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# ANTIOXIDANT POWER OF PURIFIED PROTEASE INHIBITORS FROM THE FRUITS OF *SOLANUM ACULEATISSIMUM* JACQ.

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**ABSTRACT**

Oxidative stress plays significant role in pathophysiologic events of acute and chronic diseases. Intracellular biomolecules such as lipids, proteins and nucleic acids are damaged via oxidation by excessive active oxygen species (AOS). Protease inhibitor was isolated and purified from the fruits of *Solanum aculeatissimum* Jacq. (SAPI) via four sequential step procedure i.e., salt precipitation to Sepharose affinity chromatography. Subsequently, the antioxidant power is analysed using DPPH, $H_2O_2$, $O_2^{\cdot-}$, ABTS, $OH^{\cdot}$ radical scavenging activity, reducing power potential, metal chelating ability and FRAP (Ferric reducing antioxidant power) method. SAPI exhibited significant $IC_{50}$ values for most of the AOX assays. DPPH radical scavenging, reducing power, metal chelating ability, ABTS and $OH^{\cdot}$ radical scavenging activity were comparable with the synthetic antioxidants like ascorbate and BHT. Further studies are warranted to trace the molecular mechanism of AOX activity by SAPI using *in vivo* animal models.

***Keywords:*** Chromatography: *Solanum aculeatissimum*: Protease inhibitors, antioxidant potential, Active oxygen species

## INTRODUCTION

Protease inhibitors (PI) are ubiquitous, reported in many families especially Fabaceae, Solanaceae, Poaceae and Cucurbitaceae. Serine, cysteine and aspartic proteases are the major types. Most PIs have four conserved Cys residues forming two disulfide bonds in a single or double chain polypeptide[1]. PIs are shown to be particularly abundant in storage organs, such as tubers to regulate the endogenous proteinase activities[2] and also suppress the exogenous proteinase activities from pathogens and pests[3]. They are also found to accumulate rapidly in leaves in response to mechanical wounding or insect chewing[4] suggesting its role as plant protection. Further, they are employed for curing many ailments such as inflammation, cancer, stress and pathogens[5]. Active oxygen species (AOS) are formed via endogenous and exogenous sources in living organisms. Within the cells, AOS can be generated in mitochondrial and microsomal electron transport systems, in soluble oxidase enzyme systems, and also during phagocyte activation[6]. Exogenous AOS generate from air and water pollutants, cigarette smoke, organic solvents, heavy metals, certain drugs and radiation[7]. The AOS play an important role related to the degenerative or pathological processes of various serious diseases, such as aging, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts, and inflammation[8]. Previous studies on natural products from plants revealed an association between people who have a standard diet rich in fruits and vegetable that are proved to have antioxidant activities which leads to a decrease in the risk of lifestyle diseases and certain forms of cancer. In this juncture, present study aims to isolate, purify

the PIs from fruits of *Solanum aculeatissimum* Jacq. and to evaluate its AOS scavenging potentialities.

## MATERIALS AND METHODS

### *Purification of SAPI*

*Solanum aculeatissimum* Jacq. fruits were obtained from Munnar hills of Western Ghats, Kerala. 100 g fresh fruits were homogenized with 250 ml of saline Tris buffer (20 mM Tris, pH 8.0; 0.15 M NaCl) containing 1 % polyvinylpyrrolidone (1:6 w/v) and filtered through chilled 4-fold muslin cloth and further, centrifuged for 15 min at 10,000 x g. The entire protocol was carried at 4 °C. The crude PI extract was fractionated by 20-90 % $(NH_4)_2SO_4$ precipitation. The $(NH_4)_2SO_4$ was removed by the process of dialysis using the extraction buffer stirred gently with magnetic stirrer to improve solute exchange and the dialysis buffer was changed once in 3 h for 4-5 times. The dialyzed protein showing high protease inhibition activity was subjected to DEAE cellulose exchanger column, pre-equilibrated with 20 mM Tris buffer with pH 8.0. 3 ml protein fractions were eluted using linear gradient of NaCl (0.02-0.50 M) at a flow rate of 0.5 ml /min. Fractions eluted with 0.18 to 0.24 M NaCl were pooled, dialyzed, lyophilized and loaded (1.0 mg/ml) to Sephadex G-50 superfine from Pharmacia column.

Load the dialysate in to Sephadex G-50 column and subsequently eluted using 25 mM Tris HCl buffer of pH 8.0. Collect 5 ml fractions by continuously adding buffer. The effluent emerging out of the column can be routed through a suitable spectrophotometer to monitor the absorbance and the data was recorded. The amount of protein is expressed as mg/ml. Active fractions of 0.5 ml with flow rate of 1 ml /3 min were collected. The column fractions with SAPI activity were dialyzed, concentrated and loaded onto sepharose affinity column equilibrated with 100 mM phosphate buffer (pH 7.6) containing 100 mM NaCl. The adsorbed SAPI was eluted with 100 mM HCl. The purity was checked by reverse phase HPLC (C18 column) at a flow rate of 1.0 ml /min with 100 % solvent A (0.1 % trifluoroacetic acid (TFA) in water) for 10 min and a linear gradient (0–100 %) of solvent B (0.08 % TFA in 80 % acetonitrile) over 45 min. Apparent molecular weight was checked by Sephadex G-50 gel filtration column (0.1 M phosphate buffer, pH 7.6) calibrated with known molecular weight proteins (14.3 to 43 kDa).

