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ANTI-ALBUMIN DENATURATION, TRYPSIN INHIBITION AND RBC MEMBRANE STABILIZATION AS POSSIBLE MECHANISM FOR ANTI-INFLAMMATORY ACTIVITY OF METHANOL LEAF EXTRACTS OF SOLANUM SPECIES

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ABSTRACT: In view of rising concern of problem associated with synthetic drugs against inflammation, current research has been focused towards safer, effective and natural compounds. Hence, we aimed at assessing the anti-inflammatory effect of four plants from *Solanum* species (*S. seaforthianum, S. diphyllum, S. sisymbriifollium* and *S. anguivi*) through albumin denaturation assay, trypsin inhibition and RBC membrane stabilization assays. The data showed that the methanol extracts of these plants had concentration dependent protection. *S. anguivi* extract showed maximum inhibition of albumin denaturation (26.15 ± 0.08%), whereas *S. sisymbriifollium* extract displayed maximum (16.51 ± 0.002%) trypsin inhibition. With respect to heat induced RBC lysis, *S. anguivi* extract showed highest inhibition (26.16 ± 0.08%), whereas *S. seaforthianum* extract displayed maximum inhibition (36.49 ± 0.002%) of RBC lysis induced by hypotonicity. From the study, it can be inferred that anti-inflammatory activity in the tested *Solanum* species extract varied considerably and its activity can be attributed to its richness in phytochemical compounds.

INTRODUCTION: Inflammation characterized by swelling, reddening and pain is a biological protective response towards injury or some kind of trauma, including noxious chemical and microbial agents [1]. Cells under injurious stimuli, releases inflammatory mediators like histamine, serotonin, prostaglandins and other clotting as well as fibrinolytic factors [2]. These inflammatory mediators cause vasodilation and increased permeability of blood vessels leading to release of plasma proteins and fluids to transfer leukocytes to the site of injury [3].

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Inflammatory response can be a warrant under certain conditions including allergies, microbial infections and autoimmune diseases. Steroidal and non-steroidal drugs are generally recommended to decrease long-term inflammatory responses and the associated complications. However, long term usage of these drugs has few limitations with gastrointestinal problems and in certain cases, bleeding with perforation in intestinal lining has been reported [4, 5]. Non-steroidal drugs are known to cause electrolyte imbalance, leading to liver and kidney toxicity [4, 6].

Since, ancient times, plants have remained as strong pillars in medical field because they are treasure of enormous novel compounds. Plants and their by-products have been regarded as an important means of medical therapies. Phyto-constituents have shown extreme potential as antimicrobial, anti-toxin, anti-inflammatory agents

and have been applied in curing various diseases. Secondary metabolites of plants such as tannins, phenols, steroids, alkaloids, terpenoids and flavonoids are well studied for their biological activities [1].

The plants of Solanaceae family are widely distributed in tropical and subtropical regions with more than 150 genera and >3000 species. Some of the plants of this family have high economic ranking and are used as food, for example: *S. lycopersicum, S. tuberosum* (potato), *S. melongena* (egg plant) and *S. gilo* (gilo). Traditionally, local tribes and medicinal practitioners use certain plant extracts as sedatives and in treatment of asthma, diabetes, abdominal and renal pain relied [7]. Earlier researchers have also appreciated the biological activities including antioxidant, antibacterial, larvicidal and allelopathic activity [8, 9]. Hossain *et al.*[10] have demonstrated anti-oxidant, anti-alpha amylase and anti-alpa-glucosidase activities of *S. diphyllum*. The characteristic fragrance and volatile compounds produced by flower and fruits of *S. sisymbriifollium* have appreciable therapeutic importance including antibacterial and antioxidant activity [11]. Recently, fruits of *S. anguivi* have been studies for their efficiency in treating type-2 diabetes mellitus [12].

