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Antihaemolytic, anti-lipid peroxidative potential by purified protease inhibitors from the fruits of *Solanum aculeatissimum* Jacq. In human erythrocytes against hydrogen peroxide

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Abstract
Protease inhibitor was isolated and purified from the fruits of *Solanum aculeatissimum* Jacq. (SAPI) via four sequential step procedures 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). It has deep roots in history, being one of the major botanicals used in traditional medicine to treat conditions ranging from diabetes, malaria, to snakebites. This study was conducted to quantitatively evaluate anti-haemolytic activity of purified protease inhibitor from *S. aculeatissimum*, against hydrogen peroxide induced haemolysis using human erythrocyte in an *in vitro* assay. Prior to the addition of H_2O_2 to induce haemolysis, different concentrations (50-500 µg/ml) of SAPI was added to 2 ml of 4% erythrocyte suspension and allowed to incubate for 5 minutes at room temperature. The mixture was centrifuged and the colour density of the supernatant was measured spectro photometrically. Quercetin was used as internal standard. The % haemolysis and IC_{50} values were calculated. The PI was potent against haemolysis of the erythrocyte in concentration dependent manner with IC_{50} = 368.54 µg/ml. IC_{50} value of quercetin was 224 µg/ml. The lower the IC_{50} the more protection offered against haemolysis by SAPI. These results suggest that the SAPI is ideal anti-haemolytic agent and offered significant biological action compared with standard drug employed.

Keywords: Protease inhibitors, purification, *Solanum aculeatissimum*, anti-haemolytic activity, erythrocytes, hydrogen peroxide.

1. Introduction
The practice of traditional medicine using herbals is as old as human evolution [1]. Phytochemicals found in herbals are known as the active lead molecules. These compounds have been isolated and employed in different forms such as infusions, syrups, decoctions, infused oils, essential oils and creams [2]. Plant-derived secondary metabolites such as polyphenols saponins, steroids, lactones and essential oils received considerable significance currently due to their multiple pharmacological uses, including cytotoxic and chemo-preventive effects The putative protective roles of antioxidants against deleterious oxidative stress-induced diseases have received tremendous attention in recent times, especially within biological, medical, neutraceutical, and agrochemical areas. Among the dietary antioxidants, Polyphenolics compounds, and protease inhibitors from plants are the most common natural antioxidants [3]. Haemolytic assays have long been used to measure free radical damage and counteraction by antioxidants. It is useful for screening for oxidising or antioxidising molecules. Many primary or secondary plant metabolites have been found to protect cells from oxidative damage. These compounds have been evidenced to stabilize RBC membrane by scavenging free radicals and reducing lipid peroxidation [4].
Proteinase inhibitors (PIs) are low molecular mass proteins that are ubiquitous in nature. They are natural antagonists of protease, which are present in all life forms [5]. Most PIs react with their specific active site of the proteases by resulting in the formation of stable protease-inhibitor complex that is incapable of enzymatic activity i.e., competitive inhibition [6]. Protease inhibitors have multiple functions through regulating the photolytic activity of their target proteases.
Solanaceaeous species produce diverse serine protease inhibitors (SPIs), provide anti-digestive defenses that might also regulate endogenous plant proteases. The potato serine protease inhibitors (PSPIs), represent two of the proven PI classes in plants and display a remarkable genetic and structural diversity, specifically in the Solanaceae species [7].

Solanum aculeatissimum Jacq is a sturdy perennial profusely armed with spines. Fruits are yellowish. It a native of Southern Africa but now widespread throughout Tropical Africa, Asia and also introduced in other countries like Paraguay, Brazil and Mexico [8].

The aim of this study was to investigate the anti haemolytic and anti-lipid peroxidative activities of purified PI from *Solanum aculeatissimum* and to formulate promising alternatives to cure diseases associated with haemolysis.

