Research Article

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# *ELEPHANTOPUS SCABER* LINN. ENHANCES MEMBRANE STABILITY IN HUMAN ERYTHROCYTES IN IN VITRO MODELS

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**ABSTRACT**

Blood is the medium that provides nourishment and oxygen to the tissues. Some xenobiotics entering blood can destruct RBCs. Human erythrocytes are uniquely designed to overcome this with its lipid bilayer membrane. Phyto-chemicals influence the stability of RBC membranes. Medicinal plants with antitoxic properties maybe helpful. *Elephantopus scaber Linn.*, a common herb mentioned in traditional medical systems like Ayurveda in the context of toxicity. It is distributed in tropical areas of Africa, America and Asia. The methanolic extracts at doses 480, 240, 120, 60 and 30 mcg/ml were studied for erythrocyte membrane stabilization against Acetyl Salicylic Acid (ASA) in RBCs collected from healthy volunteers, for the parameters Osmotic fragility, hypotonic saline induced hemolysis, heat induced hemolysis, hemoglobin release assay and anti-inflammatory effect by membrane stabilization effect. Antioxidant property of the drug to prevent lipid peroxidation by preventing the formation of conjugated-diene was also studied. The results showed significant dose dependent membrane stabilization activity for the drug compared to Aspirin. The results were statistically analyzed using ANOVA and Dunnett's multiple comparison tests. The study concludes that *Elephantopus scaber Linn.* methanolic extract significantly enhances the erythrocyte membrane stability and it provides hope against xenobiotic induced hemolysis.

**Keywords**: *Elephantopus scaber*, Erythrocyte membrane, RBC membrane, membrane stabilization, osmotic fragility, hemolysis, conjugated diene, anti-inflammatory

## INTRODUCTION

Blood has gained the status of an organ system due to its diverse and life sustaining functions. It is bestowed with the functions of delivery of oxygen to tissues throughout the body, maintaining vascular integrity and providing the many affecter immune functions necessary for host defense etc[1]. Blood is the primary target of all toxicants as it is the first tissue that comes into contact with it and is also the medium of transport of poison to its target site. The toxicants exert their adverse reactions to the blood at the initial stage itself [2]. Hemolysis is one of the immediate outcomes of this toxic insult. However, the unique homoeostasis coupled with the well equipped antioxidant defense mechanisms contain these toxic changes to a great extent[3]. The structural and functional integrity of erythrocytes is due to its ability to undergo extensive passive deformation and the ability to resist fragmentation[4]. For this essential qualities to persist, the erythrocytes need to be highly deformable and with a stable membrane. Erythrocyte membrane is a well characterized lipid bilayer membrane composed of integral proteins and a skeletal protein network of spectrin, actin, ankyrin, tropomyosin etc.[5]. The blood carried toxicants initiate inflammatory changes in blood itself targeting the erythrocyte membrane, which gets denatured and culminate in hemolysis. The cascade of events following this is highly complex and among them free radical generation and subsequent lipid peroxidation by extracting hydrogen from the polyunsaturated fatty acids leading to the generation of conjugated dienes is considered the key event[6]. Beyond this the reaction flares up like an uninterrupted chain of events ending up in the destruction of the cells, tissues and the organism as a whole. All the xenobiotics that enter the blood as food, drug or environmental pollutants can alter the physiology of blood. The historical description of hemolytic anemia resultant from the consumption of fava beans in people with deficient Glucose-6-phosphate dehydrogenase enzyme is a well stated example in this regard [7]. Erythrocyte osmotic fragility is the resistance of RBC hemolysis to osmotic changes[8]. Osmotic fragility testing is considered the gold standard for assessing the membrane stabilization activity of compounds. Many well documented medicinal plants and dietary items protect the toxic destruction of erythrocyte membranes. *Elephantopus scaber* is an annual herb growing wildly in India. It is documented in the ancient healing system of Ayurveda and is attributed with the property of antipyretic, bitter, acrid, astringent, constipating, diuretic and tonic[9]. *Elephantopus scaber* is rich in Sesquiterpene lactones, phenolic acids and flavonoids [10] with a wide range of therapeutic potentials[11]. The wide mentioning of this drug among toxic and inflammatory conditions makes it an ideal drug to be investigated for. In this study we investigate the efficacy of the *Elephantopus scaber* methanolic extract (ESME) in membrane stabilization and as an anti-inflammatory agent through determination of Osmotic fragility (OF), hypotonic solution induced hemolysis & heat induced hemolysis. A study has also been carried out to establish its role in preventing lipid peroxidation by reducing the levels of conjugated dienes in isolated erythrocyte membranes.

## MATERIALS AND METHODS

### Collection of Plant Materials

Fresh plants of *Elephantopus scaber* were collected from Nileswaram, Kasaragod District, Kerala, growing wildly in their natural habitat. The plant was authenticated from Department of Agadatantra, Vaidyaratnam P.S Varier Ayurveda College Kottakkal and a voucher specimen was deposited in the department herbarium (accession number AH/Ast/2016-001) for future reference. The plants were properly washed and all the foreign matter was removed and shade dried. The dried plant materials were powdered and sieved through mesh of size 40 and stored in airtight glass containers till the extraction.

**Extract Preparation**

The powdered plant materials were extracted with methanol using continuous hot percolation method in a Soxhlet extractor with 250 ml of methanol for 25 grams of drug powder. The extraction was carried for a period of eight hours. The extract was later filtered through a filter paper (Whatman No. 40) and concentrated under reduced temperature and pressure in a flash evaporator at $40^0$C. The extract was further evaporated under a continuous flow of hot air in an inert atmosphere containing nitrogen till last trace of methanol was removed and the dried extract powder was stored in an air tight container and freezed at $-20^0$C till the beginning of the experiments.

**Physico-Chemical Parameters**

Estimation of physico-chemical characteristics forms a major part of standardization and authentication of crude drugs. Parameters like loss on drying, total ash, water insoluble ash, sulphated ash, alcohol soluble extractives, water soluble extractives, Crude fiber content, Foaming index and pH were estimated based on the standard procedure laid down by the Pharmacopoeia of India[12,13]

**Fluorescence Analysis[14]**

The drug powder was treated with various reagents and solvents and viewed in visible, short UV (254 nm), mid UV (302 nm) and Long UV (356 nm) rays and the fluorescence was noted.

