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Anti-inflammatory activity of aqueous extract of *Mimusops elengi* in human whole blood and peripheral blood mononuclear cells

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Abstract

The aim of the present study is to present a simple, fast and reliable method of anti-inflammatory activity of medicinal plants especially *Mimusops elengi* in human whole blood and peripheral blood mononuclear cells using flow cytometry. To achieve this objective, aqueous extract isolated from the different parts of the plants especially leaf, stem and root and checked its anti-inflammatory activity in human whole blood and peripheral blood mononuclear cells (PBMC). Human whole blood and PBMC were pre-incubated with different concentration of aqueous extract of *Mimusops elengi* to determine the blood counts, estimation of monocyte marker CD14 and also estimate the Th1(TNF alpha) cytokine profile of *Mimusops elengi* from peripheral blood mononuclear cells using lipopolysaccharide. *Mimusops elengi* showed dose-dependently and significantly reduced a range of blood counts especially monocytes and its surface marker CD14 and also suppressed the pro-inflammatory cytokine i.e. tumor necrosis factor in peripheral blood mononuclear cells. The presented data indicate that *Mimusops elengi* has broad anti-inflammatory effects in human whole blood and PBMC.

Keywords: *Mimusops elengi*, anti-inflammatory, lipopolysaccharide, monocyte

Contents

1. Introduction 638
2. Materials and Methods 639
3. Results and Discussion 640
4. References 644

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1. Introduction

Medicinal plants are generally considered as chemical factories which are able to synthesize number of chemical compounds such as alkaloids, glycosides, saponins, lactones and oils which act on human body in different ways. The main properties of the medicinal plants related to biology are due to presence of primary and secondary

metabolites in different parts of the plant. These metabolites are used in the maintenance of health in humans and other animals [1, 2, 3, 4]. The promising results achieved from researches on various activity of the medicinal plants especially anti-cancer, anti-inflammatory, anti-arthritic, immunomodulatory etc. Due to these activities, scientists focused and search for plant derived drugs for treatment of different diseases. Therefore, there has been intense growing interest among scientists to isolate and study the pharmacological properties of the primary and secondary metabolites. Herbal medicines are employed to cure a wide variety of health related problems ranging from treatment of common colds to treatment of cancer.

Mimusops elengi Linn. (*M. elengi,* common name in different languages such as Bakul (Hindi and Bengali), Spanish cherry, Bullet wood tree (English), Bakula (Sanskrit) etc) is an evergreen ornamental tree of the family *Sapotaceae* and showed as one of the best medicinal plants and is used to cure a variety of human diseases [5, 6, 7, 8, 9, 10, 11]. Due to the complexity of the immune system, *Mimusops elengi* , medicinal plant showed number of immunopharmacological activities such as anti-viral, anti-bacterial, anti-fungal, anti-helmintic, anti-carcinogenic, anti-hyperlipidemic, antihyperglycemic, diuretic effects, free radical scavenging, antioxidant, cognitive enhancing, cytotoxic activities *etc*. due to presence of a variety of active phytochemicals.

The aim of the present study was to assess the effect of *Mimusops elengi*, medicinal plant in human whole blood. The human cell preparations, PBMCs, diluted whole blood and cell culture supernatant in human whole blood that can be used to predict anti-inflammatory effects in human whole blood. We estimate the cytokine release of tumor necrosis factor alpha (TNF) which is produced by monocytes, macrophages, and lymphocytes and used as marker for inflammatory processes in all cell preparations tested. In addition, the release of the cytokine IL-4 was determined in PBMCs.

2. Materials and Methods

Sample collection

The leaf, stem and roots of plant *Mimusops elengi* were collected from Udyan, Vidya Pratishthan School of Biotechnology (VSBT), Baramati (Pune), Maharashtra. The whole plant i.e. leaf, stem and roots of *Mimusops elengi* were washed thoroughly with distilled water and then sun dried. After that sample was macerated or grounded finely to powder form, this was then used for aqueous extract preparation and was taken for the immunological studies. The aqueous extraction was done in pre-chilled mortar and pestle in which leaf, stem and root of *Mimusops elengi* was grinded in phosphate buffered saline and the extract was centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant thus, obtained was used within four hours for various immunological assays.