2. Materials and methods

2.1 Purification of SAPI

Solanum aculeatissimum fruits were collected from Munnar hills of Western Ghats, Kerala. 100 g fresh fruits were thoroughly homogenized with 250 ml of saline Tris saline buffer (20 mM Tris, pH 8.0; 0.15 M NaCl) containing 1 % polyvinyl pyrrole done (1:6 w/v) and filtered through chilled 4-fold muslin cloth and subsequently, centrifuged at 10,000 x g for 15 min. The entire protocol was carried at 4 °C. The crude PI extract was further fractionated by 20-90 % $(NH_4)_2SO_4$ salt precipitation. The $(NH_4)_2SO_4$ in the extract was then removed by the process of dialysis using diluted extraction buffer stirred gently with magnetic stirrer to improve exchange of solutes and the dialysis buffer was regularly changed once in 3 h for 4-5 times. The dialyzate showing high protease inhibitory activity was subjected to DEAE cellulose exchanger column, pre-equilibrated with 20 mM Tris buffer (pH 8.0). 3 ml protein fractions were eluted using a linear gradient of NaCl (0.02-0.50 M) with 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 gel chromatography of 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 (pH 8.0). Elute 5 ml fractions by continuously adding buffer. The effluent leaching out of the column can be directed via 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 gel 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 validated 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 mass was checked by sephadex G-50 size elution column (0.1 M phosphate buffer, pH 7.6) calibrated with proven molecular weight proteins (14.3 to 43 kDa).

2.2 Protease inhibitor activity assay

Activity of SAPI was measured by recording 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 calculated as 1 µmol of substrate hydrolyzed/min of reaction. One inhibitor unit was recorded as the amount of inhibitor needed to inhibit 50 % of the corresponding enzyme activity. Protein content was estimated as per the method of Bradford (1976) using BSA by Coomassie blue staining.

2.3 SDS Page

Molecular mass and purity of PI was determined by SDS–PAGE [10]. The molecular mass was further compared with gel filtration chromatography.

2.4 Estimation of lipid peroxidation

Standard protocol for estimation of TBARS was employed to assay the magnitude of lipid peroxidation [11]. In order to induce lipid peroxidation, 200 ml of erythrocyte suspension (as above) was treated with different concentrations of SAPI, followed by 100 ml of 100 mM hydrogen peroxide were delivered into a test tube. The contents were incubated for 1 h at 37 °C. The reaction was stopped by the addition of 2 ml of thiobarbituric acid stock reagent (0.375% TBA, 15% TCA, 0.2 M HCl). The solution was incubated in a boiling water bath for 1 h. After cooling, the solution was centrifuged at 3 000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm.

2.5. Preparation of erythrocyte suspension and determination of anti-haemolytic activity

Human erythrocyte suspension was prepared as described by Naim. Fresh human blood sample was purchased from the blood bank of Medical College, Trivandrum, and was centrifuged at 1000 x g for 10 min. and erythrocytes were separated from the plasma and were washed thrice. The erythrocytes separated were then diluted with saline phosphate buffer (0.2M, pH 7.4) to produce 4% suspension. To 2 ml of the erythrocyte suspension, 50-500 µg/ml of SAPI was added and the volume was made up to 5ml with saline buffer. Further the mixture was incubated for 5 min at room temperature and then 0.5 ml of H_2O_2 solution in buffered saline was added to trigger oxidative degradation of the lipid membrane (haemolysis).

In another set, quercetin (50-500 µg/ml) was taken as positive control and followed the same protocol. The tubes were centrifuged at 1000 x g for 10 min and the colour intensity of the supernatant was measured at 580 nm spectrophotometrically.

To obtain 100% haemolysis (control), 2 ml of distilled water was added to 2 ml of RBC suspension. The relative haemolysis in the control, which was taken as 100%.

Inhibitory activity of SAPI on haemolysis was calculated and expressed as percent haemolysis.

% Haemolysis = Absorbance of control - absorbance of SAPI / Absorbance of control X 100.

2.6. Statistical analysis

In vitro and other assays were carried in triplicate and results were shown as mean ± SD. Statistical significance was calculated among various concentrations with one way ANOVA test. A Statistical significance of $p < 0.05$ or $p < 0.01$ was considered to be significant

4. Results and discussion

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

Protease inhibitor activity from fruits of *S. aculeatissimum* displayed significant trypsin and chymotrypsin PI inhibitory activities i.e., 54 %, 48 % respectively. Further, crude protease inhibitor (Pl) yielded from fruits was purified to homogeneity through salt precipitation followed by DEAE cellulose ion exchange, sephadex G-50 and sepharose affinity chromatography.

80-90 % saturated $(NH_4)_2SO_4$ fraction yielded 367 mg/g protein compared to lower saturation points (874 mg/g). Similarly, it yielded 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 from 0.18 to 0.24 M NaCl were dialyzed and showed 93.2 trypsin inhibitory and 90.2 chymotrypsin inhibitory unit of activities with fold of purification 52.7 and 51.8 respectively. Last step of purification was via affinity column chromatography yielded a single sharp peak. Purity of PI was further analyzed 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 lowest 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 corresponding to trypsin and chymotrypsin respectively (Table 1).