**Phytochemical Evaluation[15]**

The extract was screened for the presence of major secondary metabolites as per the accepted methodologies. The tests were performed for the presence of alkaloids, carbohydrates, glycosides, saponins, phytosterols, fixed oils & fat, Resins, Phenols, tannins, flavonoids, proteins & amino acids, anthraquinones, cholesterol, coumarins, quinines, phlobotannins etc.

**High Performance Thin Layer Chromatography (HPTLC) Profile**

***Sample preparation and application***

5 mg/ml concentration of the ESME was made with HPTLC grade methanol and filtered through Whatman filter paper No.1. 15 uL of the sample was applied on TLC aluminium sheets silica gel 60 $F_{254}$ (Merck, 1.05554.0007) with band length of 5 mm using Linomat 5 sample applicator set at a speed of 150 nL/ sec.

***Developing solvent system***

The mobile phase was fixed by trial and error after testing different combinations. The system that gave satisfactory resolution and maximum number of spots was Toluene: Ethyl acetate: Formic acid: Methanol:: 7:5:1:0.5.

***Development of Chromatogram***

The plate was developed using Camag 10 x 10 twin trough chamber with the solvent system of Toluene: Ethyl acetate: Formic acid: Methanol:: 7:5:1:0.5 for 20 minutes up to the distance of 80 mm.

***Scanning and detection of spots***

The air dried plates were visualized in Ultraviolet radiation to visible light. Spots were visualized before and after derivatization with 10% sulphuric acid spraying reagent at 254 and 366 nm and 400-600 nm wavelengths. Scanning was performed by CAMAG HPTLC Scanner 3 in absorbance mode at 254 and 366 nm wavelengths. The Rf values and fingerprint data were recorded using CAMAG TLC scanner 3.

**Erythrocyte Membrane Stabilizing Activity**

***Study participants***

Six healthy volunteers were used for the study. Ethical clearance was obtained from the Institutional Ethics Committee of Vaidyaratnam P.S Varier Ayurveda College Kottakkal (No: IEC/CL/01/15) before the beginning of the study. Only 5 ml of fasting venous blood was collected from each participant after obtaining informed consent from them.

***Determination of Erythrocyte Osmotic Fragility***

Osmotic fragility was evaluated by modified Oywale method[16]. Osmotic fragility was determined by incubating erythrocytes in serial dilutions of Phosphate Buffered Saline (PBS) after prior incubation with different concentrations of the extract. 5 ml of venous blood was drawn into tubes containing dipotassium salt of EDTA as anticoagulant from six healthy volunteers after overnight fasting. 0.5 ml of blood was incubated with 0.5 ml of various concentrations of the extract (480 mcg/ ml, 240 mcg/ ml, 120 mcg/ ml, 60 mcg/ ml and 30 mcg/ml) in PBS solution ($Na_2HPO_4.2H_2O$ – 1.71 g, $NaH_2PO_4.2H_2PO_4.2H_2O$ – 2.43 g, NaCl – 9.0 g per 1 L of distilled water at pH 7.4) for 1 hour at room temperature. At the end of the incubation time, the tubes were centrifuged for 5 minutes at 2000 x g and the supernatant was discarded. The packed erythrocytes were subjected to repeated washing in PBS for 3 more times and the mixture was centrifuged so as to remove all the traces of extract. The packed erythrocytes were finally diluted in PBS containing 0.9% NaCl and a 10% suspension of erythrocytes was prepared. Aliquots of 50 uL of the erythrocyte suspension were added to 5 ml portions of the PBS containing different concentrations of NaCl ranging from 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0% respectively and the tubes were incubated for one hour at room temperature. The tubes were centrifuged at 2000 x g for 5 minutes at the end of the incubation. The Optical density (OD) of the supernatant isolated was determined in the spectrophotometer at 540 nm. The percentage of hemolysis measured was expressed as the Osmotic fragility.

***Hypotonic Solution Induced Hemolysis***

Venous blood was collected into tubes containing EDTA as anticoagulant. The collected blood was washed free of plasma and buffy coat in cold 0.9% (w/v) sodium chloride in 5 mM sodium phosphate buffer (pH 7.5) for four times. At the end of each washing, the suspension was centrifuged at 2000 x g for 5 minutes. At the end of the fourth wash, the packed erythrocytes were reconstituted with 0.9% (w/v) sodium chloride in 5 mM sodium phosphate buffer (pH 7.5) and used immediately.

From this erythrocyte suspension 0.5 ml was mixed with 5 ml of hypotonic saline containing 50mM NaCl in 10mM sodium phosphate buffered saline (pH 7.4) containing different concentrations of the extracts and acetyl salicylic acid (0.10 mg/ml) as reference standard. The mixtures were incubated at $25^0$C for 10 minutes and then centrifuged for 10 minutes at 3000 x g and the absorbance (OD) of the supernatant was measured at 540 nm using a UV spectrophotometer. The percentage inhibition of hemolysis was computed using the following equation[17].

$$\text{Percentage Inhibition of Hemolysis} = \frac{(OD1 - OD2)}{OD1} X\ 100$$

Where OD1 is optical density of the hypotonic buffered saline solution alone (control) and OD2 is the optical density of the test sample in hypotonic solution.

***Heat Accelerated Hemolysis***

Twin centrifuge tubes containing 5 ml of isotonic buffer mixed with various concentrations of the extract was arranged. The same quantity of vehicle taken in another tube served as the control. Erythrocyte suspension as prepared in the previous experiment (30 uL) was added to each tube and mixed gently by upending. Of the twin tubes, one tube was incubated at $54^0$C for 20 minutes in a water bath and the other was placed in an ice bath at $5^0$C. After the end of the incubation, the reaction mixture was centrifuged for 3 minutes at 1300 rpm and the optical density (OD) of the supernatant was measured at 540 nm. The percentage inhibition in the tests was calculated according to the following equation[18].

$$\text{Percentage Inhibition of Hemolysis} = \frac{1 - (OD2 - OD1)}{(OD3 - OD1)} X\ 100$$

Where OD1 is the OD of unheated sample, OD2 is the OD of heated test sample and OD3 is the OD of heated control sample

***Hemoglobin Release Assay***

The assay mixture constituted a volume of 4.5 ml containing 2 ml of hyposaline, 1 ml of 5 mM PBS (pH 7.5), 0.5 ml of 2% (v/v) erythrocyte suspension in isosaline and 1 ml of the drug solution. The tubes were incubated at $56^0$C for 30 minutes. The reaction mixture was then cooled under running cold water followed by 10 minutes centrifugation at 5000 rpm. The supernatant from the tubes were collected and the optical density (OD) of the released hemoglobin was read at 560 nm. The percentage membrane stability was determined using the expression[19].

$$\text{Membrane Stability} = \frac{100 - [(OD2 - OD1)\ X\ 100]}{OD3}$$

Where OD1 = absorbance of hemoglobin released when the chemical compound where added
OD2 = absorbance of hemoglobin released when the erythrocyte suspension was omitted
OD3 = Absorbance of hemoglobin released when the test compounds were omitted.