### *Protease inhibitor activity assay*

SAPI activity was determined by estimating the residual hydrolytic activity of trypsin and chymotrypsin towards the substrates BAPNA (N-benzoyl-L-arginine-p-nitroanilide) and BTPNA (N-benzoyl-L-tyrosyl-p-nitroanilide), respectively, at pH 8.0 after pre-incubation with inhibitor[9]. One trypsin or chymotrypsin unit is referred as 1 µmol of substrate hydrolyzed per min of reaction. One inhibitor unit was recorded as the quantity of inhibitor needed to inhibit 50 % of the corresponding enzyme activity. Protein content was measured as per the method of Bradford[10] using BSA by Coomassie blue staining.

### *SDS Page*

Molecular mass and purity of PI was evaluated by SDS–PAGE[11]. The molecular mass was further compared with size elution chromatography.

### *Antioxidant activity assessment*

### *Hydrogen Peroxide Scavenging*

Scavenging activity of $H_2O_2$ was determined according to the method of Ruch *et al*.[12] with minor changes. The percentage of scavenging activity of $H_2O_2$ by the SAPI was calculated as follows:

% of $H_2O_2$ scavenging activity $[H_2O_2]$ = [Abs (control) – Abs (standard) / Abs (control)] × 100

where, Abs (control): absorbance of the $H_2O_2$ (2 mM) as control Abs (standard): absorbance of the extract/standard.

### *DPPH scavenging assay*

The purified PI extract of the fruits were mixed with 95% methanol to prepare the stock solution (1 mg/ml). AOX activity was measured as discoloration at 517 nm after incubation for 30 min in the dark. Measurements were performed at least in triplicate.

Ascorbic acid was used as a reference standard and dissolved in DDW to make the stock solution with the same concentration (1 mg/ml). The control sample was prepared, which contained the same volume without any extract and 95% methanol was used as the blank.

Percent scavenging of the DPPH free radical was measured using the following equation:

DPPH scavenging effect (%) = (Ao – A1) / Ao X 100

where, Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample. The actual decrease in absorption induced by the test compounds was compared with the positive controls. The $IC_{50}$ value was calculated using the dose inhibition curve[13].

### *Reducing Power*

Reducing power was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power[14]. Ascorbic acid and BHT were used as reference standards. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of the two parallel

experiments was taken and expressed as mean ± standard deviation.

***Metal chelating activity***

The chelation of ferrous ions by extracts was estimated by method of Dinis *et al*.[15]. The absorbance of the solution was thereafter measured at 562 nm.

The percentage inhibition of ferrozine–$Fe^{2+}$ complex formation was calculated as $[(A_0- A_s)/ A_0] X 100$,

where $A_0$ was the absorbance of the control, and As was the absorbance of the extract/ standard.

***Hydroxyl radical-scavenging activity***

Hydroxyl radical scavenging activity of extract was measured according to the method of Halliwell *et al*.[16]. The color development was measured of 532 nm against blank containing phosphate buffer.

% scavenging of hydroxyl radical = (1−Ae/Ao)×100

***Determination of superoxide radical scavenging activity***

Superoxide scavenging was determined by the nitrobluetetrazolium reduction method [17]. The OD was measured at 530 nm before and after the illumination. The percentage of inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes. The abilities to scavenge the superoxide radical were calculated by using the following formula:

% of superoxide radical scavenging scavenging = (1−Ae/Ao)×100

where, Ao is the absorbance without sample, and Ae is absorbance with sample.

***FRAP assay***

The FRAP (Ferric reducing antioxidant power assay) was assayed as per the protocol of Benzie and Strain[18]. The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37ºC for 10 min. For construction of calibration curve five concentrations of $FeSO_4$ $7H_2O$ (1000, 750, 500, 250, 125 µmol/L) were used and the absorbencies were measured.

The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4$.

***ABTS radical scavenging activity***

ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic)) acid assay was performed according to the protocol of Delgado-Andrade *et al*.[19]. The decrease in absorbance was measured exactly 1 min after mixing the solution, the final absorbance was noted up to 6 min. Data for each assay was recorded in triplicate. Ascorbic acid was used as positive control. The scavenging activity was estimated based on the percentage of ABTS radicals scavenged by the following formula:

% of ABTS radical scavenging = $[(A_0−A_s)/A_0] \times 100$

where $A_0$ is absorption of control, $A_S$ is absorption of tested extract solution.

***Statistical analysis***

SPSS 18.0 statistical software was used for data analysis. Data from different assay treatments were analyzed by one-way analysis of variance (ANOVA); The Student-Neuman-Keuls post-hoc test was used to determine significance for individual experimental conditions. Differences with $P < 0.01$ or $P < 0.05$ were regarded as significant.

## RESULTS AND DISCUSSION

***Purification of the S. aculeatissimum protease inhibitor (SAPI)***

Protease inhibitor activity from fruits of *S. aculeatissimum* was evaluated. Fruits displayed the remarkable trypsin and chymotrypsin PI inhibitory activities i.e., 54 %, 48 % respectively. Subsequently, crude protease inhibitor (Pl) obtained from fruits was purified to homogeneity through ammonium sulphate precipitation followed by chromatographies such as DEAE cellulose ion exchange, Sephadex G-50 and sepharose affinity chromatography.

SAPI was concentrated by $(NH_4)_2SO_4$ with varying concentrations ranging from 0-20, 20-40, 40-60, 60-80 and 80-90 % saturation. The concentrated proteins were desalted by dialysis against the buffer 20 mM Tris-HCl, pH 8.2 and the protein concentrations were determined by Lowry's method [20]. 80-90 % saturated $(NH_4)_2SO_4$ fraction yielded 367 mg/g protein compared to 0-20 % saturation (874 mg/g). Similarly, the concentrated and desalted $(NH_4)_2SO_4$ fractions were assayed for SAPI activity and the amount of SAPI in the 0-20 % fraction was negligible compared to fractions of 80-90%. The $(NH_4)_2SO_4$ precipitation resulted 1.49 (trypsin) and 1.51 (chymotrypsin) fold of purification compared to the crude extract (Table 1).