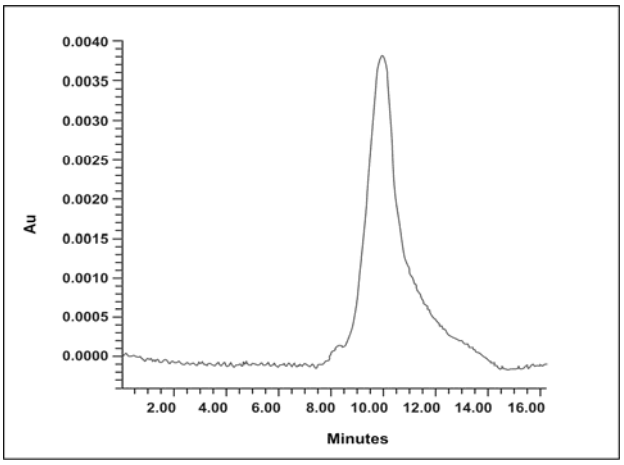


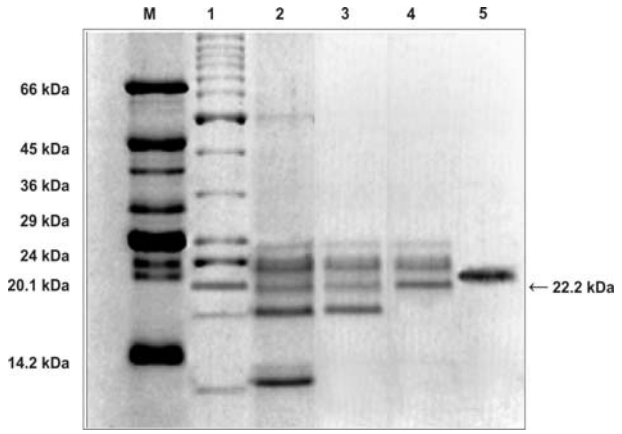
Fig 1: RP-HPLC Chromatogram of purified SAPI using C-18 column.

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

4.2 Molecular mass

The electrophoretic separation of SAPI by SDS-PAGE from crude to affinity chromatography yielded a single prominent band of 22.2 kDa mass (Fig. 2). In agreement, gel chromatography also revealed the similar mass.



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

Fig 2: SDS PAGE of purified SAPI

4.3 Anti-lipid peroxidation assay

Significant inhibitory effect of SAPI against H_2O_2 induced lipid peroxidation (LPX) was noticed in the human erythrocytes i.e., a concentration dependent inhibitory property against LPX from lower dosage onwards (Table 2). The IC_{50} value obtained was comparable ($P < 0.05$) with that of synthetic antioxidant quercetin.

Table 2: Inhibiting activity of SAPI on lipid peroxidation (mean+SD). $P < 0.01$

Conc. of SAPI (µg/ml)	% of LPX
50	70.1 ±0.16
100	62.37±0.31
200	58.36±0.17
300	50.1±0.16
400	24.21±0.25
500	4.3±0.33
H_2O_2	98.38 ±0.22
Quercetin 100	26.5±0.02

Human erythrocytes are the key target for the free radicals or reactive oxygen species because of polyunsaturated fatty acid membrane and also their oxygen transport linked redox activity [12]. H_2O_2 is a potent oxidizing molecule cause cell membrane depolarization and subsequent release of hemoglobin from the cells. Mobilization of metal ions such as Fe^{2+} through Fenton reaction leads to the production of potent OH radicals which further induces oxidative burst in the cells [13]. Anti haemolytic activity is the potentiality of antioxidant

molecules to safe guards the cells against these sorts of reactions.

RBC treated with H_2O_2 along with the SAPI displayed a marked reduction in haemolysis. i.e., haemolysis of the RBC was decreased with increase in SAPI and quercetin concentrations. Haemolytic activity by ROSs may be the result of poration in the cell membranes leading to change in membrane permeability or due to the alteration of Na-K and Ca-Mg ATPase activities. Tandan *et al*., [14] reported that administration of guava leaves extracts reduced arsenic induced blood and tissue damages through metal chelation property. This action might be due to the biomolecules in the extracts like polyphenols. They are capable of chelating iron with its C=O and C-OH moieties [15, 16]. Moreover, the compounds with structure containing two or more of the following groups: -OH, -SH, - COOH, $-PO_3H_2$, C=O, $-NR_2$, -S and -O- can show metal chelation [17]. Gas phase ligation technique shows that some flavonoids such as kaempferol, quercetin, myricetin and naringenin were able to chelate Cu^{2+} and Fe^{2+} through the functional carbonyl group [18]. In this study, protease inhibitors afford protection against oxidative damage by removing metal ions that participates in ROS generating Fenton reaction.

