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IN-VITRO MEMBRANE STABILIZING ACTIVITY OF DIFFERENT EXTRACTS OF *BAHINIA TOMENTOSA* (L.) LEAVES

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ABSTRACT

Bahinia tomentosa leaves are important medicinal plant with several ethnomedicinal properties. In this study different extracts (aqueous, methanol and ethyl acetate) of leaves of *B. tomentosa* were screened for phytochemical analysis and membrane protection against human erythrocytes (RBC). Phytochemical screening of the leaves of *B. tomentosa* showed the presence of phytosterols, glycosides, phenolic compound, saponins, alkaloids, flavonoids and tannins as major phytocompound present in all the extracts. To confirm the membrane stabilizing/protection activity of *B. tomentosa* was observed by hypotonic saline and heat induced hemolytic method. The aqueous extract of *B. tomentosa* leaf showed very less hemolytic activity in hypotonic saline induced hemolysis when compared to other two extracts. The maximum membrane protection of RBC was observed in the aqueous extract of leaf of *B. tomentosa* at concentration 500µg/ml, which has been compared to standard drug Diclofenac sodium (1mg/ml). In future recommend further *in vitro* and *in vivo* studies to evaluate the clinical efficacy of *B. tomentosa* leaf extract for treated various disease conditions.

KEY WORDS: *Bahinia tomentosa*, hemolytic activity, membrane stabilization, aqueous, methanol, ethyl acetate.

INTRODUCTION

Herbal medicines are great demand in both developed and developing countries as a source of primary health care owing to their attributes having wide biological and medicinal activities, high safety and lesser costs. Herbal molecules are safe and would overcome the resistance produced by the pathogens as they exist in a combined form or in a pooled form (Tapsell *et al*., 2006). Traditional use of medicinal plants is recognized as a way to learn about potential future medicines. Researchers have identified number of compounds used in mainstream medicine, which were derived from "ethno medical" plant sources (Fabricant and Farnsworth, 2001). Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Mahesh and Sathish, 2008).

Hemolysis is the breakage of the red blood cells (RBC's) membrane, causing the release of the hemoglobin and other internal components into the surrounding fluid. Hemolysis is visually detected by showing a pink to red tinge in sample. It can occur from two sources: *In-vivo* hemolysis may be due to pathological conditions, such as autoimmune hemolytic anemia or transfusion reaction. *In-vitro* hemolysis may be due to improper specimen collection, specimen processing, or specimen transport (Lemery, 1998). Erythrocytes are the most abundant cells in human body, possessing desirable physiological and morphological characteristics, are exploited extensively in drug delivery (Hamidi *et al*., 2003). Patients and health care providers need to be provided with lists of commonly used plants that could worsen the hemolytic conditions of patients with hemolytic disorders (Hasan *et al*., 2010).

Inflammation is a normal protective response to tissue injury and it involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane *et al.,* 1995). It is a complex process, which is frequently associated with pain and involves occurrences such as: the increase in vascular permeability, increase of protein denaturation and membrane alterations (Umapathy *et al.,* 2010).

B.tomentosa L. (Fabaceae) is commonly known as "Kanjana" in Tamil and "Phalgu" in Sanskrit. The dried leaves, buds and flowers are prescribed in dysentery. The bruised bark is applied externally to tumors and wounds. A decoction of the root-bark is administered for inflammation of the liver and also used as a vermifuge. An infusion of the bark is also used as an astringent gargle. The plant has been scientifically proved to have antimicrobial activity. Phytochemical studies, *Bauhinia tomentosa* flowers have been reported and contain Rutin, quercetin, Isoquercitrin, and glycosides of quercetin (Gopalakrishnan and Vadivel 2011).

Leaves exhibited cytotoxicity and antioxidant activity. The flowers were found to possess anti hyperglycemic and antilipedemic activity (Mannangatti *et al*., 2010). Pharmacognostic and phytochemical screening of *B. tomentosa* Linn. leaves has been reported (Rhama and Madhavan 2012) and also anticancer activity (Mukundan, 2005).

