant compared to the control.In case of liver cancer cell line HepG2 also turmeric (at 1:100 dilution), had demonstrated highest cytotoxic effect with 74% viability after 48h of treatment, followed by the ammonium sulphate precipitated day 3 sprout of horse gram with 76% viability. Many samples demonstrated cytotoxicity as shown in the table (**Table 4**). When one-way ANOVA was performed using Graph pad prism version 6, the p values were found to be significant.

Table 1: Purification summary of horse gram PI against trypsin.

Sample	Volume (mL)	Total Protein (mg)	Total protease inhibitory unit (PIU)	Specific activity (U/mg)	Yield of activity (%)	Fold purification
Crude	20	49.76	17.84	0.35	100	1
Ammonium Sulphate	4	2.31	4.76	2.05	26.68	5.85
CMC - Cellulose	3	1.59	4.06	2.54	22.75	7.25

Table 2: Purification summary of horse gram PI against chymotrypsin.

Sample	Volume (mL)	Total Protein (mg)	Total protease inhibitory unit (PIU)	Specific activity (U/mg)	Yield of activity (%)	Fold purification
Crude	20	90.85	26.9	0.29	100	1
Ammonium sulphate purified	4	1.23	9.03	7.34	33.56	25.31
CMC - Cellulose	3	0.237	3.88	16.4	14.42	56.55

Table 3: Percentage viability of HeLa cell line after 48h of treating with the chosen samples.

Samples	Viability (%)			
	Undiluted	1:10 diluted	1:50 diluted	1:100 diluted
HG-S	92.9	79.2	89.4	-
HG-3	91.4	**77.2**	82.9	-
HG-3 D	-	88	86	-
Turmeric	109.3	**76.34**	86.2	82
CP	121.83	91.22	99.16	88.15

HG-S: Horse gram soaked; **HG-3:** Horse gram day 3 sprouts;**HG-3 D:** Ammonium sulphate precipitated horse gram day 3 sprouts; **CP:** Chickpea

Table 4: Percentage viability of HepG2 cells after 48h of treating with the chosen samples

Samples	Viability (%)			
	Undiluted	1:10 diluted	1:50 diluted	1:100 diluted
HG-S	86.46	82.89	86.18	-
HG-3	95	**77**	81.1	-
HG-3 D	-	90	**76**	-
Turmeric	106.61	**76**	90	**74**
CP	94.3	**76**	86.9	79.8

HG-S: Horse gram soaked; **HG-3:** Horse gram day 3 sprouts; **HG-3 D:** Ammonium sulphate precipitated horse gram day 3 sprouts; **CP:** Chickpea

DISCUSSION

PIs have shown to be an interesting group of molecules from the viewpoint of the modulation of physiological and pathological functions. The study of PIs as anti-cancer agents has been an important subject of research since more than three decades ago. Recent studies have showed the mechanisms by which PIs exert their anti-tumor effect. Altogether, PI constitutes a promissory type of molecules with potential for their possible application in prognosis, diagnosis and therapy in cancer [15]. Horse gram, *Macrotyloma uniflorum* Lam. (Verdc.), (previously known as *Dolichos biflorus*), was reported as an

underutilized and unexplored legume[16][17].The importance of horse gram was well recognized by the folk/alternative/traditional medicine as a potential therapeutic agent to treat kidney stones, urinary diseases, piles, common cold, throat infection, fever etc.[18]. But scientific studies have not been focused to explore its potential towards cancer therapy. In a previous report, protease inhibitor from horse gram was extracted and purified to apparent homogeneity in a single step by IMAC using Zn-alginate beads, where the PI bound to the beads were eluted by imidazole [19].