Subsequent to 90% $(NH_4)_2SO_4$ precipitation, the dialyzed PI was purified by DEAE ion exchange chromatography. The elution fractions of ion exchange chromatography revealed one major and a minor protein peaks. Pooled active fractions from 0.18 to 0.24 M NaCl (fractions: 9-12) were dialyzed and showed 93.2 TlU and 90.2 ClU for trypsin chymotrypsin inhibitory activities respectively. The fold of purification for Sephadex G-50 column (52.7 and 51.8), followed by affinity column

chromatography were with single prominent elution peak. The purity of PI was further checked by RP-HPLC with retention time of 10 min in 50 mM Tris–HCl buffer, pH 8.0, coinciding with the protein peak (Fig.1). Thus, purified SAPI yielded specific activity of 502 TlU and 433.7 CIU U/mg, with low protein content of 0.95 mg. Overall, the specific activity increased about 92.6 and 82.9 folds with 9.8 and 8.77 % yield with respect to trypsin and chymotrypsin respectively (Table 1).

The present purification profile of SAPI was comparable with PI isolated from seeds of *Derris trifoliata* yielding only 57 fold of purification with Q sepharose[21]. Chaudhary *et al*.[22] also purified trypsin inhibitor from seeds of *Putranjiva roxburghii* by acid precipitation, cation-exchange and anion-exchange chromatography with low yield. Meanwhile, Prasad *et al*.[23] purified bowman-birk proteinase inhibitor from seeds of *Vigna mungo*. The fold of purification was 55.61 following DEAE cellulose, trypsin-Sepharose 4B column and sephadex G-50 chromatography. The above purification profile results suggest that the fold and recovery of protein can be increased through various chromatographies. The observation related with SAPI was commendable when compared with PIs from *D.trifoliata, P. roxburghii and V. mungo*.

***Molecular mass***

SDS-PAGE electrophoretic separation of SAPI showed a single prominent band of 22.2 kDa mass (Fig. 2). In agreement, size elution chromatography also revealed the same mass. The molecular mass of purified PI from the seeds of *Adenanthera pavonia* was 20 kDa[24]. Luo *et al*.[25] expressed an 18 kDa recombinant PIN2b PI from *S. americanum* in *Escherichia coli* and was a potent inhibitor against serine proteinases. Kansal *et al.*,[26] purified PI from *Cicer arietinum* that showed single band in SDS-PAGE corresponding to the molecular mass of 30 kDa. The mass of PI isolated from *S. aculeatissimum* was higher than *A. pavonia* and *S. americanum* but lower than *C. arietinum.*

***Antioxidant potentiality assays (AOX)***

***Ferric reducing antioxidant power (FRAP) assay***

FRAP assay measures the reducing potential of PI reacting with ferric tripyridyltriazine [$Fe^{3+}$-TPTZ] complex to form ferrous tripyridyltriazine [$Fe^{2+}$-TPTZ] [27]. Commonly, the reducing features are linked with the presence of molecules which exert their action by breaking the free radical chain by donating a hydrogen atom[28]. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction[29]. Interestingly, the absorbance of SAPI clearly increased, due to the formation of the $Fe^{2+}$-TPTZ complex with increasing dose. The $IC_{50}$ value is 74.3µg/ml suggesting its potentiality to donate electrons to free radicals and there by scavenging it from the biological system.

***Metal chelating activity***

PI of *S. aculeatissimum* had effective ferrous ions ($Fe^{2+}$) metal chelating potentiality. The metal chelating power in different concentration of PI and the control values was statistically significant at 1% level ($p < 0.01$). 60 µg/ml concentration, PI exhibited 71.5 % chelation of ferrous ion (Table 2). On the other hand, the ferrous ion chelating capacities of BHT and ascorbate (100 µg/ml ) were found to be 89.5 and 90.6%, respectively. These results show that the ferrous ion chelating effect of PI was marginally higher to BHT ($p < 0.05$) but lower than ascorbate ($p < 0.05$).

***Hydrogen peroxide ($H_2O_2$) scavenging potentiality***

Comparatively, $H_2O_2$ is a weak oxidant and can inactivate some enzymes directly, through oxidation of thiol (-SH) groups. It can cross cell membranes rapidly and can probably react with metal ions such as $Fe^{2+}$ and $Cu^{2+}$ to form hydroxyl radical and this may be the major cause for many of its toxic effects. SAPI exerted a concentration dependent scavenging potential i.e., it showed a maximum activity of 80.60 % inhibition comparable to that of BHT with an activity of 86.50 % at the same concentration of 100 µg/ml. The $IC_{50}$ value of PI was 22.4 µg/ml, whereas the standard ascorbate exerted an $IC_{50}$ of 20.5 µg/ml.

***DPPH radical scavenging assay***

The reactivity of SAPI was analyzed with the stable free radical DPPH. As DPPH picks up an electron in the presence of AOX, the absorption declines and the resulting discolouration is stechiometrically related to the number of electrons accepted. The DPPH radical scavenging (%) activity of SAPI is 71.6% and that of BHT was 82.9 % at 100 µg/ml and the $IC_{50}$ of SAPI was 53.8 µg/ml, while that of BHT was 60.4 µg/ml. $IC_{50}$ value of rhizomes *Costus pictus,* medicinal herbal was 400 µg/ml[28].

