Research Article

# Membrane Stability of Purified Protease Inhibitor from the Fruits of *Solanum aculeatissimum* Jacq. in Human Erythrocytes

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**ABSTRACT**

Membrane integrity is essential for many physiological activities against osmotic and heat-induced lyses. Protease inhibitors (PIs) are small proteins that are ubiquitous in nature. The protease inhibitor (PI) proteins are the natural antagonists of protease and reported in all life forms. Proteolytic enzymes play important roles in intracellular proteolysis by cleaving proteins. Thus, proteinases are involved in regulating many key physiological processes, such as cell-cycle progression, cell death, cell proliferation, DNA replicatin, haemostasis, immune response, tissue remodeling and wound healing. Currently, the search for PI is desired for drug development. Solanaceaeous species produce diverse protease inhibitors that control endogenous plant proteases. Protease inhibitor was isolated and purified from the fruits of *Solanum aculeatissimum* Jacq. (SAPI) via four sequential step protocols i.e., salt precipitation to Sepharose affinity chromatography. The purity was confirmed by reverse phase HPLC chromatography. The molecular mass was detected using size elution chromatography (22.2 kDa). Anti-inflammatory activity of SAPI, was investigated using stabilization of red blood cell membrane lysing technique. The percentage membrane stability exhibited by SAPI was concentration dependent and compared favourably with those of standard drugs (Diclofenac). The results revealed that protease inhibitor that protected the erythrocyte membranes effectively. Moreover, SAPI provided highest protection against induced lyses and exhibited both monophasic and biphasic responses at all the concentrations assayed. The possible mechanism of action of SAPI is described and discussed. Further studies are warranted to analyze the anti-inflammatory potential of SAPI using *in vivo* and *in vitro* models.

**Keywords:** Membrane, stabilization, protease inhibitors, purification, *Solanum aculeatissimum.*

## INTRODUCTION

Protease inhibitors (PI) are diverse group of proteins present in most of the plants. Plant PIs (PPIs) are small proteins that have commonly occurring in storage organs, such as tubers and seeds, but also found in the aerial parts of plants. PIs are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological events. They function as mediators of signal initiation, transmission and termination in many of the cellular events such as inflammation, apoptosis, blood clotting and hormone processing pathways[1,2]. Protease inhibitor II (PIN2), a serine protease inhibitor with trypsin and chymotrypsin inhibitory activities, occurs in many Solanaceaeous species like tomato, potato and tobacco. PIN2 proteins could serve an endogenous role in preventing uncontrolled proteolysis and/or a function in protecting against foreign proteolytic enzymes of pest or pathogen[3].

The membrane of the red blood cell (RBC) plays multiple roles that help in regulating their surface deformity, flexibility, adhesive feature with other cells and immune recognition.

The lipid molecules of membrane are important regulating many physical features like membrane permeability and fluidity.

Further, the role of many membrane proteins is coordinated by interactions with lipids. Meanwhile, the unsaturated fatty acids of the membrane are liable to different oxidants like free radicals or ROS. ROSs are active molecules can change RBC membrane stability therefore protective measures is critical for RBC stability. In this context, antioxidants are able to prevent ROS from damaging RBC membrane features. Plant products have high levels of antioxidants can play a potential role in RBC membrane protection and reduce oxidative damage[4]. Stabilization of lysosomal membrane is another event in limiting the inflammatory reactions of reactive species. Erythrocyte membrane is structurally analogous to the lysosomal membrane. Therefore, the application of erythrocyte membrane is an ideal model to study the protective effect of herbal phytochemicals. Many researchers used stabilization of hypo tonicity induced human red blood cell membrane (HRBC) as an *in vitro* model to analyze the cytoprotective activity of medicinal plant phytochemicals.

*Solanum aculeatissimum* is a native of Brazil. It is a stout perennial shrub armed with spines. The fruits are spherical, striped yellow when ripe. It is distributed throughout Tropical Africa, Asia and also introduced in Paraguay, Brazil and Mexico.

The present study account biological activities of purified protease inhibitor on the human red blood cells exposed to both heat and hypotonic induced lyses with a view to further evaluate the anti-inflammatory property and also determine mean corpuscular fragility and viscosity studies.

## 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 (Prasad) 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[5] using BSA by Coomassie blue staining.

### SDS Page

Molecular mass and purity of PI was evaluated by SDS–PAGE[6]. The molecular mass was further compared with size elution chromatography.