***Anti-inflammatory activity by membrane stability activity***

Anti-inflammatory activity of the extract was evaluated using in-vitro human RBC stability method. Blood sample was collected and mixed with sterilized Alsever solution (consisting of Dextrose – 2%, Sodium citrate – 0.8%, Citric acid – 0.05%, sodium chloride – 0.42% in distilled water). Blood samples were centrifuged at 3000 rpm and packed cells were washed with isotonic saline and a 10% (v/v) suspension in isosaline was made. Different concentrations of the extract were mixed with 1 ml PBS, 2 ml hyposaline and 0.5 ml RBC suspension. Aspirin was used as contrastable drug and instead of hyposaline 2 ml water was used as control. The hemoglobin content in supernatant was calculated using spectrophotometer at 560 nm[20].

$$\text{Percentage Hemolysis} = \frac{OD_{TEST}}{OD_{CONTROL}} X\ 100$$

The percent of membrane protection was calculated as

$$\text{Percentage protection} = 100 - \left[ \frac{OD_{TEST}}{OD_{CONTROL}} \right] X\ 100$$

***Determination of Conjugated Diene***

Blood samples were collected with EDTA as anticoagulant. It was centrifuged and the plasma was aspirated. 0.5 ml of the red cells was incubated with various concentrations of ESME at room temperature for one hour. The blood cells were washed three times with saline (0.9%) to remove any traces of the extract. To 0.5 ml of cells, 7 ml of ice-cold distilled water was added and left overnight at $0^0$ C, the hemolysate was separated by centrifugations in cooling centrifuge at 10,000 rpm for 20 min. The pellet was washed twice with distilled water, followed by centrifugation for 10 min and then suspended to a 10 ml of Tris-Hcl buffer and the resultant solution was then used as a membrane solution. 1 ml of solution was treated with 5 ml of chloroform: methanol (2:1) mixture and was centrifuged at 1000 rpm for 5 minutes. 3 ml of the lower layer was taken and evaporated. The lipid residue was dissolved in 1 ml of cyclohexane in reference cuvette. The conjugated diene was calculated using the molar extinction coefficient 2.5 x $10^4$ $M^{-1}$ $cm^{-1}$ [21].

**Statistical Analysis**

All values are expressed as mean $\pm$ SD or percentage. The efficacy of treatment were assessed using one way ANOVA between groups and multiple comparisons were performed using two tailed Dunnett's test. 5% ($p<0.05$) was taken as the minimum level of statistical significance.

**RESULTS**

**Physico-Chemical Parameters**

The physico-chemical parameters of *Elephantopus scaber* are as shown in Table 1.

**Fluorescence Analysis**

The powder of *Elephantopus scaber* whole plant on treating with various reagents and solvents showed the following results (Table 2).

**Phytochemical Analysis**

The phytochemical analysis of the methanolic extract of *Elephantopus scaber* showed the following results (Table: 3). The extract showed the presence of alkaloids, carbohydrates, glycosides, steroids, essential oil, phenols, tannins, flavonoids, proteins, cholesterol, quinines and phlobatannins while saponins, resins, anthraquinones and coumarins were absent.

**HPTLC Fingerprinting of *Elephantopus Scaber* Methanolic Extract**

The HPTLC profile of ESME showed best separation with Toluene: Ethyl acetate: Formic acid: Methanol (7:5:1:0.5) as solvent system. After scanning and visualizing the plates in absorbance mode at 254 nm, 366 nm and visible light range and after spraying with 10% sulphuric acid reagent showed 10 peaks in 254 nm and 7 peaks in 366 nm. At 254 nm the Rf values of the peaks ranged from 0.08 to 0.98. The first peak with Rf value 0.08 showed the maximum area of 50.66%. The peaks with Rf values 0.73 and 0.81 were having areas of 18.26 and 16.74% respectively. At 366 nm peak with Rf value of 0.08 showed an area of 52.6% followed by the peak with Rf 0.81 had an area of 29.12%. Rest of the peaks were having relatively lesser area (Table: 4, Table: 5, Fig: 2).

**Table 1: Physicochemical parameters of *Elephantopus scaber* whole plant**

| Sl.No | Parameter | Mean value |
|---|---|---|
| 1 | Loss on drying | 12.8 ± 0.1 |
| 2 | Total ash | 5.86 ± 0.34 |
| 3 | Acid insoluble ash | 4.65 ± 0.25 |
| 4 | Water soluble ash | 4.36 ± 0.12 |
| 5 | Water insoluble ash | 2.1 ± 0.21 |
| 6 | Sulphated ash | 15.37 ± 0.42 |
| 7 | Alcohol soluble extractive | 7.92 ± 0.22 |
| 8 | Water soluble extractive | 9.98 ± 0.32 |
| 9 | Crude fiber content | 15.2 ± 0.19 |
| 10 | Foaming index | < 100 |
| 11 | $P^H$ | 5.65 ± 0.26 at 28 ± 0.2$^0$C |