***$ABTS^{\cdot+}$ radical scavenging assay***

The $ABTS^{\cdot+}$ scavenging assay was measured at 734 nm within a short reaction time, can be used as marker that reflects the AOX activity of the test samples. SAPI was found to be effective in scavenging $ABTS^{\cdot}$ radicals and the increase was dose-dependent. At 100 µg/ml, the inhibition of the SAPI was 70.5% and that of BHT was 71.30%. The $IC_{50}$ of BHT was 50 µg/ml while the SAPI was 51.6 µg/ml. This shows that SAPI presents good ability to scavenge the ABTS radical. *Tamarix* methanolic

extracts of flower showed the ABTS quenching activity with $IC_{50}$ value 316.7 µg/ml[29]. The AOX activities against ABTS or DPPH were generally correlated with dose, chemical structures, and polymerization degrees of antioxidants[30].

***Superoxide radical scavenging assay***

The inhibition by SAPI against superoxide radical generation is higher than that by BHT but lower than ascorbate. As seen in table 2 the inhibition of $O2^{\bullet -}$ radical generation at the concentration of PI (100 µg/ml) was 82.5 %. On the other hand, at the same concentration, BHT and ascorbate exhibited 72.3% and 85.8% superoxide anion radical scavenging activity, respectively.

***Reducing power activity***

The AOX can donate an electron to free radicals, which leads to the neutralization of the free radical. Reducing power was evaluated by direct electron donation via the reduction of $Fe^{3+}$ $(CN^-)6$-$Fe^{2+}(CN^-)$. The product was observed as Prussian blue color complex and measured at 700 nm. As shown in table 2, a higher absorbance value indicates stronger reducing power of the samples. SAPI showed dose-dependent reducing power. Meanwhile, its reducing power was lower than that of ascorbate, which exhibited the strongest reducing power. The reducing power of extract in ethanol of tubers from *Momordica tuberosa* at 50 and 100 µg were significantly higher than the standard, sodium metabisulphate[31]. AOX molecules are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species.

***Hydroxyl radical (.OH) scavenging activity***

.OH scavenging potential was evaluated by measuring the inhibition of the degradation of 2-deoxyribose by the free radicals created by the Fenton reaction. The .OH scavenging potential of PI increased with concentration 10 - 100 µg/ml (Table 2). In the present investigation, the $IC_{50}$ value of .OH scavenging activity for the PI was 18.6 % while for BHT was 13.5 % (Table 2). The significant ($P < 0.05$) antioxidant response of PI is comparable with standards might be helpful in utilizing the significant sources of natural antioxidants.

Levine *et al*.[32] reported that in soybean cultured cells, the cell death process that is triggered by $H_2O_2$ could be arrested by synthetic PIs, such as AEBSF and leupeptin. The inhibition of PCD by PIs suggest its possible role for specific proteases in $H_2O_2$-stimulated PCD. In plants, excessive oxidative bursts have been reported in response to avirulent pathogens as part of the hypersensitive response. Formation of $H_2O_2$ concomitant with localized cell death occur as a consequence of UV irradiation, mechanical impact, salinity stress and freezing stress. Interestingly, transcriptional induction of serine and cysteine proteases was seen with xylogenesis in *Zinnia* cultured cells[33]. This event is connected with $H_2O_2$ formation and cell death. The results presented here supports the possibility of oxidative stress induced activity of PIs to scavenge the free radicals or AOS.

In plants, the proteases and the PIs are regulated by various stimuli. Expression of the PI genes is usually confined to specific regions or to particular growth periods during plant autogeny, for example, in seeds or during germination process. Some PI genes are induced during drought, wounding and also to insect attack. In plants, PI genes also are subject to regulation by intercellular signaling or elicitor molecules, like jasmonic acid, salicylic acid (SA) and systemin. With respect to regulation of protease inhibitor expression, it is interesting that SA was found to repress expression of cystatin. SA also was shown to function as potentiator of cell death induced by direct oxidative stress or pathogen attack[34]. Tibor Maliar *et al*.[35] reviewed for development of new functional food and nutraceuticals, and/or new secondary metabolites with potential as bioactive compounds.

Kumar *et al*.[36] noticed that rats treated with the trypsin inhibitor showed significantly elevated cholecystokinin levels compared with rats receiving casein or water. Further, the scavenging activities of TI against DPPH and hydroxyl radical were significantly decreased from 1.5 to 6 pmol compared to those of the controls. It is suggested that 33 kDa TI, sweet potato root storage proteins, may act as an antioxidant in roots and may be beneficial to human health.

Antioxidant mechanisms of PIs have been studied by Wen-Chi Hou *et al*.[37] in root storage protein of sweet potato. They reported purification of PI, its physicochemical and antioxidant properties. AOXs usually scavenge free radicals/ROS by an electron-transfer mechanism. The electron-donating ability is assayed by the one electron oxidation potential of the antioxidants, expressed as the reduction potential of the corresponding phenoxyl radicals. The sweet potato PI had scavenging activity against DPPH radical from 2 to 22 %. While that for hydroxyl radical were significantly decreased from 1.5 to 6 pmol[37] The SAPI showed more or less similar activities against different AOX systems. Guan-Jhong Huang *et al*.[38] purified a low molecular mass trypsin PI from the leaves of sweet potato with potential AOX activities against DPPH radical, ABTS radical, superoxide anion radical, $H_2O_2$ scavenging, total ferric ions reducing power and

metal chelating on ferrous ions activities[39]. Further, they revealed that tyrosine residue could be oxidized to a tyrosyl radical through an electron transfer process. Nitration may follow from the reaction of a tyrosyl radical with $NO_2$[40]. The present results suggest that AOX potentiality of SAPI may be due to the tyrosine residues in PI. It is generally accepted that the chemical activity of hydroxyl radical is the strongest among ROS. ·OH reacts freely with biomolecules, like amino acids, proteins and DNA. Therefore, the removal of ·OH is probably most effective defenses of living cells against various stresses. Similarly carbon-centered radicals that represent R·, RO· and ROO· could be easily scavenged by PIs.