### Blood samples

Blood samples were collected from healthy volunteers and mixed with anticoagulant (containing dextrose (2%), sodium citrate (0.8%), citric acid (0.05%) and sodium chloride (0.42%). Blood samples were centrifuged at 3000 rpm for 10 min at room temperature. The supernatants (plasma and leucocytes) were carefully removed while the packed red blood cell was washed in fresh normal saline (0.85% w/v NaCl). The process of washing and centrifugation were repeated five times until the supernatants were clear[7].

### Assay of membrane stabilizing activity

The *in vitro* HRBC membrane stabilization assay was carried out using 2% (v/v)[7,8]. The assay mixtures consisted of 2 ml of hyposaline (0.25% w/v) sodium chloride, 1.0 ml of 0.15 M sodium phosphate buffer, pH 7.4, 1 mL of HRBC suspension, equal volume of SAPI / drugs in three different concentrations, 25, 50, and 100 µg/mL, was added. Diclofenac was used as standard drug. Drugs were omitted in the blood control, while the drug control did not contain the erythrocyte suspension. The reaction mixtures were incubated at 56°C for 30 min on a water bath, followed by centrifugation at 5000 rpm for 10 min at room temperature. The absorbance of the released hemoglobin was read at 560 nm. The percentage membrane stability was estimated using the expression:

$$\% \text{ of membrane stability} = \frac{\{\text{Abs of test drug} - \text{Abs of drug control}\}}{\{\text{Abs of blood control}\}} \times 100$$

where the blood control represents 100% lysis or zero percent stability.

### Determination of osmotic fragility

This followed the procedure developed by Parpart[9] as modified by Elekwa[10]. A 10 g/l solution was made from 100 g/l NaCl stock buffered at pH 7.4 with 150 mM phosphate. Dilutions equivalent to 9.0, 7.0, 6.0, 5.5, 5.0, 4.0, 3.0 2.0 and 1.0 g/l NaCl were made to 50 ml (final volume).

A 0.05 ml aliquot of blood sample was added to 5 ml of the various hypotonic solutions, and immediately mixed by inverting several times. The tubes were allowed to stand for 30 min at room temperature. The contents were re-mixed and centrifuged for 5 min at 1500 g. The absorbance of the supernatant was read at 540 nm using 9.0 g/l NaCl tube as blank. This procedure was repeated. Each blood sample was used thrice and the mean was taken.

For the effect of SAPI, 0.5 ml extract (5 g/25 ml) was added to 4.5 ml of hypotonic solution. The final NaCl

concentrations were maintained as with the controls (no addition) by using appropriate stock NaCl solutions. A 0.05 ml aliquot of blood sample was added and the mixture treated as described earlier. The total volume was 5.05 ml, as with the control. The mean corpuscular fragility (which is the concentration of saline causing 50% haemolysis of the erythrocytes) was obtained from a plot of % lysis against NaCl concentration (g/l).

### Determination of blood viscosity

The measurement of blood viscosity was done using an Ostwald viscometer. 3 ml of distilled water was mixed with 2 ml normal saline, and the mixture was introduced into the viscometer. Using a stopwatch, the time taken for the meniscus of the liquid to fall from the top to the bottom mark on the wall of the viscometer was recorded. The test blood samples replaced the distilled water for the test experiments. All readings were carried out at laboratory temperature of 28 ±2$^{o}$ C.

### Effects of SAPI and phenylalanine on blood viscosity

Elekwa[10] methodology was adapted for viscosity study. The reaction mixture contained 0.25 ml of SAPI (10 g / 25 ml), 3.0 ml of blood sample and 1.75 ml of buffered saline. The mixture was incubated at 37$^{o}$C, and the viscosity determined at 30-, 60- and 90-min intervals using the procedure described earlier. A 0.2 ml of 10 mM phenylalanine solution replaced the extract for the determination of the effect of phenylalanine.

### Statistical analysis

Each value represented the mean ± SD of 4 consistent readings. The significance of the differences between controls, tests and fractions were analyzed using Students *t*-test and analysis of variance. Values of $P < 0.05$ were considered to be statistically 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 (PI) 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 (1951) 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 TIU and 90.2 CIU 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 TIU 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).