In the current study, which was aimed at finding a suitable protease inhibitor with promising anti-cancer potential, we have screened various plant sources as well as fungal samples for PI activity against trypsin and chymotrypsin. Many plant samples like turnip, coconut, kokum, banana, mango pulp, Phyllanthus, insulin, Nerium, turmeric, dahlia tubers, brown bean, chickpea, green peas, Ricinus, horsegram, mango seed, flax seeds, ajwain, jeera and methi seeds were collected from local market and few fungal isolates were also collected. A comparative analysis was carried out among these samples for protease inhibitory activity (trypsin and chymotrypsin), the best PI was activity was identified in 3rd day sprouted horse gram seeds, which we purified using ammonium sulphate precipitation and column purification methods. The cytotoxic activityof the promising PI was checked on HeLa and HepG2 cancer cell lines.

The highest PI activity was found in the samples of soaked Horse gram (67.32%) and day 3 sprouted horse gram (68.19%) among the various samples checked for their activity. Chickpea (23.37%) and brown bean (11.15%) extracts were next to horse gram in demonstrating protease inhibitory activities. In an earlier study, a protease inhibitor of molecular weight 23.6 kDa was reported from *Moringa oleifera*[20]. Two trypsin inhibitors with anti-proliferative effects were reported from from*Phaseolus vulgaris*cv "White Cloud Bean"[21].Among the fungal isolates, fungus 'B' was having protease inhibitor activity (15.9%). As highest PI activity was shown by soaked horse gram and 3rd day sprouted horse gram, these were taken for further purification studies. These two samples were first purified with ammonium sulfate precipitation and the sample obtained after dialysis was again checked for protease inhibition activity. The horsegram day 3 sprouted sample gave a higher activity and hence was further purified using CMC column chromatography.

Further analysis of the protease inhibition activity of Horse gram day 3 (crude), horse gram day 3 (ammonium sulphate purified) and the column fractions for the same was done against trypsin and chymotrypsin. The ammonium sulphate purified 3rd day sprouted horse gram sample showed the highest inhibition of 74.99% against trypsin and the same sample showed the highest inhibition of 86.39% against chymotrypsin. Isolation, purification and characterization of a protease inhibitor-Hayanin from Horse gram was reported earlier [22]. The antioxidant potential of the protease inhibitor isolated from the seed coats of Horse gram was reported [23].

Further to check for their potential anticancer applications, the samples were treated to HeLa and HepG2 cancer cell lines. Here again, the samples with promising PI activity have demonstrated promising cytotoxicities to the tested cell lines with percentage viability ranging from 77-82%. The cytotoxic activity was found to be highest for 3rd day sprouted horse gram precipitated by ammonium sulphate and purified (76%) and turmeric (76%) against Hep-G_2 cell line with 76% viability for both. Against HeLa cell line, highest cytotoxicity was again exhibited by 3rd day sprouted horse gram with 77.2% viability and turmeric with 76.3% viability. PI from all tested samples had higher cytotoxic activity towards Hep-G2cell line when compared to HeLa cell line. In Indian traditional medicine, horse gram seeds are used for treatment of urinary stones [24][25], piles and urinary diseases [26], in women to control abnormal menstrual cycle [27]. But to the best of our knowledge, this is the first report of the cytotoxicity of a PI from horse gram (*Macrotyloma uniflorum*) seeds. Further studies should be carried out for complete characterization of the PI from horse gram and to analyze their effects on *in vivo* animal models of cancer so as to develop a cancer therapeutic from a nutraceutical source.

CONCLUSION

Based on the results of current research on screening, protease inhibitory assay, purification, characterization and cytotoxicity studies of the promising protease inhibitor, we can conclude that the purified protease inhibitor from an important nutraceutical plant source, horse gram seed, has the potential for the development of effective medicines in pharmaceutical industries against serine protease, such as chymotrypsin and trypsin. Also, this protease inhibitor could become a suitable candidate for several biotechnological applications particularly in cancer therapy after thorough investigations. Hence, this PI molecule may serve as an effective and alternative source of anticancer agent against resistant cancers, for which further extensive studies need to be carried out.

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CONFLICT OF INTEREST DISCLOSURE
The authors state that there is no conflict of interest to declare.

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