Most of the chronic diseases, DNA breakage, mutagenesis, carcinogenesis and bactericidal events are associated with the inhibition of free radical propagation in cellular systems. AOX capacity is widely used as a parameter for accepting medicinal potentiality. In this study, the antioxidant activity of PI was compared to BHT and ascorbate. Lipid peroxidation cascade comprises series of free radical-mediated chain reaction leads to oxidative burst in biological system. The reducing capacities of AOXs are connected with reductones, which have been effective in inhibiting free radical chain by donating hydrogen atom. Further, they also react with peroxide initiators, thus inhibiting peroxide synthesis. The present results on the reducing power of SAPI reveals that reductones in PI seems to contribute towards the observed antioxidant effect. Similarly, the other diverse mechanisms includes prevention of chain initiation, binding with transition metal ions, scavenging of peroxides and hydrogen abstraction. Hydrogen peroxide is a signal cascade formed during oxidative stress in the cell system. It is formed from superoxide anion *in vivo* by dismutase activity by superoxide dismutase. It has the ability to cross-membranes and slowly oxidize biological molecules. The effective $H_2O_2$ scavenging capacity of PIs protects the cells from toxic and apoptotic cell death.

Superoxide anion produced by NADPH oxidase is an oxygen-centered radical, a weak oxidant, but produce potent singlet oxygen and hydroxyl radicals, which cause lipid peroxidation. It also reduce metallic proteins like cytochrome c. In the present study, SAPI scavenged superoxide anion comparable to the syntheticAOXs. Radulovic *et al*.[41] showed that extracts in methanol from *Geranium macrorrhizum* displayed antioxidant potential coupled with dose-dependent hepatoprotective action against $CCl_4$-induced stress in liver cells. Plastina *et al*.[42] correlated AOX potentiality of *Citrus aurantium* with the polyphenols in the plant. Md. Irshad *et al*.[43] reported that extract in methanol of *Cassia fistula* with reducing capacity. Gulati *et al*.[44] reported antioxidant activities of *Santalum spicatum* and *Pterocarpus marsupium* with hyperglycemia. Similarly, Sgherri *et al*.[45] analyzed *Salvia officinalis* and *Echinacea angustifolia* with radical decay kinetics, using EPR spectrometer. Saiba and Murugan[46] reported AOX power of *Thottea siliquosa* used by tribals for treating various ailments. Most of the AOX compounds reported belongs to polyphenols, flavonoids, alkaloids, saponin and tannin. AOX potentiality of protease inhibitors was scarse. Most of the AOX assay in the present study against free radicals and the $IC_{50}$ values obtained was commendable than by the secondary metabolites in the plants.

Owen[47] reviewed PI as AOX which regulate lung inflammation and injury in chronic obstructive pulmonary diseases. Levine *et al*.[48] analysed the mechanism of AOX potentialities using methionine and cysteine, which functions as an antioxidant and also as a regulators of cellular metabolism.

Methionine is easily oxidized to methionine sulfoxide by ROS/ free radicals and there by protecting from the oxidative damage. Reversible covalent medication of amino acids in PIs provides the functional basis of its role in cellular events. So, the present mode of action of PIs may be due to the active amino acids. Maliar *et al*.[49] also confirmed AOX potentiality of PIs of *Trifolium pretense*[50]. In addition, biopolymers from *Moringa oleifera* pods and gum of *Acacia* plant have been suggested as good antioxidants for controlling ROS. Gum of *Acacia* showed better superoxide scavenging ability while *Moringa* seed associated biopolymer had more potential to scavenge hydrogen peroxide, hydroxyl and nitric oxide radicals. Both the biopolymers had sufficient reducing power and thus have great potential as wound management aids[51].

Nooman *et al*.[52] analyzed antioxidant activity of *Camellia sinensis*, *Eugenia caryophyllus*, *Piper cubeba*, *Zingiber officinale* and *Piper nigrum*. *Trigonella foenumgraecum* and *Elettaria cardamomum* showed weak free radical scavenging activity with the DPPH method. All the other plants exhibited significant antioxidant activity. The $IC_{50}$ of the methanolic extracts ranged between 6.7 ± 0.1 and 681.5 ± 8.4 µg/ml and that of ascorbic acid was 8.9 ± 0.1 µg/ml. The PI fistulin from *Cassia fistula* was found to possess remarkable antioxidant and protective activity against hepatocellular toxicity[53].

## CONCLUSION

*S. aculeatissimum* PIs was found to be an effective antioxidant as revealed by the different *in vitro* assays like reducing power, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O2^{\bullet-}$,

FRAP, hydrogen peroxide scavenging and metal chelating compared to synthetic antioxidant compounds such as BHT and ascorbate. Free radicals scavenging activity of PI may be by the H-atom abstraction from the free hydroxyl group. Based on the results, SAPI can be employed for minimizing or inhibiting lipid oxidation and also the formation of toxic products. Further studies are planned to analyze the anticancer potentialities of SAPI using various malignant cell lines.

