Trypsin inhibitors demonstrate antioxidant activities, inhibit A549 cell proliferation, and increase activities of reactive oxygen species scavenging enzymes

Tooba Naz Shamsi, Romana Parveen, Sadaf Fatima

Abstract:
OBJECTIVES: Protease inhibitors are one of the most promising and investigated subjects for their role in pharmacognostical and pharmacological studies. This study aimed to investigate antineoplastic and antioxidant activity of trypsin inhibitors (TIs) isolated from three plant sources and their inhibitory role in the cell line.

MATERIALS AND METHODOLOGY: TIs were obtained from different plant sources. Antineoplastic potential on adenocarcinoma human alveolar basal epithelial cell line (A549) and normal Human Embryonic Kidney (HEK) was determined using MTT assay. Activities of antioxidant enzyme, nitric oxide scavenger, superoxide dismutase, glutathione S-transferase, and glutathione peroxidase were assessed in cell lines incubated with and without TIs. The outcome was analyzed by spectrophotometer.

RESULTS: TIs showed the higher cytotoxicity on A549 cells as compared to normal HEK cell line. TIs exhibited fair increase in antioxidant enzyme activity in A549 cells as compared to control. This might be one of the strategies of antineoplastic effect in cancer cells.

CONCLUSIONS: This study has reported the antioxidant and antineoplastic properties of these TIs for the first time in A549 cells (to the best of our knowledge). The results show that TIs possess ability to prevent cancer and diseases caused due to oxidative stress. Therefore, we conclude that TIs can be used as supplements along with the conventional drugs for increased efficacy in the treatment of diseases such as cardiovascular disease, atherosclerosis, and cancer.

Keywords: Antioxidant, lung cancer, protein, protein protease inhibitors

Introduction
Protease inhibitors (PI) are small peptides capable of inhibiting proteolytic enzymes. Proteinaceous PIs in plants may help in regulating and keeping a check on endogenous proteases and in acting as protective agents against insect and/or microbial proteases.[1] In living cells, protease signaling pathways are strictly regulated, and the dysregulation of protease activity can lead to pathologies, important being cancer. The dysregulated proteases cause the dysregulation of cell cycle, apoptosis, cell growth and activation, cell-cell adhesion, cellular interactions, and signal transduction. These all suggest that proteases are hallmarks of cancer.[2]

Cancer progression and metastasis begin with tumor cell invasion. This is a multistep...
process in case of humans and animals involving various events of adherence, migration, and degeneration of extracellular matrix, and establishment of cancerous cells at the new site.\textsuperscript{[1]} Metastasis of cancer cells requires action of proteases including serine and matrix metalloproteinases (MMP) family that functionally comprises complex interacting protease cascade system.\textsuperscript{[2]} MMPs along with activated trypsin are coexpressed to facilitate cell invasion and metastasis. A complex array of cellular and molecular transformations involved in cancer formation is conciliated by a range of exogenous and endogenous stimuli. For example, oxygen (dioxygen) reduction intermediates, i.e., reactive oxygen species (ROS) such as hydroxyl radicals and hydrogen peroxide ($\text{H}_2\text{O}_2$) invade deoxyribose backbone of DNA along with the attacked bases and hence are considered as an important class of carcinogens.\textsuperscript{[3]}

Biochemical redox reactions such as mitochondrial respiration continuously produced ROS as by-products. To counterbalance these ROS, human body has inbuilt defense mechanism but increase in ROS level due to imbalance between ROS generation and antioxidant machinery may lead to oxidative stress. This oxidative stress is the root cause of various pathologies such as hypertension, inflammation, diabetes, atherosclerosis, Parkinson’s and Alzheimer’s, cardiovascular diseases, and cancer.\textsuperscript{[4]} Furthermore, ROS are said to be associated with mutations, DNA damage, and genetic instability, which can lead to cancer development which results in metastasis causing cancer deaths and ineffectiveness of chemotherapeutic drugs.\textsuperscript{[5]}

Food containing phytochemicals with the antioxidant properties, such as phenolic compounds and Vitamin C, may help in strengthening the antioxidant potential.\textsuperscript{[6]} At the same time, plant derivatives may also be used as chemotherapeutic agents to counterbalance cellular antioxidant system leading to apoptosis.\textsuperscript{[7]} Preventing formation of ROS or scavenging them may be the key step in combating cancer and other diseases.