During cascade of inflammatory response, excessive amount of free radicals, hydroxyl radicals are released causing lipid peroxidation and membrane destruction. Hence medicinal plants with potential radical scavenging activity are preferred as natural source to reduce complications related to inflammatory response. In this context, Solanum species (*S. seaforthianum, S. diphyllum, S. sisymbriifollium* and *S. anguivi*) previously demonstrated for potential antioxidant activity was evaluated for anti-inflammatory property [13].

MATERIALS AND METHODS:

Plant Material and Processing: Four wild species of Solanum (*S. seaforthianum, S. diphyllum, S. sisymbriifollium* and *S. anguivi*) were collected from different areas of Mysore district, Karnataka, India. The identity was confirmed by taxonomists Dr. K. N. Amruthesh, from the Department of Botany, University of Mysore and the voucher specimen has been deposited at Herbarium stock, Department of studies in Botany, Mysore. The shade dried leaf powder of each plant was homogenized into fine powder and the phytochemical was extracted with methanol using Soxhlet apparatus. The dried residue was dissolved in DMSO (dimethyl sulfoxide) and stored at -20°C until use.

Albumin Denaturation Assay: The method of Gunathilake *et al.*[4] was followed with minor modification. Briefly, different concentration of the extract (0.5, 1.0, 1.5 mg/mL) was mixed with 0.2 mL of 1% bovine albumin solution and the volume was made up to 5 ml using phosphate buffered saline (PBS, pH 6.4). The reaction mixture was incubated in a water bath at 37°C for 15 min, and then heated at 70°C for 5 min. The final turbidity was measured at 660 nm using a UV-VIS spectrometer. Phosphate buffer saline was used as the blank. Sample in buffer was kept as sample control. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\text{Percentage inhibition} = \text{Ab control} - \text{Ab test} / \text{Ab control} \times 100$$

Trypsin Inhibition Assay: The inhibition of trypsin activity by the prepared extract was evaluated by the method described by Gunathilake *et al.*[14]. The test extract at different concentration (0.5, 1.0, 1.5 mg/mL) was mixed with 0.06 mg trypsin (prepared in 20 Mm Tris-HCl buffer, pH 7.4). The volume was made upto 2 mL with same buffer. The reaction mixture was incubated at 37°C for 5 min, and then 1 mL of 0.8% (w/v) casein was added. The mixture was further incubated for an additional 20 min at 37°C. At the end of incubation, 2 ml of 70% perchloric acid was added to terminate the reaction. The mixture was centrifuged, and the absorbance of the supernatant was measured at 210 nm against buffer as the blank. Sample blank with buffer was kept as control. The percentage inhibition of protease activity was calculated by using the following formula:

$$\text{Percentage inhibition} = \text{Ab control} - \text{Ab test} / \text{Ab control} \times 100$$

Membrane Stabilization Assay: The effect of extract on HRBC membrane stabilization was assessed accordingly Sak at *et al.*[15]. Blood from

healthy human volunteer was collected and centrifuged at 3000 rpm for 10 min. Cells were washed thrice with equal volume of normal saline and reconstituted as 10% (v/v) suspension. This cell suspension was used to examine heat and hypotonic induced membrane stabilization.

Heat Induced Hemolysis: The reaction mixture (2 mL) consisting 1 ml of extract at different concentrations (0.5, 1.0 and 1.5 mg/mL) and 1 ml of 10% RBCs suspension was incubated in a water bath at 56°C for 30 min. Saline was used in control tube. At the end of the incubation, the tubes were cooled and centrifuged at 2500 rpm for 5 min. The absorbance of the supernatants was taken at 560 nm using UV-Vis spectrophotometer. The percentage inhibition of hemolysis was calculated as compared to control.

Hypotonicity-Induced Hemolysis: Different concentration of the extract (0.5, 1.0 and 1.5 mg/mL) were made up to 1 ml with phosphate buffer and mixed with 2 ml of hyposaline (0.5%) and 0.5 ml of RBC suspension. The mixtures was incubated at 37°C for 30 min and centrifuged at 3000 rpm. The supernatant absorbance was measured by a spectrophotometer at 560 nm. Sample without RBC was considered as sample blank. The percentage hemolysis was estimated by the hemolysis produced in the control as 100%.