**Table 2: Fluorescence analysis of *Elephantopus scaber* whole plant powder**

| SL NO. | TREATMENT | UV 254 nm | UV 356 nm | UV 302 nm | VISIBLE LIGHT |
|---|---|---|---|---|---|
| 1 | Powder alone | Black | Black | Greenish brown | Greenish brown |
| 2 | Powder + Glacial acetic acid | Black | Black | Olive green | Greenish brown |
| 3 | Powder + Conc $H_2SO_4$ | Black | Black | Henna green | Greenish brown |
| 4 | Powder + Conc $HNO_3$ | Black | Black | Olive green | Reddish brown |
| 5 | Powder + Conc HCl | Black | Black | Olive green | Greenish brown |
| 6 | Powder + 50% $HNO_3$ | Black | Black | Olive green | Greenish brown |
| 7 | Powder + 1N HCl | Black | Black | Olive green | Greenish brown |
| 8 | Powder + 1 N $H_2SO_4$ | Black | Black | Olive green | Greenish brown |
| 9 | Powder + Distilled water | Black | Black | Olive green | Brownish green |
| 10 | Powder + $CHCl_3$ | Black | Black | Greenish black | Brownish green |
| 11 | Powder + Ethyl acetate | Black | Black | Olive green | Greenish brown |
| 12 | Powder + Acetone | Black | Black | Greenish black | Greenish brown |
| 13 | Powder + Benzene | Black | Black | Greenish brown | Greenish brown |
| 14 | Powder + Methanol | Black | Black | Olive green | Greenish brown |
| 15 | Powder + 1 N $AgNO_3$ | Black | Black | Greenish brown | Greenish brown |
| 16 | Powder + $NH_4OH$ | Black | Black | Greenish brown | Greenish brown |
| 17 | Powder + 1 N NaOH | Black | Black | Olive green | Greenish brown |
| 18 | Powder +1N NaOH in Methanol | Black | Black | Greenish black | Greenish brown |
| 19 | Powder + 50% $FeCl_3$ | Black | Black | Olive green | Pale green |
| 20 | Powder + Picric acid | Black | Black | Greenish brown | Greenish brown |
| 21 | Powder + 5% Iodine solution | Black | Black | Olive green | Brown |

**Table 3: Phytochemical analysis of methanolic extract of *Elephantopus scaber***

| | Test | | Reaction |
|---|---|---|---|
| | Test for Alkaloid | | |
| | | Mayer's test | + |
| | | Wagner's test | + |
| | | Dragendroff's test | + |
| | | Hager's test | + |
| | | Marque's test | + |
| | | Froehde's test | + |
| | Test for Carbohydrates | | |
| | | Molisch's test | + |
| | | Bernard's test | + |
| | | Fehling's test | + |
| | Test for Glycosides | | |
| | | Modified Bontrager's test | - |
| | | Legal's test | + |
| | | Keller-killiani test | + |
| | Test for saponins | | |
| | | Froth test | - |
| | | Foam test | - |
| | Test for phytosteroids | | |
| | | Salkowski's test | + |
| | | Lieberman Burchard test | + |
| | Test for fixed oils and fat | | |
| | | Stain test | + |
| | Test for Resins | | |
| | | Acetone-water test | - |
| | Test for Phenols | | |
| | | Ferric chloride test | + |
| | Test for Tannins | | |
| | | Gelatin test | + |
| | | Braemer's test | + |

| | | | |
|---|---|---|---|
| | Test for flavonoids | | |
| | | Alkaline reagent | + |
| | | Lead acetate test | + |
| | | Shinoda test | + |
| | Test for proteins and amino acids | | |
| | | Xanthoproteic test | + |
| | | Ninhydrin test | - |
| | | Biuret's test | + |
| | Test for Anthraquinones | | - |
| | Test for Cholesterol | | + |
| | Test for Coumarins | | - |
| | Test for Quinones | | + |
| | Test for Phlobatannins | | + |

**Table 4: Area and peaks of *Elephantopus scaber* methanolic extract at 254 nm**

| PEAK NO | Rf VALUE | AREA (AU) | % AREA (AU) |
|---|---|---|---|
| 1 | 0.08 | 7511.5 | 50.66 |
| 2 | 0.33 | 398.4 | 2.69 |
| 3 | 0.39 | 301.6 | 2.03 |
| 4 | 0.43 | 279.4 | 1.88 |
| 5 | 0.52 | 390.6 | 2.63 |
| 6 | 0.59 | 348 | 2.35 |
| 7 | 0.73 | 2708.2 | 18.26 |
| 8 | 0.81 | 2480.5 | 16.74 |
| 9 | 0.92 | 354.3 | 2.39 |
| 10 | 0.98 | 55 | 0.37 |

**Table 5: Area and peaks of *Elephantopus scaber* methanolic extract at 366 nm**

| PEAK NO | Rf VALUE | AREA (AU) | % AREA (AU) |
|---|---|---|---|
| 1 | 0.08 | 10157.8 | 52.6 |
| 2 | 0.34 | 647.5 | 3.35 |
| 3 | 0.38 | 177.9 | 0.92 |
| 4 | 0.52 | 826.8 | 4.28 |
| 5 | 0.77 | 1293.5 | 6.7 |
| 6 | 0.81 | 5622.3 | 29.12 |
| 7 | 0.88 | 585.4 | 3.03 |

**Table 6: Percentage protection of *Elephantopus scaber* methanolic extract on human erythrocytes at various concentrations of sodium chloride**

| **Concentration** | **0.9** | **0.8** | **0.7** | **0.6** | **0.5** | **0.4** | **0.3** | **0.2** | **0.1** | **0** |
|---|---|---|---|---|---|---|---|---|---|---|
| **480** | $97.20^{a,3}$ | $97.20^{a,3}$ | $97.20^{a,3}$ | $94.41^{a,3}$ | $93.01^{a,3}$ | $86.71^{a,3}$ | $27.27^{d,4}$ | $12.59^{d,4}$ | $7.69^{d,4}$ | 0 |
| **240** | $98.23^{a,3}$ | $99.12^{a,3}$ | $97.35^{a,3}$ | $96.46^{a,3}$ | $91.15^{a,3}$ | $72.57^{a,3}$ | $13.27^{d,4}$ | $8.85^{d,4}$ | $4.42^{d,4}$ | 0 |
| **120** | $100^{a,3}$ | $99.16^{a,3}$ | $97.48^{a,3}$ | $94.96^{a,3}$ | $94.96^{a,3}$ | $87.39^{a,3}$ | $23.53^{d,1}$ | $16.81^{d,4}$ | $14.29^{d,4}$ | 0 |
| **60** | $98.20^{a,3}$ | $99.10^{a,3}$ | $98.20^{a,3}$ | $95.50^{a,3}$ | $92.79^{a,3}$ | $76.58^{a,3}$ | $9.91^{d,4}$ | $9.91^{d,4}$ | $8.11^{d,4}$ | 0 |
| **30** | $98.18^{a,3}$ | $98.18^{a,3}$ | $91.82^{a,3}$ | $97.27^{a,3}$ | $92.73^{a,3}$ | $74.55^{a,3}$ | $7.27^{d,4}$ | $6.36^{d,4}$ | $3.64^{d,4}$ | 0 |
| **CTRL** | $82.54^{4}$ | $76.98^{4}$ | $76.98^{4}$ | $73.81^{4}$ | $73.02^{4}$ | $42.86^{4}$ | $12.70^{4}$ | $7.94^{4}$ | $7.14^{4}$ | 0 |

Values expressed as percentage protection from hemolysis exhibited by various concentrations of ESME, a- $p < 0.05$, b -$p < 0.01$, c- $p < 0.001$, d- $p > 0.05$ when compared with control and 1- $p < 0.05$, 2- $p < 0.01$, 3- $p < 0.001$ and 4 -$p > 0.05$ when compared to the percentage protection in 0 % NaCl concentration.