Plant serine PI, particularly trypsin inhibitors (TIs), have been extensively studied.\textsuperscript{[8,9]} At present, phytodrugs are a promising complementary and alternative medicine in health care for treatment of diseases like cancer. Our study reveals antineoplastic and antioxidant activity of TIs from three different plant sources to examine their suppressive effects on normal and cancerous cell lines.

Materials and Methods

Chemicals

Dimethyl sulfoxide (DMSO), 1-Chloro-2, 4-Dinitrobenzene (CDNB), sodium bicarbonate, and Hydroxyl ethyl piperazineethanesulfonic acid, trypsin (bovine pancreatic trypsin), Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA), and diethylaminoethyl cellulose were purchased from Sigma-Aldrich (USA). Trichloroacetic acid (TCA), sodium pyrophosphate, phenazine methosulfate, nitroblue tetrazolium, NADH, sulfanilamide, N-1-naphthylethylenediamine dihydrochloride (NEDD), orthophosphoric acid, dithiobis-2-dinitrobenzoic acid (DTNB), and ethylenediaminetetraacetic acid were procured from SRL, India. All other reagents and chemicals were of analytical grade.

Sample preparation

TIs were purified from \textit{Vigna unguiculata} “lobia” and \textit{Allium sativum} “garlic” by the ammonium sulfate precipitation followed by chromatographic techniques. The trypsin inhibitory activity was determined by chromogenic assay of Patil et al.\textsuperscript{[9]} using BAPNA as substrate. They were named as \textit{V. unguiculata} TI (VUTI) and \textit{A. sativum} TI (ASTI). TI from \textit{Phaseolus limensis} “lima bean” was obtained commercially from Sigma-Aldrich as lima bean TI (LBTI).

Cell lines and cell culture

Human Embryonic Kidney (HEK) and adenocarcinomic human alveolar basal epithelial cells (A549) were maintained in RPMI-1640 grown in 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were cultured at 37°C, 5% CO$_2$ humid condition in CO$_2$ incubator (Thermo). All the other chemicals were procured from Sigma-Aldrich and Merck.

\textit{In vitro} antineoplastic activity

MTT assay was carried out as described by van Meerloo et al.\textsuperscript{[10]} Cells were seeded at a density of 5 × 10$^4$ cells/well in 96-well plate supplemented with 2.5% FBS. After treatment with the TIs, cells were incubated at 37°C with two different concentrations, i.e., 5 μg/ml and 10 μg/ml for 24 h and 48 h. After the required period, 20 μl of 5 mg/ml MTT solution in Phosphate-buffered saline (PBS) (pH 7.4) was added to each well and incubated for 4 h. 150 μl of DMSO was added to dissolve the formazan crystals which were formed by the cellular reduction of MTT reagent. The change in absorbance was recorded at 540 nm wavelengths on an ELISA plate reader.

All measurements were done in triplicates. The percent cytotoxicity values were determined by

\[
\% \text{Cytotoxicity} = \left(\frac{[A]_i - [A]_c}{[A]_c}\right) \times 100
\]

Where $[A]_i$ is absorbance of the test sample and $[A]_c$ is absorbance of control.

Antioxidant potential of trypsin inhibitors in cell lines

A549 and HEK cells (1 × 10$^6$) were cultured in T-25 flasks maintained in RPMI 1640 medium containing...
10% FBS. The cells were allowed to adhere for 24 h before treatment with TIs sample. The incubation was done with TIs at concentration 10 μg/ml and respective controls at the same concentration for 48 h. The controls used for SOD assay, nitric oxide (NO) assay, Glutathione S-Transferase (GST) assay, and glutathione peroxidase (GPx) assays were trolox, gallic acid, Vitamin C, and reduced glutathione (GSH), respectively. The cells were washed with PBS after incubation and detached using scraper. These cells were then harvested, sonicated, and centrifuged at 10,000 g for 20 min at 4°C. The resulting supernatant was used for the following antioxidant enzyme assays.