For discovery and development of novel drugs, scientists are looking forward to the alternative sources and in last few decades, medicinal plants have been extensively studied for their bioactive principles to develop new lead molecules for pharmaceutical use. Toxicity of the active molecule is a key factor during drug designing, and hemolytic activity represents a useful starting point in this regard, it provides the primary information on the nitration between molecules and biological entities at cellular level (Eric Da *et al.*, 2004)

The aim of the present study was to identify phytochemical compounds and membrane stabilizing activity of different extracts of *B. tomentosa* leaves

MATERIALS AND METHODS

Plant materials

The fresh leaves of *B. tomentosa* were collected in November 2014 from the village of Thiruvizhimizhalai, Thiruvarur District, Tamilnadu. The collected leaves were cleaned well and air dried under shade at room temperature, the sample was powdered in an electric grinder, sieved with coarse powder and stored in air tight container.

PREPARETION OF EXTRACTS

Organic solvent extract

25 g of powdered material were soaked in the 250 ml of ethyl acetate and ethanol for 3 days and then filtered through a cotton plug followed by Whatmann No. 1 filter paper. The filtrate was concentrated by boiling water and then crude extracts was stored at 4 – 8^{o}Cin air tight container.

Aqueous extract

To one part of the plant material (25 g) three parts of water was added and then boiled, extract was reduced to one third of original volume and filtrate was evaporated to dryness. Paste form of the extract was obtained and stored at 4 - 8^{o}C.

QUALITATIVE METHOD OF PHYTOCHEMICAL SCREENING (Sofowara, 1993),

The different extracts of leaf *B. tomentosa* were analyzed for alkaloids flavonoids, pholabatannins, glycosides, phenols, saponins, lipids and fat, tannins, anthraquinones, quinines, cardiac glycosides, coumarines acids, steroids, phytosterols, proteins, carbohydrates etc.

Detection of Alkaloids

About 50 mg of solvent free extract was stirred with 3 ml of dilute hydrochloric acid and then filtered thoroughly. The filtrate was tested carefully with various alkaloid reagents as follows.

Mayer's test

To a 1 ml of filtrate, few drops of Mayer's reagent are added by the side of the test tube. The white or creamy precipitate indicated test as positive.

Wagner's test

To a 1 ml of filtrate, few drops of Wagner's reagent are added by the side of the test tube. The color change was observed. A reddish-brown precipitates confirms the test as positive.

Dragendorff's test

To a 1 ml of filtrate, 2 ml of Dragendorff's reagent are added and the result was observed carefully. A prominent yellow precipitate confirms the test as positive.

Detection of Carbohydrate

Fehling's test:

One ml of extract was boiled on water bath with 1 ml each of Fehling solutions A and B. The color change was observed. A red precipitates indicated presence of sugar.

Barfoed's test

To 1 ml of extract, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 minutes. The color change was noted and recorded. A red precipitates indicated presence of sugar.

Benedict's test

To 0.5 ml of extract, 0.5 ml of Benedict's reagent was added. The mixture is heated on a boiling water bath for 2 minutes and the result was observed. A red precipitates indicated presence of sugar.

Detection of Glycosides

Legal's test

Chloroform (3ml) and ammonia solution (10%) was added to 2ml plant extract. Formation of pink color indicated the presence of glycosides.

Detection of Proteins

The extract was dissolved in 10 ml of distilled water and filtered through Whatman No.1 filter paper and the filtrate is subjected to tests for proteins and amino acids.

Million's test

To 2 ml of filtrate, few drops of Millon's reagent are added. The result was observed. A white precipitates indicated presences of proteins.

Biuret test

An aliquot of 2 ml of filtrate was treated with drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. The pink color in ethanol layer indicated presences of proteins.