Statistical Analysis: The experiments were conducted in triplicates and the data are presented as average ± standard deviation. The results were analyzed using one-way ANOVA in SPSS software. P value <0.05 was accepted as significant.

RESULTS AND DISCUSSION: Inflammation is a beneficial, defensive response towards injurious agents. However, this response also contributes towards release of reactive free radicals and various active cells by immune system which causes considerable discomfort as well as lipid peroxidation and tissue damage [16]. Steroidal and non-steroidal agents are generally recommended to treat inflammation, but these have been reported with side-effects [17]. This made many researchers, globally to search for natural, safe and effective drug that can neutralize the inflammatory response. In this regard, several plant extracts have been examined for their inflammatory activities. We conducted this study to evaluate the *in-vitro* anti-inflammatory activity in four Solanum species extract which were previous reported for potential antioxidant property. Albumin denaturation, trypsin inhibition and HRBC membrane stabilization assays were carried out to examine the anti-inflammatory potential.

Albumin Denaturation Assay: Table 1 shows the effect of difference concentration of four Solanum species methanol leaf extract on albumin denaturation. A dose dependent rise in percentage inhibition was noticed in all the four samples. Aspirin used as reference standard also showed the similar trend **Fig. 1A**.

The anti-denaturation activity ranged between 9.94% to 26.15% depending on the sample and concentration. Comparatively, *S. anguivi* was effective with highest (26.15 ± 0.08%) inhibition at 1.5 mg/mL concentration. During inflammation process, extensive disruption of protein structure through breakage of hydrogen bond, electrostatic and disulphide bonds occurs. Consequently, enzyme activators and mediators are released with cell migration and tissue breakdown which finally leads to protein conformational change and denaturation [18, 19]. This protein denaturation is considered as a marker for inflammatory response. In this view, BSA denaturation is an ideal method to determine anti-inflammatory potential and the compound which has anti-denaturation activity would be a potential inhibitor of inflammatory response.

In the similar line, Pungle *et al.* [20] explored ethanol extracts of seeds and leaves from *Solanum xanthocarpum* for anti-inflammatory activity by percent inhibition of albumin denaturation, membrane stabilization and protease inhibition. The ethanol leaf extract showed 25% albumin denaturation at a concentration of 0.1 mg/ml. Bailey-Shaw *et al.*[21] demonstrated inhibition of BSA denaturation in 99 plants and reported the ethanol extracts of *Cajanuscajan, Cinnamomum zeylanicum, Cordia alba, Mangifera indica,* and *Tecoma stans* as effective inhibitors with 62.31%, 49.61%, 65.47%, 72.60% and 61.50% respectively. Modi *et al.*[22] studied 25 different medicinal plants from Junagadh region of Gujarat for *in-vitro* anti-

inflammatory activities. Water extracts of *Adansonia digitata* L. leaves, *Flueggea leucopyrus* Willd. leaves, and Solanum xanthocarpum Schrad. & H. Wendl. showed an inhibition of 87.54, 80.23, and 80.38%, respectively. While methanol extracts of *Adansonia digitata* L. leaves and Solanum xanthocarpum demonstrated 87.54 and 81.79% inhibition at 0.5 mg/mL concentration, respectively. They suggested that the two glycol-alkaloids, Solasodine and solasonine present, may be the reason for anti-inflammatory effects. Aqueous extract of *Solanum aethiopicum* has been reported to show an IC_{50} value 31.5 µg/ml [23]. In the present study, *S. anguivi* has IC_{50} value of 2.59 mg/mL.