Superoxide dismutase (SOD) assay
SOD assay was performed according to the protocol used by Xu et al.\[13\] First, the reaction mixture was prepared which consisted of 0.025 M sodium pyrophosphate (pH 8), 180 μM phenazine methosulfate, and 300 μM nitroblue tetrazolium. To the final assay mixture of 100 μl, 20 μl of cell supernatant and 60 μl of reaction mixture were added, and the reaction was initiated by the addition of 20 μl of 780 μM NADH. The absorbance was recorded kinetically at 560 nm for 5 min. SOD activity was calculated as units/mg of protein.

Nitric oxide radical scavenging activity
NO assay was performed as described by Abdel-Moneim et al.\[12\] Reagent A–1% sulfanilamide in 2.5% orthophosphoric acid and Reagent B–0.1% NEDD in 2.5% orthophosphoric acid were taken and mixed in equal amounts (1:1). To the final assay mixture of 200 μl, 100 μl of cell supernatant was mixed with Griess Reagent in equal ratio of (1:1). The absorbance was recorded immediately at 540 nm using ELISA plate reader. The results were expressed in terms of concentration (μM) of NO enzyme produced.

Glutathione S-transferase activity
The supernatants were used for analysis of GST activity according to the method of Mateen et al. with few changes.\[13\] The reaction mixture contains 9.8 ml of phosphate buffer (pH 6.5), 100 mM CDNB (100 μl), and 100 mM reduced GSH (100 μl). The absorbance was measured at 350 nm, and the results were expressed in μM/min/mg protein.

Glutathione peroxidase assay
GSH estimation was performed as per the protocol of Mateen et al. with some few modifications.\[13\] Briefly, 100 μl of mitochondrial fraction was precipitated with 20 μl of 5% TCA. The assay tubes were centrifuged at 1200 rpm for 5 min at room temperature to remove the precipitated proteins. To the final assay mixture of 110 μl, 45 μl of cell supernatant (treated with TCA), 45 μl of sodium phosphate buffer (0.2 M, pH 8), and 20 μl of dinitrobenzoic acid (DTNB-10 mM) were added. The absorbance was taken at 412 nm after 10 min. The results were expressed in μM/min/mg protein.

**Results and Discussion**

Antineoplastic activity assay
We report herein the functional properties of TIs isolated from various plant sources, i.e., Vigna unguiculata and A. sativum and P. limensis in cell lines. We aimed to investigate VUTI, ASTI, and LBTI for their antineoplastic and free radical scavenging ability in cell lines. As drug resistance has become a major problem in the cancer therapy. Therefore, new drug candidates are needed to be explored to meet these upcoming challenges. Several TIs have been reported for antineoplastic, chemopreventive, and apoptosis-inducing factors against human cancer.\[7,14\] Our results also suggest that ASTI, VUTI, and LBTI show appreciable suppressive activity on adenocarcinomic cells which is explained in detail further. These evidence have made TIs to be considered as nutraceuticals due to their capability in cancer prevention and hence its treatment.

MTT assay studies of TIs were performed on normal and cancer cell lines, i.e., HEK and A549 cell lines. A comparative analysis of LBTI, VUTI, and ASTI activity was performed. Our results clearly proved that the TIs show more suppression in A549 cells as compared to HEK cells in time- and concentration-dependent fashion. The MTT endpoints showed low cytotoxic effects of TIs in HEK cells; LBTI showing maximum cytotoxicity followed by VUTI and ASTI. This cytotoxicity was much higher in A549 cells where LBTI showed maximum value followed by VUTI and ASTI. These values were higher when cells were incubated with 10 μg/ml TIs [Figure 1a] as compared to the values obtained with 5 μg/ml [Figure 1b]. All these results show higher cytotoxicity in the presence of TIs when compared with control. Hence, we can conclude that TIs may be used as natural anticancer drugs which would inhibit the growth of cancerous cells without much harm to the normal cells in vicinity.