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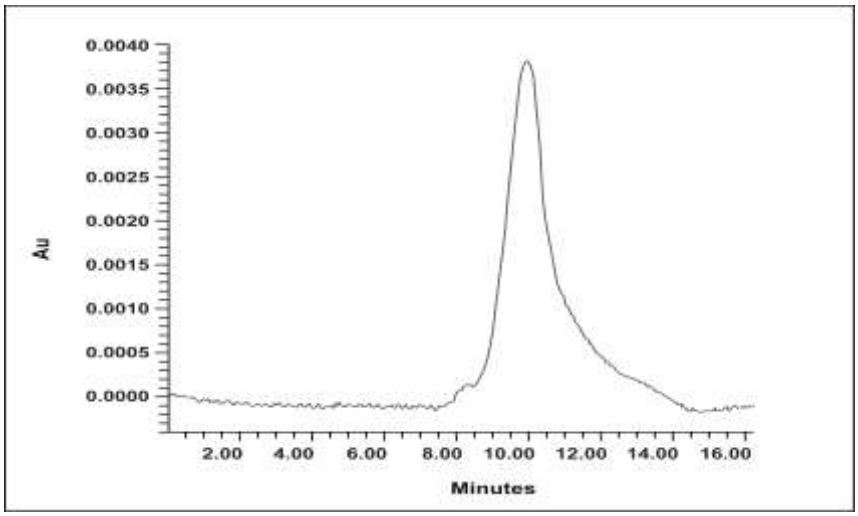


**Figure 1. RP-HPLC Chromatogram of purified SAPI using C-18 column.**

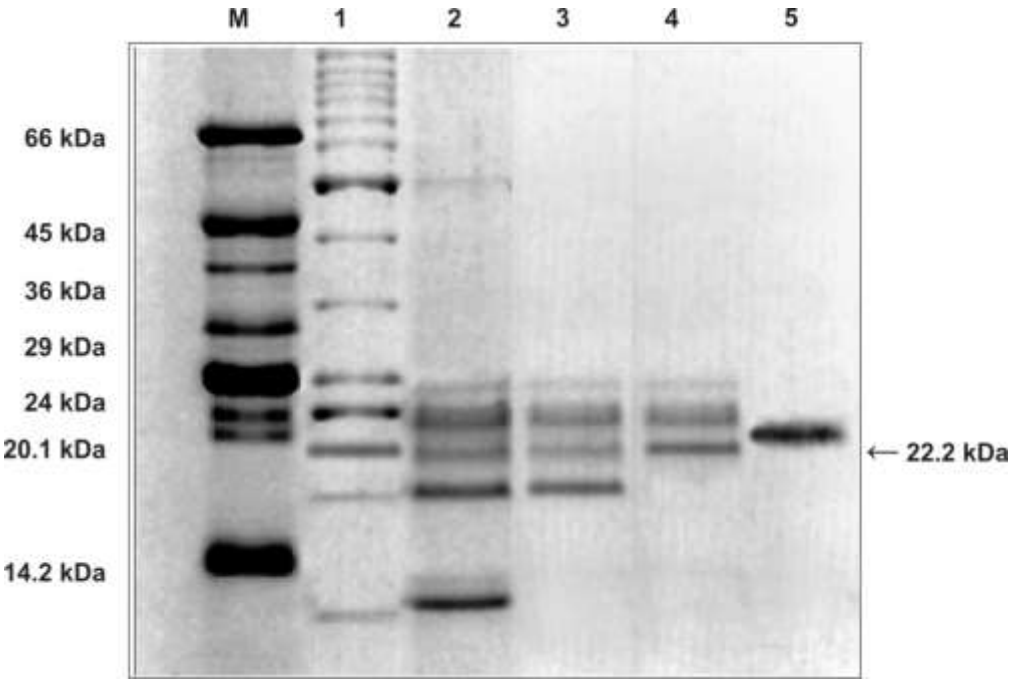


**Figure 2. SDS PAGE of purified SAPI**

M- Marker; 1: Crude; 2: Salt precipitated; 3: Ion Exchange; 4: Gel filtration; 5: Affinity chromatography.

**Table1. Purification profile of *S. aculeatissimum* PI**

| Purification steps | Total activity U/g tissue | | Yield (%) | | Total protein (mg/g tissue) | Specific activity (U/mg protein) | | Fold of purification | |
|---|---|---|---|---|---|---|---|---|---|
| | T | CT | T | CT | | T | CT | T | CT |
| Crude inhibitor | 4865 | 4698 | 100 | 100 | 898 | 5.42 | 5.23 | 1 | 1 |
| Ammonium sulphate 90% | 2960 | 2895 | 60.8 | 61.6 | 367 | 8.07 | 7.89 | 1.49 | 1.51 |
| DEAE cellulose ion exchange | 1528 | 1479 | 31.4 | 31.5 | 16.4 | 93.2 | 90.2 | 17.1 | 17.24 |
| Sephadex G-50 | 657 | 623 | 13.5 | 13.3 | 2.3 | 285.7 | 270.9 | 52.7 | 51.8 |
| Sepharose | 477 | 412 | 9.8 | 8.77 | 0.95 | 502 | 433.7 | 92.6 | 82.9 |