**Table 7: Efficacy of ESME on various parameters of membrane stabilization assay, anti-inflammatory effect and anti-oxidant parameters**

| **CONCENTRATION** | **480 mcg** | **240 mcg** | **120 mcg** | **60 mcg** | **30 mcg** | **ASA** | **CONTROL** |
|---|---|---|---|---|---|---|---|
| Mean Corpuscular Fragility($CIH_{50}$) | 2.77 | 3.54 | 2.50 | 2.94 | 3.32 | -- | 4.04 |
| Hypotonic solution induced hemolysis | $86.6 \pm 2.4^{d}$ | $82.6 \pm 3.8^{d}$ | $78.5 \pm 3.2^{d}$ | $67.4 \pm 2.8^{d}$ | $52.9 \pm 4.5^{d}$ | $93.6 \pm 2.8$ | 0 |
| Heat-induced hemolysis | $73.3 \pm 8.4^{d}$ | $53.3 \pm 6.5^{c}$ | $37.6 \pm 4.4^{c}$ | $33.8 \pm 9.4^{c}$ | $16.9 \pm 7.1^{c}$ | $86.3 \pm 7.3$ | -- |
| Hemoglobin release assay | $47.1 \pm 9.9^{d}$ | $39.7 \pm 8.5^{d}$ | $38.2 \pm 5.8^{d}$ | $32.4 \pm 5.0^{d}$ | $14.7 \pm 7.8^{d}$ | $64.7 \pm 4.2$ | -- |
| Anti-inflammatory effect by membrane stabilization (Percentage Protection) | $68.1 \pm 12.5^{d}$ | $67.2 \pm 11.9^{d}$ | $66.4 \pm 8.3^{d}$ | $63.3 \pm 13.9^{d}$ | $56.9 \pm 4.5^{d}$ | $73.7 \pm 15.6$ | 0 |
| Anti-inflammatory effect by membrane stabilization (Percentage Hemolysis) | $31.9 \pm 12.5^{d,3}$ | $32.8 \pm 11.9^{d,3}$ | $33.6 \pm 8.3^{d,3}$ | $36.7 \pm 13.9^{d,3}$ | $43.1 \pm 4.5^{d,3}$ | $26.3 \pm 15.6^{3}$ | $100^{c}$ |
| Conjugated-diene levels | $0.36 \pm 0.07^{2}$ | $0.34 \pm 0.08^{2}$ | $0.26 \pm 0.06^{4}$ | $0.23 \pm 0.04^{4}$ | $0.31 \pm 0.09^{2}$ | $0.39 \pm 0.04^{2}$ | $0.15 \pm 0.04$ |

Values expressed as percentage protection from hemolysis exhibited by various concentrations of ESME, a- $p < 0.05$, b -$p < 0.01$, c- $p < 0.001$, d- $p > 0.05$ when compared with ASA and 1- $p < 0.05$, 2- $p < 0.01$, 3- $p < 0.001$ and 4 -$p > 0.05$ when compared with Control.

**Figure 1: *Elephantopus scaber Linn.* habit**

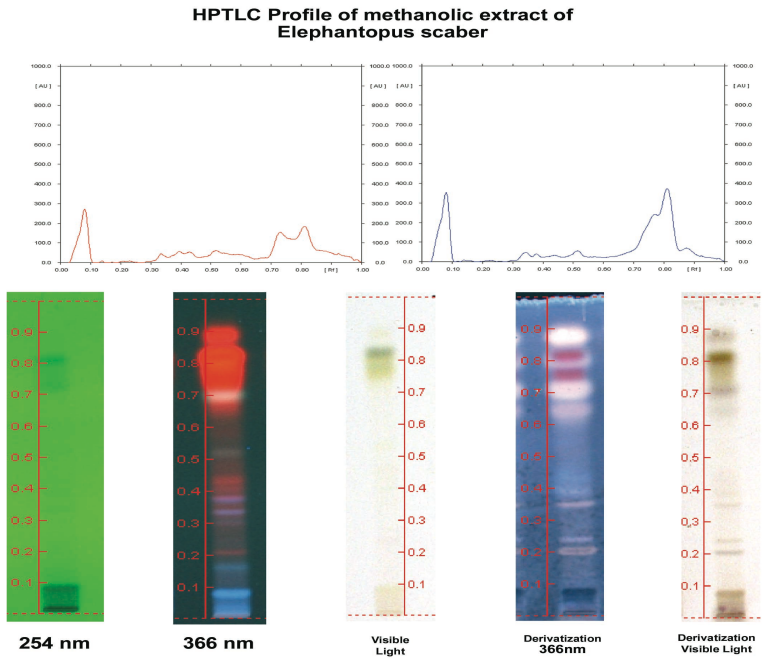


**Figure 2: HPTLC profile of *Elephantopus scaber* methanolic extract**

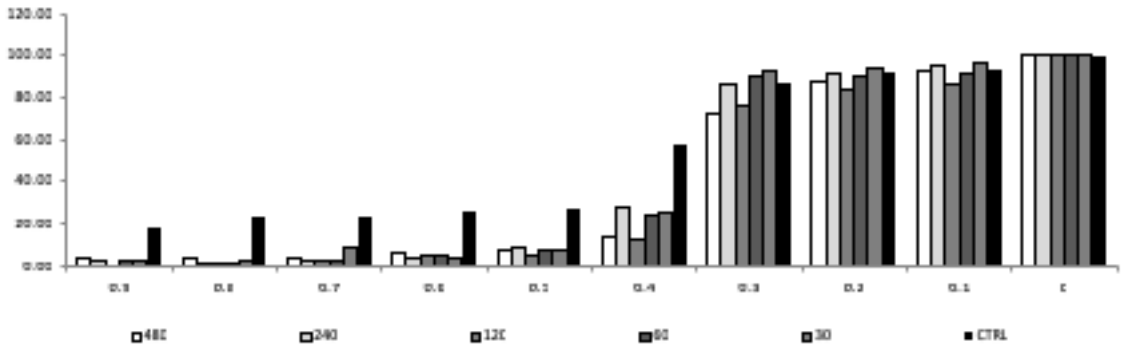

**Figure 3: Percentage hemolysis in erythrocyte suspension treated with various concentrations of ESME in different graded concentrations of sodium chloride**