Detection of amino acid

Ninhydrin test

Two drops of ninhydrin solution (5 mg of ninhydrin in 200 ml of acetone) are added to two ml of aqueous filtrate. The color change was observed. A characteristic purple color indicated the presence of amino acids.

Detection of Phytosterols

Liberdmann-Burchard's test

The extract (5 mg) was dissolved in 2 ml acetic anhydride and one or two drops of concentrated sulphuric acid was added slowly along the sides of the test tube. The formation of blue green color indicated the presence of triterpenoids and Phytosteroids.

Detection of Tannins

Ferric chloride test

The extract (5 mg) was dissolved in 5 ml of distilled water and few drops of neutral 5% ferric chloride solution were added. The formation of blue green color indicated the presence of tannins.

Detection of Phenols

Lead acetate test

The extract (5 mg) was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white precipitates indicated the presence of phenols.

Detection of flavonoids

An aqueous solution of the extract was treated with ammonium hydroxide solution. The yellow fluorescence indicated the presence of flavonoids.

Detection of coumarins

10% NaOH (1ml) was added to 1 ml of the plant extracts formation of yellow color indicated presence of coumarines.

Detection of Saponins

Distilled water 2ml was added of each plant extracts and shaken in a graduated cylinder for 15 mins lengthwise. Formation of 1cm foam indicates the presence of saponins.

Detection of Quinone

Concentrated sulphuric acid (1ml) was added to 1ml of each of the plant extract. Formation of red color indicated the presence of Quinones.

Detection of Cardiac glycosides

Glacial acetic acid (2ml) and few drops of 5% ferric chloride were added to 0.5% of the extract. This was under layered with 1ml of concentrated sulphuric acid. Formation of brown ring at the interface indicated presence of cardiac glycosides.

Detection of Terpenoid

Chloroform (2ml) and concentrated sulphuric acid was added carefully to 0.5 ml of extract. Formation of red brown color at the interface indicated the presence of terpenoid.

Detection of Phlobatannins

Few drops of 10% ammonia solution were added to 0.5 ml of root extract. Appearance of pink color precipitates indicated the presence of phlobatannins.

Detection of Anthraquinones

Few drops of 2% HCL were added to 0.5 ml of root extract. Appearance of red color precipitate indicated presence of anthraquinones.

Detection of steroids and Phytosteroids

To 0.5 ml of the plant extract equal volume of chloroform was added and subjected with few drops of concentrated sulphuric acid. Appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicated the presence of Phytosteroids.

***In-vitro* hemolytic assay**

Hemolytic assay was carried out by adopting the method of Bulmus *et al*., (2003) Freshly collected human red blood cells were taken and allow clotting for 30 minutes. The serum was removed by 2500 rpm for 15 minutes, and then pellet was washed three times by 150 Mm NaCl (2500 rpm for 10 minutes). 0.5% RBC cells were suspended in 100 mM sodium phosphate buffer. Four different concentrations (250µg, 500 µg, 750 µg and 1000µg) of different extracts were mixed with200 µL (or) 0.2 ml of RBC solutions and the final reaction mixture volume was made up to 1 ml by adding sodium phosphate buffer. The reaction mixture was then placed in water bath for 1 hour at 37 °C. After the incubation time the reaction mixture was centrifuged again at 1500 rpm for 10 minutes. The supernatant was collected and the optical density was measured at 541 nm keeping sodium phosphate buffer as blank. Deionized water was used as a positive control. The experiment was done in triplicate and mean ± S.D. was calculated.

$$\text{Percentage hemolysis} = \frac{\text{(Absorbance of sample- Absorbance of blank)}}{\text{Absorbance of positive control}} \times 100$$

Heat induced hemolysis

The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis (Chippada *et al*., 2011).