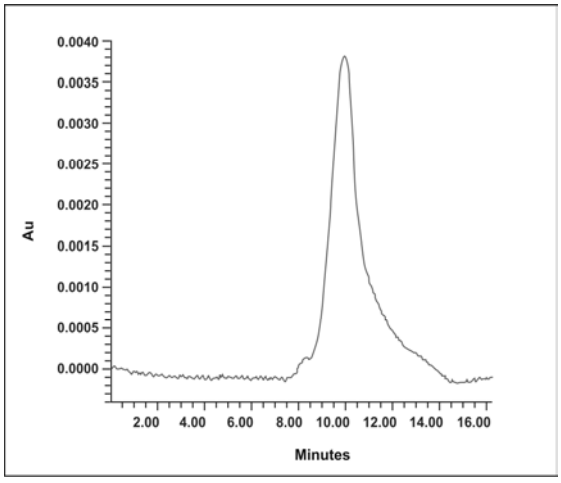


**Figure 1:** RP-HPLC Chromatogram of purified SAPI using C-18 column.

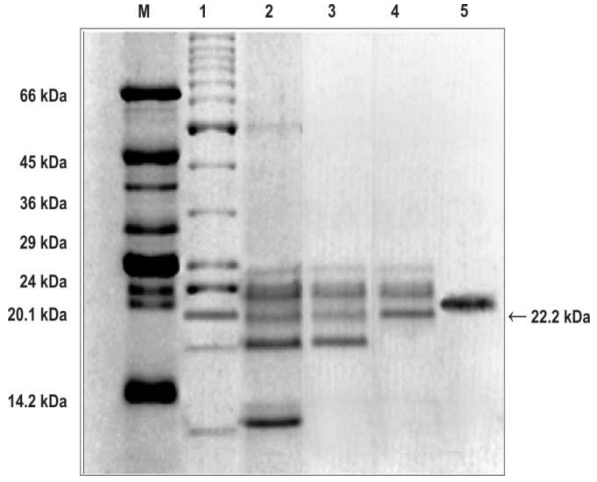


**Figure 2:** SDS PAGE of purified SAPI

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

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[11].

Chaudhary[12] also purified trypsin inhibitor from seeds of *Putranjiva roxburghii* by acid precipitation, cation-exchange and anion-exchange chromatography with low yield.

Meanwhile, Prasad[13] 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[14]. Luo and Ding[15] expressed an 18 kDa recombinant PIN2b PI from *S. americanum* in *Escherichia coli* and was a potent inhibitor against serine proteinases. Kansal[16] 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.*

**Membrane stability, Median Corpuscular Fragility (MCF) and viscosity**

SAPI showed dose dependent stabilizing activity towards HRBC. 79.21% membrane stability was observed at 25 µg/ml concentration, and 89.8% membrane stability at 50 µg/ml concentration. Beyond this concentration, activity was reduced marginally (Table 2). SAPI gave membrane stability of 13.52 ± 5.20 (10 µg/ml) and 89.8 ± 3.15% as minimum and maximum percentage activity respectively. The response of the red blood cells was monophasic and biphasic to SAPI. Generally inflammation occurs through two distinct phases and is a biphasic, the first phase (up to 1 h) involves the release of serotonin and histamine and in the second phase (after 1 h) is mediated by prostaglandin, the cylooxygenase products, and the continuity between the two phases is provided by bradykinins.

The results of the median corpuscular fragility (MCF) (the concentration of saline causing 50% haemolysis of the erythrocytes) determinations are presented in Table 3. The SAPI increased the MCF values from 3.7 + 0.1 to 4.48 + 0.19. The results are statistically significant at 5% level ($p < 0.05$). Phenylalanine, a known anti-sickling agent also increased the MCF values from 3.7 + 0.1 to 4.6 + 0.24. The % of stabilizations, calculated from the MCF values, for the effects of both phenylalanine and SAPI showed increased stabilizations (Table 4).

Blood viscosity determinations are shown in Table 4. At 0 min, the viscosity ($x10^{-3}$ Pa-S) values were 1.7. In the presence of SAPI the observed viscosities at 30 min were similar to the control bloods.

**Table 1:** 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:** Membrane stability (%) potential of SAPI

| | Concentration (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 10 | 25 | 50 | 75 | 100 |
| **Diclofenac** | 15.3 | 21.4 | 76 | 83.5 | 80 | 77.6 |
| **SAPI** | 17.8 | 13.52 | 79.21 | 89.8 | 84.3 | 80.1 |

**Table 3:** Median Corpuscular Fragility (MCF) values of erythrocytes with or without Phenylalanine and SAPI. (MCF expressed as (NaCl) g/L)

| | Concentration (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | **5** | **10** | **25** | **50** | **75** | **100** |
| **Control** | 3.7 | | | | | |
| **PA** | 3.8 | 3.9 | 4 | 4.2 | 4.5 | 4.6 |
| **SAPI** | 3.37 | 4. | 4.1 | 4.4 | 4.4 | 4.48 |