Antioxidant potential of trypsin inhibitors in cell lines
In a living system, numerous biochemical redox reactions are carried out during cellular metabolism which generates ROS such as H₂O₂, superoxide (·O₂⁻), nitric oxide free radical (·NO), and hydroxyl radical (·OH).\[6\] ROS can be detrimental when produced in high amounts in intracellular compartments. The cells generally respond to ROS by upregulating ROS scavengers such as SOD, NO, GST, and GSH which protects living system by converting harmful free radicals to harmless molecules like water to prevent from various pathologies.\[13\]
Damage caused due to oxidative stress can be overcome through the action of antioxidants. Therefore, TI might contribute toward complete or partial treatment of such clinical disorders caused due to oxidative stress.

**Superoxide anion radical scavenging activity**

SOD molecules convert superoxide free radicals to $\text{H}_2\text{O}_2$ and molecular oxygen. It plays a major role in toxic effects of superoxide radicals in aerobic cells. They are a part of big isoenzyme family that responds to oxidative stress and hence represents a main enzymatic source of peroxides.\(^{[11]}\)

As presented in Figure 2, the activity of SOD was significantly increased in case of TIs-treated A549 cells when compared with HEK cells. Furthermore, control showed lesser activity in comparison of TIs-treated cells. Enhanced SOD levels were observed in LBTI-treated cells (in HEK - 108.3639 ± 0.98 units/mg TI and in A549-328.4926 ± 1.2 units/mg TI) followed by VUTI (in HEK - 124.32 ± 1.34 units/mg TI and in A549-243.543 ± 1.25 units/mg TI) and ASTI (in HEK - 75.12 ± 1.28 units/mg TI and in A549-140.47 ± 1.75 units/mg TI) indicating increased SOD level in cancerous cells than noncancerous cells and higher SOD activity of LBTI followed by VUTI and ASTI.

**Nitric oxide radical scavenging activity**

In general, free radicals are required in a very small amount in the cell system. Some immune responses are mediated through macrophage and neutrophils which involve production and release of NO, which diminish viruses, pathogens, and tumor proliferation.\(^{[14,15]}\) NO can also act as detoxifier or ROS scavenger as it reacts with other ROS and finally scavenges them. NOS produces NO by catalyzing the conversion of L-arginine to L-citrulline, with concomitant oxidation of NADPH, which has slow down the velocity of the electrons in the respiratory chain to inhibiting the breath and reducing the consumption of oxygen, causing a state of stress leading to mitochondrial lipid peroxidation.\(^{[16]}\)

In our study, we observed NO levels were slightly enhanced in LBTI treatment (2314 ± 1.2 μM), as compared to VUTI (2290 ± 1.2 μM) and ASTI (2288 ± 1.2 μM) in HEK cells. However, significant increased levels of NO were observed in A549 cells treated with LBTI (3724 ± 1.2 μM), VUTI (3572 ± 1.2 μM), and ASTI (2968 ± 1.2 μM). Here too, control showed lesser activity in comparison of TIs-treated cells [Figure 3].

**Glutathione S-transferase activity**

Increased GST levels indicate no oxidative stress in the body. However, the levels of GST were significantly higher when compared to GSH. It may be concluded that there is an immense effect to revert the oxidant-antioxidant balance as the body strives to overcome the stress.\(^{[17]}\) Our results revealed that the GST level was significantly higher in LBTI-treated cells (HEK-3.8997919 ± 1.5 μM/min/mg protein, A549-13.246951 ± 0.9 μM/min/mg protein) over VUTI (HEK - 3.6112737 ± 1.5 μM/min/mg protein, A549-6.555012 ± 0.9 μM/min/mg protein) and ASTI (HEK - 2.7017966 ± 1.5 μM/min/mg protein, A549-3.5092115 ± 0.9 μM/min/mg protein). However, higher GST levels were observed in TIs-treated groups than control (HEK - 4.8950246 ± 1.5 μM/min/mg protein, A549-5.4939092 ± 0.9 μM/min/mg protein) [Figure 4].
Shamsi, et al.: Antioxidant and anticancer activity of trypsin inhibitor