The assay mixture contains 1 ml phosphate buffer [pH 7.5, 0.15mM], 2ml hypo saline [0.36%], 0.5 ml HRBC suspension [10% v/v] with 0.5 ml of plant extracts and standard drug Diclofenac sodium of various concentration (100, 200, 300, 400, 500 µg/ml) and control (distilled water instead of hypo saline to produce 100% hemolysis) were incubated at 37^{o}C for 30 min and centrifuged respectively.

The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage of hemolysis of HRBC membrane can be calculated as follows:

% Hemolysis = (optical density of test sample / optical density of control) × 100

% Protection=100-[(Optical density of test sample / Optical density of control) ×100]

RESULTS AND DISCUSSION

The three different extracts (aqueous, ethyl acetate, & methanol) of phytochemical screening of the *B. tomentosa* (leaf) revealed the presence of alkaloids, carbohydrate, proteins, phenol, Quinone, cardiac glycosides, terpenoid, steroids, Phytosteroids in all the extracts and while amino acids, saponins and coumarins was present only in aqueous extract.

Table1: Phytochemical constituents of aqueous, ethyl acetate and methanol extract of *Bahinia tomentosa*(L.) Leaf

Phytochemical test's	Different extracts of *B. tomentosa*		
	Aqueous	Ethyl acetate	Methanol
Alkaloids	+++	+++	+++
Carbohydrate	++	++	++
Glycosides	_	_	_
Proteins	++	++	++
Amino Acid	++	_	_
Tannins	_	_	_
Phenols	+++	+++	+++
Flavonoids	_	+	+
Coumarins	+	_	+
Saponins	++	_	_
Quinone	+++	+++	++
Cardiac Glycosides	+++	+++	+++
Terpenoid	++	++	++
Phlobatanins	_	_	_
Anthraquinone	_	+	_
Steroids	+	+	+
Phytosteroids	+	+	++

+++ = (Highly present); ++ = (Moderate); + = (mild); - = absent

Medicinal plants will continue to provide a source for generating novel drug compounds. Plants may become the base for the development of a new medicine or they may be used as phytomedicine for the treatment of disease (Iwu *et al*., 1999). It are the rich source of medicinally important compounds and since ancient time, plants and plant derived products are used as medicine in traditional and folk medicinal system. Initially the herbal drugs were used in the form of dried powder, gums, extracts or formulations of more than one plant

products. Advanced scientific techniques brought a revaluation in herbal medicine industry and focus is concentrate on bioactive molecule (kumar *et al.*, 2011).

The phytochemical screening of methanolic extract of *B. tomentosa* (Linn.) showed the presence of carbohydrate, flavonoids, glycosides, phytosterols, and saponins were identified (Kishore Kumar *et al*., 2010). The author reported the presence of carbohydrates, glycosides, flavonoids, fixed oils and phenolic compounds and tannins in the extracts. The relative potencies of extracts are in the order of aqueous > ethyl acetate > methanol extract of *B. tomentosa* leaf (Varshatiwari *et al.*, 2011).

***In- vitro* hemolytic activity**

Hemolytic activity of different extracts (aqueous, methanol and ethyl acetate) of *B .tomentosa* leaf was screened against normal human erythrocytes. All the extracts exhibited low to high hemolytic effect toward human erythrocytes. Hemolytic activity of the plant is expressed in %. The aqueous extract possess minimum hemolytic activity (25±0.88%) at the concentration 500 µg/ml, whereas ethyl acetate extract possess highest hemolytic activity at the concentration 500µg compared to methanolic extract of leaf. Hemolysis was found to be increasing with increased concentration of plant extracts (Table-2 and Figure-1).