TABLE 1: PERCENTAGE INHIBITION OF ALBUMIN DENATURATION

Methanol extracts	Percentage anti-albumin denaturation activity at different concentrations			IC50 (mg/mL)
	0.5 mg/mL	1 mg/mL	1.5 mg/mL	
S. seaforthianum	9.94 ± 0.05[a]	13.38 ± 0.01[a]	14.79 ± 0.01[a]	4.32
S. sisymbriifollium	12.29 ± 0.00[b]	15.68 ± 0.10[b]	17.29 ± 0.04[b]	3.66
S. diphyllum	12.86 ± 0.06[b]	19.59 ± 0.02[c]	21.10 ± 0.02[c]	3.03
S. anguivi	14.58 ± 0.00[c]	20.94 ± 0.10[d]	26.15 ± 0.08[d]	2.59

Values are average three independent experiments. Values with different superscripts in a column are significantly different ($p<0.05$).

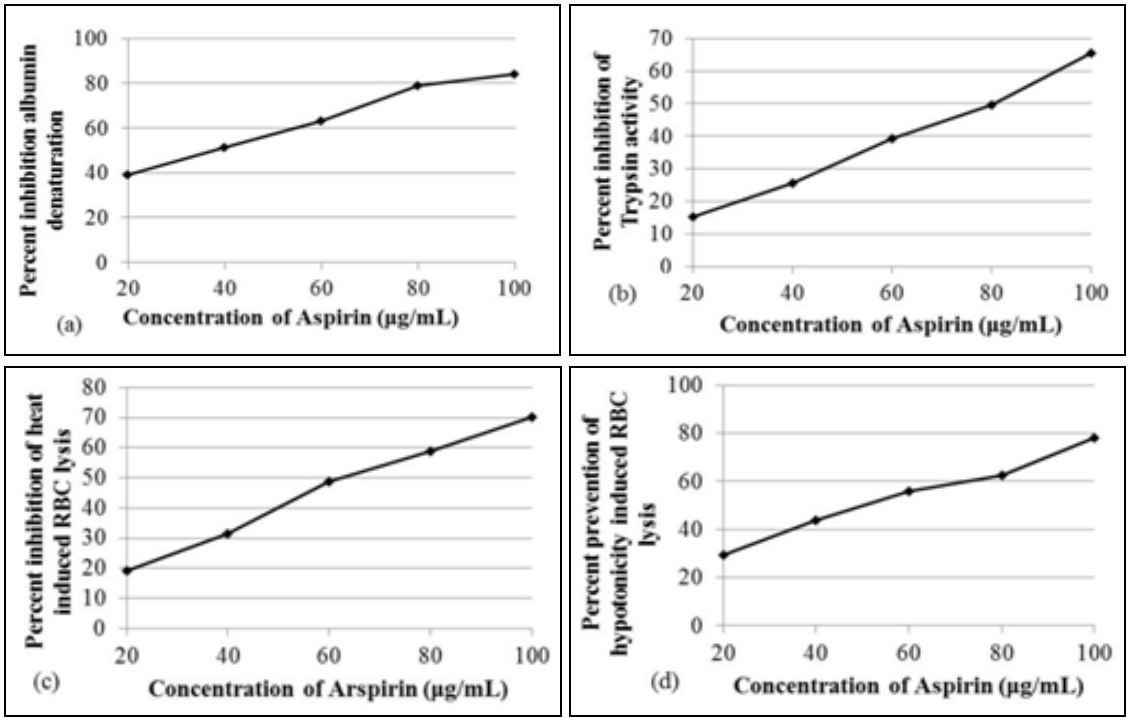

FIG. 1: *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY OF STANDARD ASPIRIN. (A) PROTEIN DENATURATION ASSAY; (B) TRYPSIN INHIBITION ASSAY; (C) HEAT INDUCED RBC LYSIS ASSAY; (D) HYPOTONICITY INDUCED RBC LYSIS ASSAY