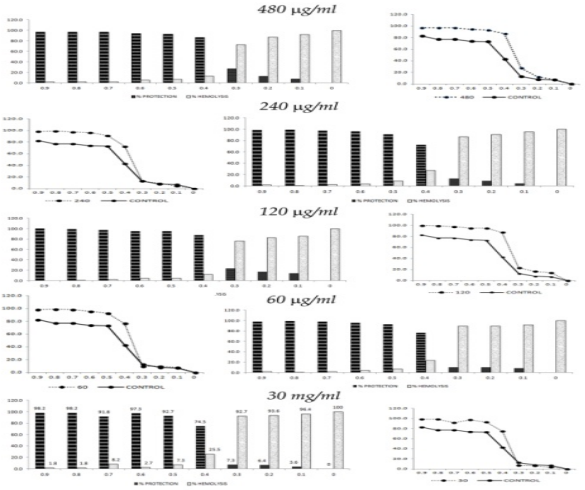


**Figure 4: Percentage protection from hemolyisis at various concentrations of ESME in comparison with the control**

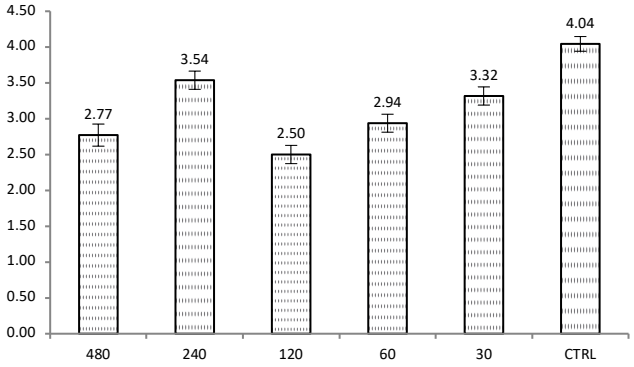


**Figure 5: Mean Corpuscular Fragility ($CIH_{50}$) of ESME in comparison with the control**

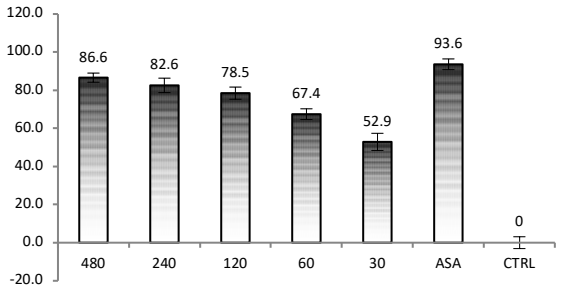


**Figure 6: Percentage inhibition of hemolysis in hypotonic solution exhibited by various doses of ESME**

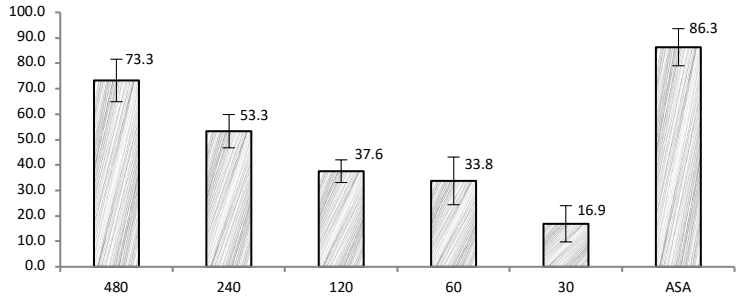


**Figure 7: Percentage inhibition of hemolysis in heat induced hemolysis exhibited by various doses of ESME**

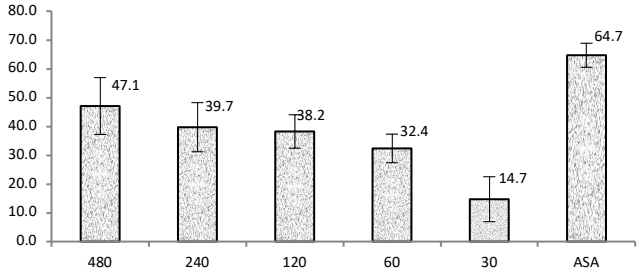


**Figure 8: Percentage membrane stability exhibited by various doses of ESME**

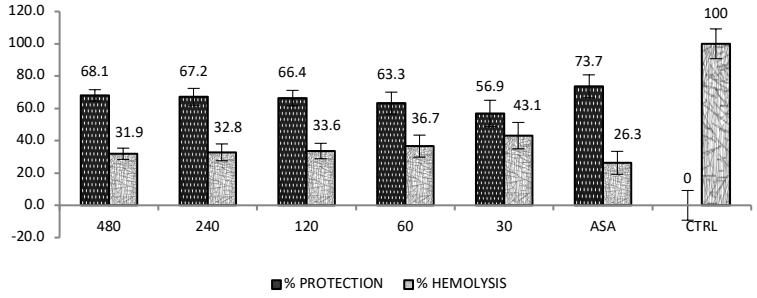


**Figure 9: Percentage inhibition and percentage of hemolysis exhibited by various doses of ESME**

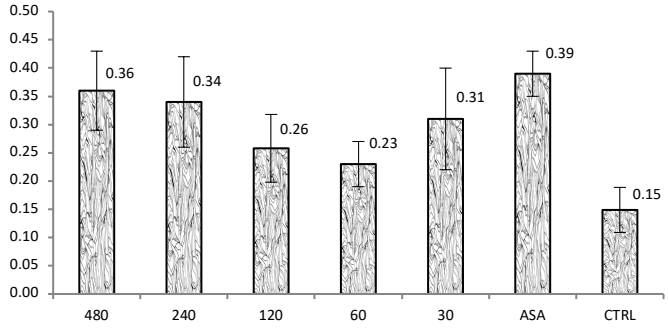


**Figure 10: Level of Conjugated diene in RBC membranes treated with various doses of ESME**

**Erythrocyte Membrane Stabilizing Activity**

***Osmotic Fragility in Human Erythrocytes with ESME***

Osmotic fragility of human erythrocytes treated with different concentrations of *Elephantopus scaber* methanolic extract are shown in Table: 6, Fig: 3 and Fig: 4, the hemolysis in 0% conc. NaCl was taken as 100%. The percentage of protection to erythrocyte fragility contributed by the plant extracts were calculated with respect to this control and expressed as percentage. The study showed a significant decrease in the osmotic fragility with increasing concentrations of NaCl. In comparison with the control group, all concentrations of ESME provided protection up to 0.4% NaCl which was statistically significant at $p<0.001$.