Table 2: *In -vitro* hemolytic activity of different extracts of *B.tomentosa* leaf

Concentration of samples (µg)	Different extracts of plant /% of hemolysis		
	Aqueous	Ethyl acetate	Methanol
100	6.13±0.28	13±3.53	12.9±0.24
200	12.9±0.24	28±1.41	25±3.40
300	19.8±1.76	31.7±0.77	31.3±9.49
400	20.1±1.80	44±2.29	40±1.4
500	25±0.88	58.3±5.12	44.3±1.76
CONTROL	73.2±1.93		

The Data was represented as Mean ± SD. Experiment was done in triplicates; n=3

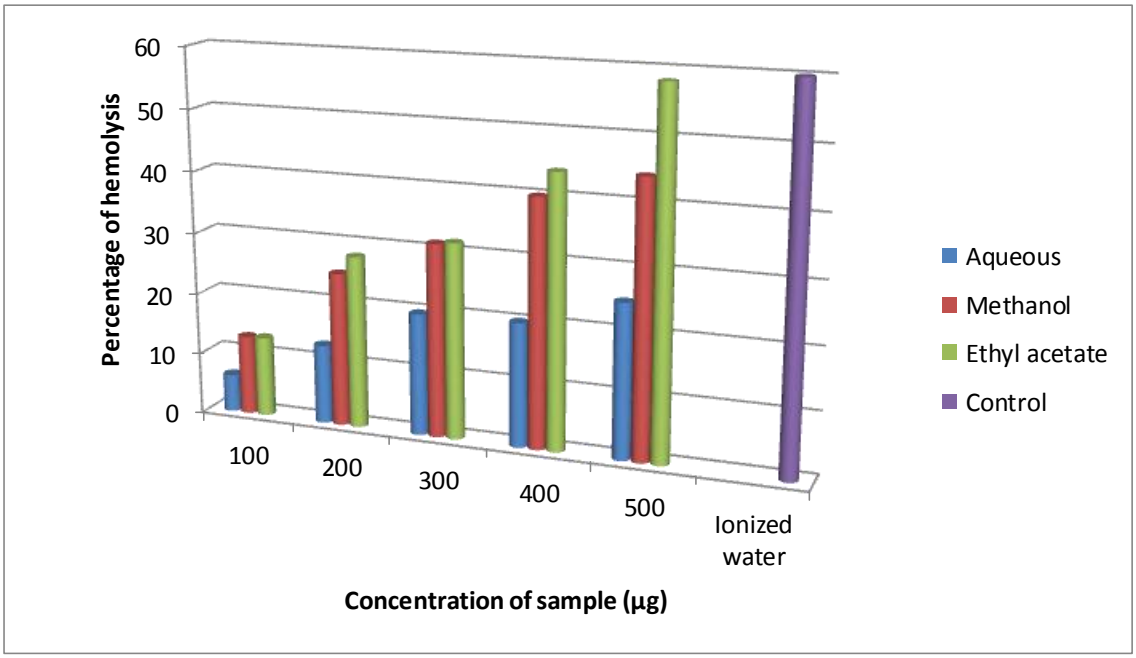

Figure 1: The hemolytic active aqueous, methanol and ethyl acetate extracts of leaf of *B.tomentosa*

The erythrocyte model has been widely used as it presents a direct indication of toxicity of injectable formulations as well as general indication of membrane toxicity. Another advantage of human erythrocytes model is the very good and easily available. Red Blood Cells are easy to isolate from the blood because, its membrane has similarities with other cell membrane (Robertis *et al*., 1995).

Ralph *et al*., (2009) through testing for hemolytic activity rated the degree of *in vitro* toxicity according to the observed mortality rate: 0 to 9% = non-toxic, 10 to 49 % = slightly toxic, 50 to 89 % =toxic; 90 to 100 % highly toxic. Therefore, for new studies to be conducted, the use of non-toxic concentrations (LC 0-9) is suggested. Erythrocytes have been used as a model system by a number of workers for the study of interaction of drugs with membranes. Hemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer. This hemolysis relates to various concentration and potency of extract. Furthermore the hemolytic activity of plant extracts is related to their phytochemical composition (Mohammed *et al.*, 2014). The aqueous extract of *B. tomentosa* not affect stability of RBC membrane, these data suggested the non-toxic effect of the plant extract thus making it suitable for the preparation of drug involved in the treatment of various diseases.