Glutathione peroxidase or glutathione assay

GPx catalyzes the hydroperoxide reduction using reduced GSH and hence protects cells from oxidative damage.\(^{[18]}\) GSH is the important part of nonenzymatic antioxidants. GSH can remove peroxynitrite (ONOO\(^{-}\)) by forming oxidized glutathione (GSSG). GSSG is converted back to GSH by the NADPH-dependent enzyme GSH reductase.\(^{[19]}\) A deficiency of GSH puts the cell at risk for oxidative damage whereas increased GSH levels indicate healthy condition prevailing in the body. Decreased GSH levels indicate decreased detoxification capacity, a mechanism by which cell gains a selective advantage over their surrounding normal cells. Figure 5 illustrates the GPx activity of the treated cells (test) over reduced GSH-treated cells (control). GSH levels were found very high with LBTI treatment in A549 cells (0.002783 ± 1.2 μM/min/mg protein), as compared to HEK cells (0.0016361 ± 0.9 μM/min/mg protein). Similar GSH level was observed in VUTI (A549-0.00265±1.2μM/min/mg protein and HEK-0.0014409±0.7 μM/min/mg protein) and ASTI (0.0020995 ± 1.2 μM/min/mg protein and HEK-0.0015848 ± 0.7 μM/min/mg protein) on 24-h posttreatment indicating low oxidative stress in cancerous cell. The high levels of GSH in A549 cells were observed than in HEK cells favoring low oxidative stress in cancerous cells than noncancerous cells [Figure 5].

As oxidative stress is a key biomarker in cancer progression and its initiation,\(^{[20]}\) we hereby report the ROS scavenging activity of TIs in cancer cells as compared to normal cells. The assays performed confirm the previous studies which show that TIs play a vital role in free radical scavenging and thus act as antioxidant and anticancer agent.\(^{[2,21]}\)

Conclusions

Based on the results obtained from the studies conducted with the TIs, it can be concluded that it has tremendous potential for the development of suitable drugs in pharmaceutical industries against diseases due to generation of ROS and cancer. Furthermore, results indicate that TIs exhibit high antioxidant, free radical scavenging activities, which might be helpful in preventing the progression of various oxidative stress-mediated disorders by ceasing the generation of toxic ROSS. As TIs are known to have antioxidant and anticancer properties, they may help to prevent in vivo oxidative damage associated with diseases and illnesses and also it can be used to replace the modern synthetic drugs with safe phytodrug. Nevertheless, a better understanding on the detailed mechanism of how these TIs suppress the proliferative activity of cancerous cells would be valuable thus demands further investigation.

Acknowledgments

The authors are thankful to Anita Kamra Verma, Kirori Mal College, Delhi University, Delhi, Cyanobacterial Biotechnology Lab, Central Instrumentation Facility, Department of Biotechnology, Department of Biosciences, Jamia Millia Islamia, New Delhi, to allow the access to instruments for the research work.
Financial support and sponsorship
The authors acknowledge financial support from DST-SERB (SR/FT/LS-194/2009).

Conflicts of interest
There are no conflicts of interest.

References

1. Shamsi TN, Parveen R, Fatima S. Characterization, biomedical and agricultural applications of protease inhibitors: A review. Int J Biol Macromol 2016;91:1120-33.

2. Wilkins-Port CE, Higgins SP, Higgins CE, Kobori-Hotchkiss I, Higgins PJ. Complex regulation of the pericellular proteolytic microenvironment during tumor progression and wound repair: Functional interactions between the serine protease and matrix metalloproteinase cascades. Biochem Res Int 2012;2012:454368.

3. Ding S, Li C, Cheng N, Cui X, Xu X, Zhou G. Redox regulation in cancer stem cells. Oxid Med Cell Longev 2015;2015:750798.

4. Abou-Zeid L, Baraka HN. Combating oxidative stress as a hallmark of cancer and aging: Computational modeling and synthesis of phenylene diamine analogs as potential antioxidant. Saudi Pharm J 2014;22:264-72.

5. Jiménez-Estrada M, Velázquez-Contreras C, Garibay-Escobar A, Sierras-Canchola D, Lizcano-Vázquez R, Ortiz-Sandoval C, et al. In vitro antioxidant and antiproliferative activities of plants of the ethnopharmacopeia from northwest of Mexico. MC Complement Altern Med 2013;13:12.