***Mean Corpuscular Fragility***

Mean corpuscular fragility as the concentration of NaCl that showed inhibition of hemolysis by 50% ($CIH_{50}$) was calculated by logistic regression. The $CIH_{50}$ concentration for the various doses of ESME are shown in Table: 7 and Fig: 5. Statistical analysis with one way ANOVA followed by Dunnett's post hoc test showed insignificant results showing that all the concentrations of ESME was almost equivalent to the control but the mean values shows promising decrease in the NaCl concentration that would bring 50% hemolysis.

***Hypotonic Solution Induced Hemolysis***

In hypotonic solution induced hemolysis, ESME showed dose dependent protection comparable to ASA Table: 7 and Fig: 6. ESME at 480 mcg/ml, 240 mcg/ml and 120 mcg/ml showed 86.6, 82.6 and 78.5% protection respectively against hypotonic solution induced hemolysis while the standard drug ASA produced 93.6% protection. On statistical analysis they showed insignificant results ($p>0.05$) showing that they have equivalent activity compared to ASA.

***Heat Induced Hemolysis***

The study showed dose dependent protection to heat induced hemolysis by ESME. 480 mcg/ml ESME showed percentage protection equivalent to that of ASA ($p>0.05$) (Table: 7 and Fig: 7). All other doses of ESME were significantly different than ASA ($p<0.001$).

***Hemoglobin Release Assay***

All the concentrations of ESME showed decrease in hemoglobin release which was dose dependent. ESME at concentrations 480 mcg/ml, 240 mcg/ ml and 120 mcg/ ml showed 73.3, 53.3 and 37.6 % protection respectively while the standard drug ASA showed 86.3% protection. Though they showed statistically insignificant results ESME at 480 mcg/ml showed closeness towards the value of ASA (Table: 7 and Fig: 8).

***Anti-Inflammatory Assay***

The ESME showed significant anti-inflammatory effect in comparison with ASA. All the doses showed comparable anti-inflammatory effect to ASA which was statistically significant (Table: 7 and Fig: 9).

***Conjugated Diene levels***

The levels of conjugated dienes formed as a result of lipid peroxidation were significantly reduced dose dependently by ESME. ESME at concentrations 480, 240 and 120 mcg/ml showed statistical insignificance with the values of control indicating that the conjugated diene levels were significantly reduced by these concentrations of ESME (Table:7 and Fig:10).

**DISCUSSION**

Medicinal plants display a wide variability in pharmacological activities due to its ecological variations. Determination of physico-chemical constants of a drug is an important step in the standardization of the drug and to detect adulteration. A drug gets devoid of the traces of all organic matter when it is reduced to ashes. The constituents of the ash are generally the carbonates, silicates and phosphates of magnesium, sodium, potassium and calcium. Extractive values represent another criterion of standard for medicinal plants. The extractive values suggest the chemical composition of the drug and in this study water soluble extractives were slightly higher than the alcohol soluble extractives. The moisture content, $p^H$, Water insoluble ash, foaming index and crude fiber content along with the fluorescence of the drug powder with various reagents in 254 nm, 356 nm, 302 nm and visible light were also ascertained. All these parameters aids in establishing the standards for identification of the drug[14].

Secondary metabolites present in a drug are its signature of pharmacological and therapeutic activities. In this study the methanolic extract of *Elephantopus scaber* showed the presence of alkaloids, carbohydrates, glycosides, phytosteroids, oils and fats, phenols, tannins, flavonoids, proteins, cholesterol, quinines and phlobatannins. These vast phyto-constituents created attraction towards this drug for various ailments in traditional medicines[22].

The HPTLC studies on the methanolic extract of *Elephantopus scaber* in the mobile phase Toluene: Ethyl acetate: Formic acid: Methanol in the ratio of 7:5:1:0.5 produced the maximum number of spots with good separation. The plate was visualized in 254 nm, 366 nm and visible light and derivatized with 10% sulphuric acid. At 254 nm 10 spots were visualized with Rf values 0.08, 0.33, 0.39, 0.43, 0.52, 0.59, 0.73, 0.81, 0.92 and 0.98. The maximum concentration was obtained for the spot with Rf value of 0.08 (50.66%) followed by Rf values 0.73 and 0.81 having area of 18.26 and 16.74 respectively. In 366 nm 7 spots were visualized with Rf values 0.08, 0.34, 0.38, 0.52, 0.77, 0.81, 0.88. Among these the maximum concentration was for the spot of Rf value 0.08 (52.6%) followed by Rf value of 0.81 having an area of 29.12 %. All other spots showed very less concentrations.

The osmotic fragility test compares the resistance of normal erythrocytes to various osmotic pressures. Erythrocytes suspended in hypotonic solutions take up water, swell, become spheroid and after reaching a critical volume bursts. It is a measure of the tensile strength of the erythrocyte membrane[23]. Osmotic fragility assay is also used to verify the toxicity of chemicals and environmental pollutants on membrane integrity of erythrocytes[24]. Incubating the packed erythrocytes with the extract stabilizes the erythrocyte membranes. In this study all the concentrations of the ESME (viz. 480 mcg/ ml, 240 mcg/ ml, 120 mcg/ ml, 60 mcg/ ml and 30 mcg/ ml) showed statistically significant osmotic resistance against hypotonic saline. Compared to control, ESME produced significant protection against hyposaline up to a concentration of 0.4% NaCl solution. This result shows that ESME stabilizes the erythrocyte membrane against osmotic fragility. The alterations on the erythrocyte membrane integrity could be related to components present in the methanolic extract of *Elephantopus scaber* capable of interacting with membrane components that could modify the erythrocyte membrane ions transport or the osmotic transport balance.