Different solvent extracts of *Syzigum Cuminii* seeds and *Crateva nurvula* bark were reported to possess no hemolytic effect on sheep erythrocytes (Mathur *et al*., 2011). *Achranthes aspera* was reported to possess very low hemolytic activity towards human erythrocytes (Priya *et al*., 2010). Aqueous extract of *Lantana camera* and its various solvent fractions were reported to possess moderate hemolytic activity towards human erythrocytes (Kalita *et al*., 2011). Oliveiral *et al*., (2009) screened the hemolytic activity of seventy one extracts from twelve plants. Only three extracts prepared from *Elegia nuda* showed significant hemolytic activity. Mukherjee and Rajasekaran (2010) reported the high hemolytic activity of the different solvent extracts of *Allium stracheyi Baker* towards the rabbit red blood cells. (Thirunavukarasu *et al*., 2011)

***In -vitro* membrane stabilizing activity**

Stabilization of RBCs membrane was studied for anti-inflammatory activity of different extracts of *B. tomentosa* (leaf) by heat induced hemolysis. All the extracts were effectively inhibiting RBC membrane lysis. The results are showed in table 3. The maximum RBC membrane lysis (61±2.94 %) and minimum protection was recorded in methanolic extract followed by ethyl acetate extract (48±3.63 %) of leaf of *B. tomentosa* at the concentration 500µg. The minimum hemolysis and maximum protection was observed in the aqueous extract (28.2±7.98 %) of *B. tomentosa* at the concentration 500 µg / ml. This has compared with standard anti-inflammatory drug Diclofenac sodium at 1 mg / ml. These results provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. This effect may be possibly inhibiting the release of lysosomal content of neutrophils at the site of inflammation.

Table 3: *In vitro* membrane stabilizing activity of different extract of *B. tomentosa* leaves

Various concentration of plant extracts	Heat induced hemolysis					
	% of hemolysis			% of membrane stabilization		
	Aqueous	Ethyl acetate	Methanol	Aqueous	Ethyl acetate	Methanol
100	7±7.53	16.8±0.82	22.2±5.16	92±1.41	83.2±5.61	78.2±1.44
200	9.56±4.87	20.03±3.25	30.9±6.25	90.3±1.84	80.7±1.44	69.2±5.80
300	18.2±1.93	27.5±1.41	42.3±5.66	81.1±4.44	72.3±5.12	58.2±0.77
400	23.1±5.12	37.5±3.53	55.1±3.5	76.3±2.83	62.1±3	44.4±1.59
500	28.2±7.98	48±3.63	61±2.94	71.4±1.61	52±7.33	39±53
CONTROL	18.3±2.44			80.4±2.46		

The Data was represented as Mean ± SD. Experiment was done in triplicates; n=3

The ethanolic extract of *Cassia occidentals* significantly prevented the degranulation at 250 mg/kg dose. The highest dose of plant extract (500 mg/kg) showed lesser protection from degranulation than the lower dose. This could be due to the fact that at the higher dose *Cassia occidental* may even be cytotoxicity to the mast cells (Sreejith *et al*., 2010).