**Table 2. AOX potential of purified SAPI**

| Concentration (µg/ml) | Assays (% of scavenging activity) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Hydrogen Peroxide Scavenging** | **DPPH scavenging effect** | **Hydroxyl radical-scavenging activity** | **Superoxide radical scavenging activity** | **FRAP assay (mmol/L FeSO4)** | **ABTS radical scavenging activity** | **Metal chelating activity** | **Reducing Power** |
| 10 | 30.10 | 28.40 | 30.20 | 28.50 | 18.70 | 20.40 | 29.60 | 17.80 |
| 20 | 49.60 | 39.60 | 52.30 | 35.60 | 25.50 | 33.30 | 50.20 | 36.30 |
| 40 | 52.30 | 42.50 | 60.30 | 45.30 | 36.80 | 48.50 | 62.60 | 42.50 |
| 60 | 69.80 | 59.60 | 65.80 | 60.20 | 46.40 | 53.9 | 71.50 | 55.60 |
| 80 | 72.50 | 65.70 | 71.20 | 78.60 | 58.80 | 64.20 | 83.30 | 60.30 |
| 100 | 80.60 | 71.60 | 74.40 | 82.50 | 69.60 | 70.50 | 98.20 | 65.40 |
| ASC (100 µg/ml) | 90.60 | 85.30 | 80.30 | 85.80 | 94.20 | 74.20 | 90.60 | 80.30 |
| BHT (100 µg/ml) | 86.50 | 82.90 | 75.60 | 72.30 | 85.50 | 71.30 | 89.50 | 62.60 |
| SE | 0.296 | 0.578 | 0.892 | 0.746 | 0.489 | 0.550 | 1.230 | 0.985 |
| F ratio I | 385.53** | 469.26** | 371.39** | 396.65** | 299.54** | 525.12** | 498.69** | 503.67** |
| ID | 7.58** | 9.45** | 7.17** | 6.93** | 9.52** | 10.26** | 8.94** | 7.56** |
| $CD_{(0.05)}$ I | 0.113 | 0.127 | 0.213 | 0.175 | 0.167 | 0.128 | 0.123 | 0.138 |
| ID | 1.31 | 1.17 | 1.12 | 1.15 | 1.17 | 1.16 | 1.32 | 1.09 |

**REFERENCES**

1. Habib H, Fazili KM. Plant protease inhibitors: a defense strategy in plants, Biotechnol Mol Biol Rev, 2007; 2 (3): 068-085.
2. De Leo F, Volpicella M, Licciulli F, Liuni S, Gallerani R, Ceci LR. Plant-PIs: a database for plant protease inhibitors and their genes. Nucleic Acid Res, 2002; 30 (1): 347-348.

3. Eckelkamp C, Ehmann B, Schopfer P. Wound-induced systemic accumulation of a transcript coding for a Bowman-Birk trypsin inhibitor-related protein in maize (*Zea mays* L.) seedlings, FEBS Lett, 1993; 323: 73-76.
4. Ryan CA. Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Hystopathol, 1990; 28: 425-449.
5. DeClerck YA, Imren S. Protease inhibitors: role and potential therapeutic use in human cancer. Eur J Cancer, 1994; 30A (14): 2170-2180.
6. Kehrer JP. Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol, 1993; 23: 21-48.
7. Valko M, Rhodes CJ, Moncol J, Izakovic M. Free radical, metals and antioxidants in oxidative stress-induced cancer. Minireview Chem.-Biol Interact, 2006; 160: 1-40.
8. Lai HY, Kim KH. *Blechnum orientale* Linn - a fern with potential as antioxidant, anticancer and antibacterial agent. BMC Complement Altern Med, 2010; 10:15-22
9. Prasad ER, Dutta-Gupta A, Padmasree K. Purification and characterization of a Bowman-Birk proteinase inhibitor from the seeds of black gram (*Vigna mungo*), Phytochem, 2010; 71: 363–372.
10. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem, 1976; 72: 248-254.
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage, Nature, 1970; 227: 680-685.
12. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis, 1989; 10: 1003-1008.
13. Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L., BMC Complement Altern Med, 2012; 12: 1-12.
14. Oyaizu M. Antioxidaive activities of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography. Nippon Shokuhin Kogyo Gakkaishi, 1988; 35: 771-775.
15. Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys, 1994; 315: 161-169.
16. Halliwell B, Guttridge JMC, Aruoma OI. The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Analytical biochem, 1987; 165: 215- 219.
17. Liu F, Ooi VE, Chang ST. Free radical scavenging activity of mushroom polysaccharides extract. Life Sci, 1997; 60:763-771.
18. Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Method Enzym, 1999; 299: 15–27.
19. Delgado-Andrade C, Rufián-Henares JA, Morales FJ. Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. J Agric Food Chem, 2005; 53: 7832-7840.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent, J Biol Chem, 1951; 193 265–75.
21. Segel IH. Enzyme Kinetics. John Wiley and Sons, New York 1975; pp 86-90.
22. Chaudhary NS, Shee C, Islam A. Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds, Phytochem, 2008; 69: 2120–2126.
23. Prasad ER, Dutta-Gupta A, Padmasree K. Purification and characterization of a Bowman-Birk proteinase inhibitor from the seeds of black gram (*Vigna mungo*), Phytochem, 2010; 71: 363–372.
24. Andrea NM, Marta MC, Xavier FJ. The complete amino acid sequence of the major Kunitz trypsin inhibitor from the seeds of *Prosopsis juliflora*, Phytochem, 2001; 30: 2829-2833.
25. Luo M, Ding L. The Characterization of SaPIN2b, a Plant Trichome-Localized Proteinase Inhibitor from *Solanum americanum*, Int J Mol Sci, 2012; 13: 15162-15176.
26. Kansal R, Kumar M, Kuhar K. Trypsin protease inhibitor from *Cicer arietinum* L. and its efficacy against *Helicoverpa armigera*, Brazilian Soc Plant Physiol, 2008; 20 (4): 313-322.
27. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal Biochem, 1996; 239: 70–76.
28. Jayasri MA, Lazar Mathew, Radha A. A report on the antioxidant activity of leaves and rhizomes of *Costus pictus* D. Don., Int J Int Biol, 2009; 5: 20-26.
29. Riadh Ksouri, Hanen Falleh, Wided Megdiche, Najla Trabelsi, Baya Mhamdi, Kamel Chaieb, Amina Bakrouf, Christian Magne, Chedly Abdelly. Antioxidant and antimicrobial activities of the edible medicinal halophyte *Tamarix gallica* L. and related polyphenolic constituents. Food Chem Toxicol, 2009; 47: 2083–2091.