The mean corpuscular fragility calculated from the optical densities also showed reduction in the NaCl concentration correspondingly required for 50% hemolysis in erythrocyte suspension treated with various concentrations of the ESME. The control samples showed a $CIH_{50}$ value of 4.03 while the ESME at 120 mg/ml showed the least value of $CIH_{50}$ of 2.5. But these reductions were statistically insignificant ($p>0.05$).

ESME was studied for its membrane stabilizing property by inhibiting hypotonic solution induced hemolysis. As evident from Table: 7, hypotonic saline produced extensive hemolysis. ESME at a concentrations 480 mcg/ml, 240 mcg /ml and 120 mcg/ml exhibited 86.3, 82.2 and 78.3% protection respectively while the standard drug ASA produced 93.3% protection. The membrane stabilizing activity of ESME at these concentrations were statistically equivalent to that of protection exhibited by ASA as signified by the p value greater than 0.05 ($p>0.05$).

In heat induced hemolysis also ESME at a dose of 480 mcg/ ml showed 73.3% protection compared to the standard drug ASA which produced 86.3% protection. ESME at 480 mcg/ml was found to be statistically equivalent to the standard drug ASA ($p>0.05$). All other concentrations were statistically different than ASA.

In hemoglobin release assay also ESME at a concentration of 480 mcg/ ml showed a significant membrane protection equivalent to that of ASA. The concentration of 480 mcg/ ml produced 47% protection while ASA provided 64.7% protection.

Anti-inflammatory assay also showed a significant effect provided by the ESME at all concentrations. On comparison with ASA which produced a membrane protection of 73.6%, all concentrations of ESME were equivalent to the standard drug. The inflammatory process is accompanied by lysis of lysosomal membrane with subsequent release of a variety of enzymes producing different disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) exert their beneficial effects by either inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes[25]. Erythrocyte membranes exposed to stressors like hypotonic medium and heat results in the lysis of membrane leading to hemolysis and oxidation of hemoglobin[26]. Human erythrocyte membranes are similar to lysosomal membrane. The hemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Injury to red cell membrane render the cell more susceptible to secondary damage through free radical induced lipid peroxidation[27].

During hypotonic insult, excessive accumulation of fluid within the cell results in the rupture of the erythrocyte membrane. Membrane stabilization activity of the drug prevents the rupture of the cell membrane to a greater extent when compared to the control. Membrane stabilization in other cells prevents the serum protein leakage and that of other fluids in to the tissues triggered by the increased permeability caused by inflammatory mediators[28]. ESME stabilized the erythrocyte membrane by preventing the release of lytic enzymes and active mediators of inflammation. During inflammation, the chief event is the disruption of lysosomal membrane and the resultant release of hydrolytic enzymes that trigger the cascade of events leading to the tissue destruction. Erythrocyte membranes are similar in structure to the lysosomal membranes. Membrane stabilization exerted by ESME acts in two way by stabilizing the erythrocyte membrane and thereby preventing the hemolysis induced by circulating toxicants and by the presence of genetically variant forms of aberrant membrane or they induce anti-inflammatory effect by stabilizing the lysosomal membrane by preventing the release of lytic enzymes. This study highlights the erythrocyte membrane stabilization and anti-inflammatory activity of methanolic extract of *Elephantopus scaber* by inhibiting heat induced and hypotonic saline induced erythrocyte membrane lyses. Heat induced hemolysis, hemoglobin release and anti-inflammatory assay by membrane stabilization provides evidence for membrane stabilization by additional mechanism of their anti-inflammatory effect. The present study reveals that methanolic extract of *Elephantopus scaber* provides significant membrane stabilization activity at 480 mcg/ml, 240 mcg/ ml and 120 mcg/ ml.

The inflammatory process initiated by the release of lytic enzymes in turn initiates lipid peroxidation and free radical damages to other cell membranes. Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids[29]. Polyunsaturated fatty acids are more sensitive, and the activated methylene bridge represents a critical target site. The presence of a double bond adjacent to a methylene group makes the methylene C-H bond weaker and therefore the hydrogen is more susceptible to abstraction. This leaves an unpaired electron on the carbon, forming a carbon-centered radical, which is stabilized by a molecular rearrangement of the double bonds to form a conjugated diene which then combines with oxygen to form a peroxyl radical. This peroxyl radical initiates a chain reaction by extracting a hydrogen atom from another polyunsaturated fatty acid so on and so forth[30,31]. Thus conjugated diene formation is the preliminary step in free radical induced membrane damage. In the present study, the methanolic extract of *Elephantopus scaber* significantly reduced the levels of conjugated dienes in isolated erythrocyte membranes. These results suggest the effectiveness of the methanolic fraction of *Elephantopus scaber* as a potent erythrocyte membrane stabilizer, anti-inflammatory agent and its phyto-constituents prevents lipid peroxidation by quenching the conjugated diene formation.

Phytochemicals are proved to be containing potential to stabilize biological membranes when exposed to many osmotic stressors. Presence of phytosterols has been attributed to anti-inflammatory activity in several in-vitro and in-vivo studies by modulating the pro-inflammatory cytokines like C-reactive proteins. Tannins, flavonoids are reported to have profound erythrocyte membrane stabilizing and lysosome membrane stabilizing activity in both in vitro and in vivo models[10]. The probable modes of actions of phytochemicals are attributed to the binding of these agents to the charged surface moieties of the membrane and the subsequent change in the surface charge of the cell membranes. This event is thought to be preventing the physical interaction of the membrane with hemolytic agents by the process of mutual repulsion of like charges. Methanolic extract of *Elephantopus scaber* has tannins, flavonoids and phytosterols as their constituents.

## CONCLUSION

The medicinal property ascribed to drugs mentioned in Ayurvedic texts provides pharmacological evidence with respect to their therapeutic uses. The findings of this study supported the use of *Elephantopus scaber* for the purpose of stabilization of biological membranes and as an anti-inflammatory agent in toxicity related membrane damage and hemolysis. It acts by hampering lipid peroxidation at its early stage itself. Utilization of such herbs with unearthed potentials should be popularized widely among Ayurveda fraternity.

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