6. Li F, Wang H, Huang C, Lin J, Zhu G, Hu R, et al. Hydrogen peroxide contributes to the manganese superoxide dismutase promotion of migration and invasion in glioma cells. Free Radic Res 2011;45:1154-61.

7. Chan YS, Zhang Y, Ng JTB. Brown kidney bean Bowman-Birk trypsin inhibitor is heat and pH stable and exhibits anti-proliferative activity. Appl Biochem Biotechnol 2013;169:1306-14.

8. Fang EF, Bah CS, Wong JH, Pan WL, Chan YS, Ye XJ, et al. A potential human hepatocellular carcinoma inhibitor from Bauhinia purpurea. L. seeds: From purification to mechanism exploration. Arch Toxicol 2012;86:293-304.

9. Patil DN, Preeti, Chaudhry A, Sharma AK, Tomar S, Kumar P. Purification, crystallization and preliminary crystallographic studies of a Kunitz-type proteinase inhibitor from tamarind (Tamarindus indica) seeds. Acta Crystallogr Sect F Struct Biol Cryst Commun 2009;65(Pt 7):736-8.

10. van Meerloo J, Kater J, Cloos J. Cell sensitivity assays: The MTT assay. Methods Mol Biol 2011;731:237-45.

11. Xu S, Zhang Y, Jiang K. Antioxidant activity in vitro and in vivo of the polysaccharides from different varieties of Auricularia auricula. Food Funct 2016;7:3868-79.

12. Abdel-Moneim AM, Al-Kahtani MA, El-Kersh MA, Al-Omair MA. Free radical-scavenging, anti-inflammatory/anti-fibrotic and hepatoprotective actions of turmeric and silymarin against CCl4 Induced Rat Liver Damage. PLoS One 2015;10:e0144509.

13. Mateen S, Moin S, Khan AQ, Zafar A, Fatima N. Increased reactive oxygen species formation and oxidative stress in rheumatoid arthritis. PLoS One 2016;11:e0152925.

14. Clemente A, Carmen Marín-Manzano M, Jiménez E, Carmen Arques M, Domoney C. The anti-proliferative effect of TII1B, a major Bowman-Birk isoinhibitor from pea (Pisum sativum L.), on HT29 colon cancer cells is mediated through protease inhibition. Br J Nutr 2012;108 Suppl 1:S135-44.

15. Marvibaigi M, Amini N, Supriyanto E, Abdul Majid FA, Kumar Jaganathan S, Jamil S, et al. Antioxidant activity and ROS-dependent apoptotic effect of Scutellaria ferruginea (Jack) danser methanol extract in human breast cancer cell MDA-MB-231. PLoS One 2016;11:e0158942.

16. Katakar P, Metgud R, Naik S, Mittal R. Oxidative stress marker in oral cancer: A review. J Cancer Res Ther 2016;12:438-46.

17. Solien J, Haynes V, Giulivi C. Differential requirements of calcium for oxoglutarate dehydrogenase and mitochondrial nitric-oxide synthase under hypoxia: Impact on the regulation of mitochondrial oxygen consumption. Comp Biochem Physiol A Mol Integr Physiol 2005;142:111-7.

18. Rocha FR, Arruda AF, Siqueira EM, Arruda SF. Phytochemical compounds and antioxidant capacity of tucum-do-cerrado (Bactris setosa Mart), Brazil’s Native Fruit. Nutrients 2016;8:110.

19. Chandra N, Pandey N. Influence of sulfur induced stress on oxidative status and antioxidative machinery in leaves of Allium cepa L. Int Sch Res Notices 2014:2014;2014:568081.

20. Kodykova J, Vavrova L, Stankova B, Macasek J, Krechler T, Zak A. Antioxidant status and oxidative stress markers in pancreatic cancer and chronic pancreatitis. Pancreas 2013;42:614-21.

21. Chunmei GU, Shujun LI, Linlin ZH, Xinxin SO, Guixin QI. The effects of soybean trypsin inhibitor on free radicals levels in pancreatic mitochondria of mice. J Food Nutr Res 2014;2:357-62.