Plant protease inhibitors are utilized for the treatment of various health problems from ancient time onwards. Plants are unique sources of drug discovery and development. *Solanum* species have been excessively used in traditional medicine to cure variety of disorders. Rajesh *et al*., [19] (2012) analyzed *Aerva lanata* extracts against helminthic infection, diabetes, inflammation, skin diseases, kidney stone, headache, cough, cholera, dysentery and diarrhea. It is also reported for diuretic properties. Similarly, Kumar *et al*., [20] employed *Calotropis gigantea* as curative against paralysis, swellings, intermittent fevers, asthma, catarrh, anorexia, helminthic infections, inflammations, fever, infections, cough, asthma, bronchitis and dyspepsia. Das gupta *et al*., [21] reviewed the medicinal properties of *Elaeocarpus. ganitrus* against stress, anxiety, depression, palpitation, neuro pain, epilepsy, migraine, lack of concentration, asthma, hypertension, arthritis and liver disorders.

In the present study, haemolytic activity of SAPI was screened against normal human erythrocytes. Haemolytic activity of *S. aculeatissimum* is expressed in percentage of haemolysis and reported as mean ± standard deviation of three replicates. Direct application of SAPI exhibited no haemolytic effect toward human erythrocytes. In addition, SAPI showed dose dependant decrease in haemolytic activity in RBC cells treated with H_2O_2 (Table 3). Many herbals have been screened for the haemolytic activity towards humans or animal erythrocytes. Different solvent extracts of *Syzigum cuminii* seeds and *Crateva nurvula* bark were reported no haemolytic impact on sheep erythrocytes [22]. *Achyranthes aspera* was reported to possess margibal haemolytic activity towards human erythrocytes [23]. Water extract of *Lantana camara* and its other solvent fractions were reported to possess moderate haemolytic activity towards human erythrocytes [24]. Oliveira1 *et al*., [25] screened the haemolytic activity of many herbal extracts. Only three extracts prepared from *E. nuda* showed significant haemolytic activity. Karanja oil of *Pongamia glabra* was reported to display concentration dependent increase in the haemolysis towards the rabbit RBCs [26]. (Mukherjee and Rajasekaran [27] reported the high haemolytic activity of various solvent extracts of *Allium stracheyi* towards human RBCs. Chloroform and aqueous extract of leaves of *Acanthus ilicifolius* were reported to possess significant haemolytic potential. Medicinal plants are potential in boosting the immune system of resistance against infections, healing the allergies, raising and renewing the body's vitality [28].

Primary and secondary metabolites present in commonly consumed plant foods are normally non-toxic and have the potential of preventing chronic disorders. The plant extracts encompasses high concentration of secondary metabolites. The synergistic characters of these phytochemicals have made them more unique, as they can mitigate many diseases like atherogenesis, thrombosis, carcinogenesis, hepatotoxicity and variety of disease via inhibiting lipid peroxidation [29]. The biomolecules are responsible for the free radical scavenging and antioxidant activity of the plants. PIs possess many biological effects, mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelation of transition metals.

The magnitude of haemolysis was found to be higher, when RBCs were treated with hydrogen peroxide (oxidant). This could be attributed by the oxidizing potential of H_2O_2 or its chain reactions connected with metal ions break cell membrane and subsequent release of haemoglobin. The anti-haemolytic activity of drugs is the expression of synergistic action of the diverse antioxidant mechanisms.

The application of SAPI showed generally remarkable effect in inhibiting haemolysis. When RBCs were treated with SAPI along with H_2O_2, marked reduction in haemolysis was seen (Table 3). This may be because of radical scavenging activity of the PIs of the extracts showing potent anti-haemolytic nature through donating electrons to H_2O_2, thus neutralizing it to water molecule [30]. The result shows that the protective effect of SAPI against haemolysis increased with increase in concentration. The SAPI exhibited the highest anti-haemolytic activity with IC_{50} value of 368.54 µg/ml.