Trypsin Inhibitory Activity: Percentage of Trypsin inhibition by methanol extracts of four Solanum plants at different concentration has been presented in **Table 2**. The inhibition in concentration dependent, with 1.5 mg/mL having highest inhibition in all the samples. The standard drug aspirin shows a maximum of 65.35% inhibition at a final concentration of 100 µg/Ml **Fig. 1B.** The trypsin inhibitory activity in the extracts varied considerably ($p<0.05$) ranging from 5.50% to 16.51%. *S. sisymbriifollium* displayed maximum (16.51 ± 0.002%) followed by *S. anguivi* and *S. diphyllum* with 15.59% inhibition each. Protease inhibitors including trypsin play a major role in protein interaction and are necessary regulators and modulators of inflammatory response. Neutrophils located near lysosomes are rich source of serine proteases. Nugteren and Samsom [24] reviewed the role of leukocytes proteases in the progression of tissue damage during inflammatory reaction and the use of protease inhibitors had significant protective effect. Pungle *et al.*[20] studied serine

protease inhibition of *S. xanthocarpum* seeds and leaf with ethanol, acetone, ethyl acetate and water extracts. Around 4% inhibitions with acetone and aqueous extract of seed have been illustrated while 1% inhibition with ethyl acetate extract. The anti-trypsin activity was noticed more for seed ethanol extract (3.14%) than for Leaf ethanol extract (1.26%). *Solanum xanthocarpum* aerial part water extract has been demonstrated to have 67% protease inhibition at a concentration of 0.5 mg/mL concentration [22]. For proteinase inhibitory action, aqueous extract of *Solanum aethiopicum* has an IC_{50} value of 19.85 µg/ml compared to the standard aspirin with IC_{50} value of 9.35 µg/ml [23]. In our study, better IC_{50} value of 4.02 mg/mL was observed in *S. anguivi* extract.

TABLE 2: PROTEINASE INHIBITION BY METHANOL LEAF EXTRACTS OF SOLANUM SPECIES

Methanol extracts	Percentage of Trypsin inhibitory activity at different concentrations			IC_{50} value (mg/mL)
	0.5 mg/mL	1 mg/mL	1.5 mg/mL	
S. seaforthianum	5.50 ±0.00[a]	10.09 ±0.01[a]	11.00 ±0.00[a]	5.96
S. sisymbriifollium	5.50 ±0.00[a]	12.84 ±0.00[c]	16.51 ±0.00[c]	4.34
S. diphyllum	9.17 ±0.01[b]	11.92 ±0.00[b]	15.59 ±0.00[b]	4.39
S. anguivi	11.00 ±0.01[c]	14.67 ±0.00[d]	15.59 ±0.00[b]	4.02

Values are average three independent experiments. Values with different superscripts in a column are significantly different ($p<0.05$).

HRBC Membrane Stabilization: Inhibition of heat and hypotonicity induced RBC lysis was considered as a measure to study anti-inflammatory activity of Solanum sp extract.

Heat Induced Hemolysis: Each extract at various concentration tested significantly ($p<0.05$) prevented the lysis of HRBC membrane **Table 3**. The percentage inhibition varied from the lowest 9.95% to highest of 26.16%. The dose-dependent percentage increase in the prevention of lysis, support for the capacity of the extract in membrane protection. At a final concentration of 1.5 mg/mL, *S. anguivi* extract showed highest inhibition (26.16 ± 0.08%) followed by *S. diphyllum* (21.10 ± 0.02%).

TABLE 3: PREVENTION OF HEAT INDUCED HEMOLYSIS WITH SOLANUM LEAF EXTRACTS

Methanol extract	Percentage heat induced hemolysis inhibition (%) at different concentrations			IC_{50} value
	0.5 mg/mL	1 mg/mL	1.5 mg/mL	
S. seaforthianum	9.95 ± 0.05[a]	13.39 ± 0.01[a]	14.80 ± 0.01[a]	4.31
S. sisymbriifollium	12.30 ± 0.00[b]	15.69 ± 0.10[b]	17.30 ± 0.04[b]	3.66
S. diphyllum	12.87 ± 0.06[b]	19.59 ± 0.02[c]	21.10 ± 0.02[c]	3.03
S. anguivi	14.59 ± 0.00[c]	20.95 ± 0.10[d]	26.16 ± 0.08[d]	2.59

Values are average three independent experiments. Values with different superscripts in a column are significantly different ($p<0.05$).