**Table 4:** Effects of Phenylalanine (400 µM) and SAPI on the viscosity of erythrocytes (Viscosity ($x10^{-3}$ Pa-S)

| PA | | | | SAPI | | |
|---|---|---|---|---|---|---|
| Incubation time (min) | | | | | | |
| 0 | 30 | 60 | 90 | 30 | 60 | 90 |
| 1.7 | 1.6 | 1.4 | 1.4 | 1.6 | 1.5 | 1.5 |

Since human red blood cell membranes are similar to lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis was taken as a measure of anti-inflammatory activity of drugs. It was observed in the present study that the stabilization by SAPI was at par with phenylalanine. This finding differs from the observation of Elekwa[10] where the stabilization by aqueous extracts of *Garcinia kola*, a popularly consumed seed in Nigeria, was higher than that produced by phenylalanine. This suggests that the extract from *G. kola* could be a better agent in the stabilization of human erythrocytes than that from SAPI.

Awe[16] analyzed membrane stabilizing activity of *Russelia equisetiformis* aqueous and methanolic extracts. Elekwa[17] revealed that aqueous extracts of *Zanthoxylum macrophylla* roots on membrane stability of human erythrocytes of different genotypes. Antioxidant and anti-inflammatory activities of the methanolic leaf extract of traditionally used medicinal plant *Mimusops elengi* substantiate the present results[18].

Oyedapo[20] analyzed red blood cell membrane stabilizing potentials of the extracts of *Lantana camara* and its fractions. Amujoyegbe[21] reported *in vitro* evaluation of membrane stabilizing activities of leaf and root extracts of *Calliandra portoricensis* on sickle and normal human erythrocytes. Sreejith[22] analyzed anti-allergic, anti-inflammatory and anti-lipidperoxidant effects of *Cassia occidentalis*. Similarly, Siju[23] reported anti-Inflammatory activity of *Pimenta doica* by membrane stabilization. Yoganandam and Sucharitha[24] revealed deStabilizing properties of various extracts of *Punica granatum* L.(Lythraceae). Zohra and Fawzia[25] confirmed hemolytic activity of different herbal extracts used in Algeria.

The exact mechanism of action, responsible for the membrane stabilizing activity of SAPI could not be established in this study. Protease inhibitor in the fruit might be responsible for the observed membrane stabilizing action. However, a number of investigators have shown that, flavonoids, triterpenoids, and other secondary plant metabolites, exhibited analgesic, anti-inflammatory effects as a result of their membrane stabilizing action in various experimental animal models[26,27].

It has also been reported that, there is production of free radicals, such as lipid peroxide and superoxide in various conditions, such as heat-induced stress haemolysis, due to cell membrane destabilization[28].

Flavonoids, triterpenoids and other phenolic compounds are good scavengers of free radicals due to their antioxidant properties[29,30]. The RBC membrane stability test is based on the finding that a number of non-steroidal anti-inflammatory agents inhibit heat induced lysis of erythrocytes, presumably by stabilizing the membrane of the cell. The erythrocyte membrane may be considered a model of the lysosomal membrane which plays an important role in inflammation[31].

The compounds which prevent the lysis of membrane caused by the release of hydrolytic enzymes contained within the lysosomes may relieve some symptoms of inflammation.

When the human RBC is subjected to hypotonic stress, the haemoglobin release from RBC, will be prevented by anti-inflammatory drugs because of the membrane stabilization.

It has been demonstrated that certain herbal preparations were capable of stabilizing the red blood cell membrane and this may be indicative of their ability to exert anti-inflammatory activity[7]. The mode of action of the proteins or drugs, may bind to the erythrocyte membranes with subsequent alteration of the surface charges of the cells. It has been reported that certain saponins and flavonoids exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins and saponins possess ability to bind cations, thereby stabilizing erythrocyte membranes and other phytochemicals. Since studied protease inhibitor is responsible for the observed membrane stabilizing action, and probably as a result of their antioxidant activity, thereby acting as free radicals scavengers.

To conclude, the purified protease inhibitors from *S. aculeatissimum* that showed high membrane stability activity, MCF, viscosity in the present study. Therefore, PI could serve as easily accessible sources of natural antioxidants for the pharmaceutical industry. Further studies are warranted to analyze the anti-inflammatory potential of the PIs.

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