Table 3: Inhibiting activity of SAPI on haemolysis (mean+SD). $P < 0.01$

Conc. of SAPI (µg/ml)	% of haemolysis
50	75.66±0.12
100	67.58±0.54
200	63.43±0.31
300	55.91±0.11
400	27.35±0.09
500	5.78±0.08
H_2O_2	98.87±0.04
Quercetin 100	64.7±0.21

Although quercetin being the standard had lower haemolytic activity with IC_{50} = 224 µg/ml compared with SAPI it also showed good anti-haemolytic activity. Anti-haemolytic activity of quercetin [31] and the relation between irons chelating activity against oxidative damage to erythrocyte membrane by plants was previously reported [32]. Also, binding of PIs to the RBC membrane remarkably inhibits LPX and at

the same time, enhances their integrity against lysis [33]. Further, SAPI quenched H_2O_2 before it oxidizes the biomolecules of the RBC membrane to cause oxidative haemolysis as reported by on the effects of green tea.

Although H_2O_2 itself is not reactive, it can bring cytotoxicity through hydroxyl radical in the cell. Thus, removing H_2O_2 is important in the cell system of organisms. The actual mechanism by which the SAPI reduced H_2O_2 induced haemolysis remains to be traced.

SBTI (Soybean trypsin inhibitor) and aprotinin retard haemolysis of red blood cells by inhibiting activation of some components of the complement system [34, 35]. Similarly, the cells of human peripheral blood did not suffer any cytotoxic effect when analyzed by blood cells counting or by haemolytic assay using the *Erythrina velutina* trypsin inhibitors [36].

5. Conclusion

The present study showed that the SAPI have anti-haemolytic activity comparable to the reference drug quercetin. SAPI exhibited concentration dependent inhibitory activity towards H_2O_2 induced haemolysis of RBCs attributed to the SAPI which offer protective effects against oxidative damage to biological macromolecules like lipids and proteins in the RBC membrane. Further work is needed to confirm the results obtained by *in vivo* animal models.

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7. References

1. Trease GE, Evans WC. Pharmacognosy. 15th ed. Edinburg: Saunders WB, 2002, 585.
2. Sofowora A. Medicinal plants and traditional medicine in Africa. Ibadan: Spectrum Books, 1993, 153.
3. Zbigniew Sroka Z. Antioxidative and Antiradical Properties of Plant Phenolics Naturforsch 2005; 60:832-843.
4. Ebrahinzadeh MA, Ehsanifar S, Eslami B. Sambucus ebulus elburensis fruits: A good source for antioxidants. Pharamacognosy Magazine 2009; 4(19):213-218.
5. Habib H, Fazili KM. Plant protease inhibitors: a defence strategy in plants. Biotechnology and Molecular Biology Review 2007; 2(3):068-085.
6. Norton G. Proteinase Inhibitors. Toxic Substances in Crop Plants. The Royal Society of Chemistry, 1991, 68-106.
7. Shu Guo FAN, Guo Jiang WU. Characteristics of plant proteinase inhibitors and their applications in combating phytophagous insects. Bot. Bull. Acad. Sin 2005; 46:273-292
8. Bothalia The genus Solanum (Solanaceae) in southern Africa: subgenus Leptostemonum, the introduced sections Acanthophora and Trove 2003; 33(1):1-18.
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*), Phyto chemistry 2010; 71:363-372.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage, Nature 1970; 227:680-685.
11. Devasagayam TP, Boloor KK, Ramasarma T. Methods for estimating lipid peroxidation: An analysis of merits and demerits, Indian Journal of Biochemistry & Biophysics. 2003; 4:300-308.
12. Ebrahimzadeh MA, Ehsanifar S, Eslami B. Sambucus edulis elburensis fruits: A good source for antioxidants. Pharmacognosy Magazine 2009; 4(19):213-218.
13. Devjani C, Verma RJ. Ameliorative effect of *Emblica officinalis* aqueous extract against ochratoxin – induced lipid peroxidation in the kidney and liver of mice. International Journal of Occupational Medicine and Environmental Health. 2010; 23:1-11.
14. Tandan N, Roy M, Roy S. Ameliorative Potential of *Psidium guajava* on Hemato-biochemical Alterations in Arsenic-exposed Wistar Rats. Toxicology International 2012; 19:121-124.
15. Ak T, Gulcin I. Antioxidant and radical scavenging properties of curcumin. Chemico Biological Interactions 2008; 174:27-37
16. Srichairatanakool S, Thephinlap C, Phisalaphong C, Porter JB, Fucharoen S. Curcumin contributes to in vitro removal of non transferrin bound iron by deferiprone and desferrioxamine in thalassemic plasma. Medicinal Chemistry 2007; 3:469-474.
17. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 1996; 20:933-956.
18. Kazazic SP, Butkovic V, Srzic D, Klasinc L. Gas-phase ligation of Fe+ and Cu+ ions with some flavonoids. Journal of Agricultural and Food Chemistry. 2006; 54:8391-8396.
19. Rajesh R, Chitra K, Paarakh PM. In vitro anthelmintic activity of aerial parts of Aerva lanata Linn Juss. International Journal of Pharmaceutical Sciences and Drug Research. 2010; 2(4):269-271.
20. Kumar G, Karthik L, Rao KVB. A review on pharmacological and phytochemical profile of *Calotropis gigantean* Linn. Pharmacology online Newsletter 2011a; 1:1-8.
21. Dasgupta A, Agrawal SS, Basu DK. Anticonvulsant activity of the mixed fatty acids of the *Elaeocarpus ganitrus* Roxb. Indian Journal of Physiology and Pharmacology. 1984; 28(3):245-246.
22. Mathur A, Purohit R, Mathur D, GBKS Prasad, Dua VK. Pharmacological investigation of methanol extract of Syzygium cuminii seeds and Crateva nurvula bark on the basis of antimicrobial, antioxidant and anti-inflammatory properties. Der Chemica Sinica 2011; 2(1):174-181.
23. Priya CL, Kumar G, Karthik L, Rao KVB. Antioxidant activity of *Achyranthes aspera* Linn stem extracts. Pharmacology online Newsletter 2010; 2(2):228-237.
24. Kalita S, Kumar G, Karthik L, Rao KVB. Phytochemical composition and in vitro haemolytic activity of *Lantana camara* L. (Verbenaceae) leaves. Pharmacology online Newsletter 2011; 1:59-67.