30. Oszmianski J, Wojdylo A, Lamer-Zarawska E, Swiader K. Antioxidant tannins from Rosaceae plant roots. Food Chem, 2007; 100: 579–583.
31. Pramod kumar, Deval rao, Lakshmayya, Ramachandra settey. Antioxidant and hepatoprotective activity of tubers of *Momordica tuberosa* Cogn. against $CCL_4$ induced liver injury in rats. Indian J Exp Biol, 2008; 46: 510-513.
32. Levine A, Pennell R., Alvarez M, Palmer R, Lamb CJ. Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr Biol, 1996; 6: 427–437.
33. Ye ZH, Varner JE. Induction of cysteine and serine proteases during xylogenesis in *Zinnia elegans*. Plant Mol Biol, 1996; 30:1233–1246.
34. Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. Plant Cell Reports, 1997; 9: 261–270.
35. Tibor Maliar, Jarmila Drobna, Jan Kraic, Mária Maliarova, Jana Jurovata. Proteinase inhibition and antioxidant activity of selected forage crops Biologia, 2011; 66/1: 96—103.
36. Kumar S, Teotia UVS, Sanghi A. Antioxidative Property of Cow Milk Caseinates Hydrolyzed With Different Proteases Int J Pharm Pharm Sci, 2013; 5(2), 418-422.
37. Wen-Chi Hou, Yen-Chou Chen, Hsien-Jung Chen, Yaw-Huei Lin, Ling-Ling Yang, Mei-Hsien Lee. Antioxidant Activities of Trypsin Inhibitor, a 33 KDa Root Storage Protein of Sweet Potato (*Ipomoea batatas* (L.) Lam cv. Tainong 57), J Agric Food Chem, 2001; 49: 2978-2981.
38. Guan-Jhong Huang, Ying-Chih Lin, Jeng-Shyan Deng, Hsien-Jung Chen, Jung-Chun Liao, Shyh-Shyun Huang, Shu-Jen Chang, Yaw-Huei Lina. A novel trypsin inhibitor from sweet potato (*Ipomoea batatas* Lam.) leaves and its synthesized peptides with antioxidant activities *in vitro*. Bot Studies, 2012; 53: 215-222.
39. Gulcin I. Comparison of *in vitro* antioxidant and antiradical activities of L-tyrosine and L-Dopa. Amino Acids, 2007; 32: 431-438.
40. Lin HL, Kent UM, Zhang H, Waskell L, Hollenberg PF. Mutation of tyrosine 190 to alanine eliminates the inactivation of cytochrome P450 2B1 by peroxynitrite. Chem Res Toxicol, 2003; 16: 129-136.
41. Radulovic NS, Stojkovic MB, Mitic SS, Randjelovic PJ, Ilic IR, Stojanovic NM, Stojanovic-Radic ZZ. Exploitation of the antioxidant potential of *Geranium macrorrhizum* (Geraniaceae): hepatoprotective and antimicrobial activities. Nat Prod Commun, 2012; 7(12):1609-1614.
42. Plastina P, Fazio A, Gabriele B. Comparison of fatty acid profile and antioxidant potential of extracts of seven *Citrus* rootstock seeds. Nat Prod Res, 2012; 26(23):2182-2187.
43. Md. Irshad, Md. Zafaryab, Man Singh, Moshahid M, Rizvi A. Comparative analysis of the antioxidant activity of *Cassia fistula* extracts. Int J Med Chem, 2012; 12: 1- 6.
44. Gulati V, Ian Harding H, Enzo Palombo A. Enzyme inhibitory and antioxidant activities of traditional medicinal plants: Potential application in the management of hyperglycemia BMC Complement Altern Med, 2012; 12: 1-8.
45. Saiba AN, Murugan K. In vitro analysis on bactericidal screening and antioxidant potentiality of leaf and root extracts of *Thottea siliquosa* (Lam.) Ding Hou. An ethnobotanical plant. Asian Pac J Trop Biomed, 2013; 3(11): 859–865.
46. Sgherri C, Pinzino C, Quartacci MF. Antioxidant potential in lipophilic and hydrophilic extracts from medicinal herbs *Salvia officinalis* and *Echinacea angustifolia*. a comparison between assays based on electron paramagnetic resonance and spectrophotometry BMC Complement Altern Med, 2012; 7 (4): 417-424.
47. Owen CA. Proteinases and oxidants as targets in the treatment of chronic obstructive pulmonary disease. Proc Am Thorac Soc, 2(4): 373–385.
48. Levine RL, Moskovitz J, Stadtma ER. Oxidation of methionine in proteins: Roles in antioxidant defense and cellular regulation. IUBMB Life, 2000; 50: 301–307.
49. Maliar T, Drobna J, Kraic J, Maliarova M, Jurovat J. Proteinase inhibition and antioxidant activity of selected forage crops, Biologia, 2011; 66(1): 96-103.
50. Fakhriya S, Taha, Samira S, Suzanne, Wagdy M, Gamal F. Antioxidant and Antimicrobial Activities of enzymatic hydrolysis products from Sunflower protein isolate. World Appl Sci J, 2013; 21 (5): 651-658.
51. Parwani L, Bhatnagar M, Bhatnagar A, Sharma V. Reactive oxygen species control by plant biopolymers intended to be used in wound dressings. Int J Pharm Pharm Sci, 2012; 4(5), 506-510.
52. Nooman AK, Ashok KS, Atif AL-Othman, Zaha E, Husni F. Antioxidant Activity of Some Common Plants. Turk J Biol, 2008; 32: 51-55.
53. Arulpandi I, Sangeetha R. Antibacterial Activity of Fistulin: A Protease Inhibitor Purified from the Leaves of *Cassia fistula*, ISRN Pharm, 2012; 55-59.