Hypotonicity Induced Hemolysis: The data from table 4 confirm that the tested extracts significantly ($p<0.05$) inhibited RBC lysis induced by hypotonicity. The observed rise in the inhibition with dosage confirms their protective effect. *S. seaforthianum* extract displayed maximum inhibition (36.49 ± 0.002%) at a concentration of 1.5 mg/mL.

TABLE 4: PREVENTION OF HYPOTONICITY INDUCED HEMOLYSIS BY SOLANUM LEAF EXTRACTS

Methanol extracts	Percentage hypotonicity induced hemolysis inhibition (%) at different concentrations			IC_{50} value
	0.5 mg/mL	1 mg/mL	1.5 mg/mL	
S. seaforthianum	12.80 ±0.017[a]	26.25 ±0.006[d]	36.49 ±0.002[c]	2.00
S. sisymbriifollium	14.21 ±0.012[b]	24.33 ±0.008[b]	27.78 ±0.009[a]	2.39
S. diphyllum	15.24 ±0.018[c]	21.51 ±0.006[a]	34.44 ±0.005[b]	2.17
S. anguivi	15.75 ±0.017[c]	25.86 ±0.003[c]	34.96 ±0.000[b]	2.03

Values are average three independent experiments. Values with different superscripts in a column are significantly different ($p<0.05$).

During inflammatory cascade, hydrolytic enzymes are released from lysosomal cells at the site of trauma. These enzymes are also known to cause damage of other organs and tissues surrounding the

site of infection. Erythrocyte membrane is an analogous to the lysosomal membrane [25] and stabilization of lyosomal membrane can limit the inflammatory response. Erythrocyte membrane has similar component as that of liposomal membrane, hence prevention of hypotoxicityor thermal induced HRBC lyses has been extensively used to measure anti-inflammatory potential [26]. In the present study, concentration dependent decrease in the lysis of human RBC induced by heat and hypotonic solution was observed. This implies that the extracts also stabilizelysosomal membrane thereby inhibiting efflux of intracellular components.

The rich polyunsaturated fatty acid content in RBC membrane makes the cells vulnerable to oxidative stress. The reduced hemolysis in presence of the extract indicates the protective effect. *S. aethiopicum* methanol extract at a dose of 100-800 µg/ml showed HRBC protection against heat induced lysis with 86.67% inhibition at a concentration of 800 ug/ml [27]. Chirumamilla *et al.*[28] analyzed *in-vitro* anti-inflammatory effect of *S. khasianum's* leaf, fruit, and root. They reported inhibition of hemolysis by 83.97±0.36% at 1 mg/mL concentration, as opposed to fruit (81.68 ± 0.82%) and leaf (70.93 ±0.38%) extracts.

In the similar line, leaf ethanol extract of *S. xanthocarpum* at a concentration of 12 mg/ml has been reported to show 73.66% protection of erythrocytes in hypotonic solution [29]. In another study, ethanol extract of *S. xanthocarpum* at 6 mg/ml concentration showed 50.1% protection of HRBC in hypotonic solution [30]. *S. aethiopicum* methanol extract showed HRBC protection against hypotonic induced lysis with a maximum of 50.8% inhibition at a concentration of 800 ug/ml [27]. RBC lysis due to hypotonic solution is related to excessive accumulation of fluid inside the cell leading to membrane rupture [31]. Hence the present extract with an ability to stabilize membrane can prevent leakage of proteins and fluids from the cells during inflammatory process.

CONCLUSIONS: The results of the investigation show that the plant extracts tested exhibited moderate level of anti-inflammatory activity than reported in the earlier literature. The phytochemical constituents and the potential anti-oxidant activity reported from these plant extracts may be the reason for these outcomes. The study supports the scientific basis for the folkloric claim for the use of this plant in preventing inflammatory cascade.

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