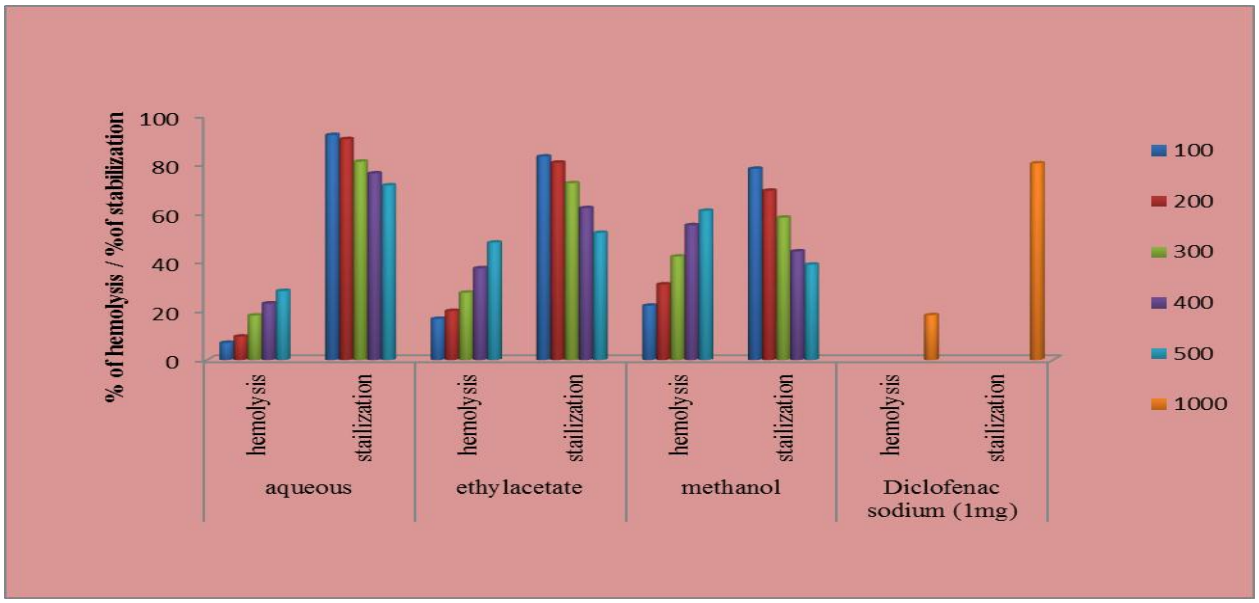

Figure 2: *In vitro* membrane stabilizing activity aqueous, methanol & ethyl acetate extract of leaf of *B. tomentosa*

Inflammation is the pathology to make people suffer. Numerous anti-inflammatory drugs are available in the market but all remain with some side effects more or less. If dietary agents can provide some side effects, it might be very beneficial in the time of need. Membrane-stabilizing experiment can serve as an indicator to screen out the anti-inflammatory agents. Compounds with membrane stabilizing properties can prevent the release of phospholipases that initiate the formation of inflammatory mediators (Shinde *et al*., 1999).

The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorders. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The anti-inflammatory constituent act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal

membrane (Govindappa *et al*., 2011). Plant possess a lot of secondary metabolite such as phenolics and flavonoids (Tenore *et al*., 2012), which may be considered as causative agents to show substantial anti-inflammatory activity (Jean-Gilles *et al*., 2012).

The mode of the extracts and standard anti-inflammatory drugs could be connected with binding to the erythrocyte membrane with subsequent alteration of the surface charges of the cells (Hess *et al*., 1972). This might have prevented physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the hemolysis of red blood cells. It has been reported that certain saponins and flavonoids exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins and saponins possess ability to bind cations, thereby stabilizing erythrocyte membrane and other biological macromolecules (Veena Sharma *et al*., 2013).

CONCLUSION

Since ancient time, plant products have been utilized for the treatment of various health problems. Plants are one of the most important sources of drug discovery and development. Plants used in this study have been excessively used in traditional medicine to cure a variety of disease. Medicinal plant contains biological active compound that possess May ability to facilitate the stability of RBC membranes when exposed to hypotonic saline and heat induced hemolysis. *B. tomentosa* plant was found to possess good membrane stabilizing property which is one of the preliminary steps involved in the screening of *in vitro* anti-inflammatory property. Further studies can be done on the isolation of bioactive compound form the leaves extracts of *B. tomentosa*, to elucidate the possible mechanism(s) of action of plant.

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