25. Oliveira VM, Carneiro AB, Glaucia Socorro BC, Pohlit AM. In vitro screening of Amazonian plants for haemolytic activity and inhibition of platelet aggregation in human blood. Acta Amazonica 2009; 39(4):973-980.
26. Gandhi VM, Cherian KM. Red cell haemolysis test as an *in vitro* approach for the assessment of toxicity of karanja oil. Toxicology in Vitro. 2000; 14:513-516.
27. Mukherjee A, Rajasekaran C. *In vitro* haemolytic activity of *Allium stracheyi* Baker. Journal of Pharmacy Research. 2010; 3(5):1160-1162.
28. www.nlm.nih.gov/herbalmedicine.html.
29. Tivari AK. In balance in antioxidant defence and human diseases: multiple approach of natural antioxidant therapy. Current Sciences 2001; 81(9-10):1179-1187.
30. Alinezhad A, Baharfar R, Zare M, Azimi R, Nabavi SF, Nabavi SM, 2012. Doi: 10.3109/13880209.2012.655859.
31. Walker PD, Shah SV. Gentamicin enhanced production of hydrogen peroxide by renal cortical mitochondria. American Journal of Physiology. 1987; 253:495-499.
32. Nabavi SF, Nabavi SM, Abolhasani F, Moghaddam AH, Eslami S. Cytoprotective Effect of Silymarin against Sodium Fluoride-Induced Oxidative Stress in Rat Erythrocytes. The Bulletin of Environmental Contamination and Toxicology 2012; 88:846.
33. Chaudhuri S, Banerjee A, Basu K, Sengupta B, Sengupta PK. Interaction of flavonoid with red blood membrane lipids and proteins: antioxidant and anti-haemolytic activity. International Journal of Biological Macromolecules. 2007: 41:42-48.
34. Eugene A, Borodin Igor E, Pamirsky Mikhail A, Shtarberg, Vladimir *et al*. Effects of Soybean Trypsin Inhibitor on Hemostasis http://dx.doi.org/10.5772/52600
35. Yamile González-González, Aida Hernández-Zanuy, Mariana da Silva Araujo, María Luiza V Oliva, María A Chávez Planes, Claudio AM Sampaio. Screening of serine proteinase inhibitors from marine organisms Biotecnología Aplicada 2003; 20:111-114.
36. Richele Machado JA, Norberto Monteiro KV, Ludovico Migliolo, Osmar Silva N, Michele Pinto FS, Adeliana SO *et al*. Characterization and Pharmacological Properties of a Novel Multifunctional Kunitz Inhibitor from *Erythrina velutina* Seeds. PLOS One 2013; 8(5):635-671.