High performance thin layer chromatography (HPTLC) fingerprinting

The aqueous extract was used for HPTLC studies to detect and quantify the primary as well as secondary metabolites in the extract. All the solvents used were of HPTLC grade from Qualigens and HPTLC plate is of silica gel GF254 (Merck) 10 X 10 cm and solvents used in mobile phase i.e. Toluene: Ethyl Acetate: Formic acid (5:4:1) and detect its wavelength at 366 nm. A stock solution of *Mimusops elengi* (1 mg/ml) was prepared by dissolving 5 g of accurately weighed *Mimusops elengi* and diluting it to 50 ml with phosphate buffered saline or with different solvents. Further dilutions were made with aqueous extract to obtain working standards 100, 30, 10 and 0.5 mg/ml. These phytochemical investigations of the aqueous extract of leaf, stem and root of *Mimusops elengi* showed the presence of glycosides, terpenoids, and flavonoids. The RF value of glycosides, terpenoids and flavnoids are < 1.8 µg, 0.92 µg and 0.34 µg.

Human blood samples

Venous blood samples were collected from 10 adults (age 20 – 30 years) directly into Vacutainer tubes containing sodium heparin anticoagulant. Donors were screened to guarantee that none had ingested drugs such as aspirin or other anti-inflammatory agents for a period of at least 10 days prior to donation. Blood samples were stored at room temperature (22°C) in the dark. Informed consent letter was obtained from all subjects or their guardians prior to blood collection only if the participants are healthy and does not show any signs or symptoms of asthma exacerbation or respiratory infection or any other illness.

Flow Cytometric analysis using whole blood and peripheral blood mononuclear cells (PBMC)

In first set of experiment, whole blood (100 µl) was pipetted directly into a 12 x 75 mm fluorescence-activated cell sorting tube containing 1000 µl of phosphate buffered saline containing different concentrations of aqueous extract i.e. 0.5, 1, 10 and 30 mg/ml and then incubated at carbon dioxide incubator (37 °C, 5 % CO_2) for 2 h. After incubation, RBCs were lysed using 2 ml of FACS lysing solution and incubate for 30 minutes. After centrifugation at 2000 rpm for 10 minutes, the supernatant was aspirated and washed two times with phosphate buffered saline.

After centrifugation, pellet dissolved in PBS and studied the lymphocytes, monocytes and granulocytes count using flow cytometry [12, 13].

In second set of experiment, the numbers of leukocytes in peripheral blood samples were analyzed by the flow cytometer (Facs Calibur) using 3 µl of mouse anti- human CD14 FITC lymphoid marker monoclonal antibodies to the 100 µl of human peripheral blood sample, incubated for 30 minutes at room temperature, and then lysed with 2 ml of FACS lysing solution by centrifuging for 5 minutes at 2000 rpm. After centrifuging the supernatant was removed and then washed by centrifuging for 5 minutes at 2000 rpm with 2 ml of PBS and then samples were analyzed for 10000 cells on the flow cytometer [12, 13].

TNFr Release from PBMCs

PBMCs were extracted from heparinized blood of healthy donors by means of density gradient centrifugation using Ficoll reagent (density 1.077 g/l). Cells were harvested and washed three times and then resuspended in RPMI 1640 medium supplemented with 10% FCS, HEPES 25 mM, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol (50 µM). 100 µl of PBMCs were cultured in 96-well tissue-culture plates (10^6 cells/ml for TNF α) at 37°C in a humidified atmosphere with 5% CO_2 and 37 °C for 24 h. Aqueous extract of different concentrations were diluted in RPMI 1640 medium, and added in triplicates to wells at a range of concentrations (0.5 – 100 mg/ml, 50 µl). Viability as determined by trypan blue exclusion was uniformly < 95%. Cells were stimulated with 50 µl/well of stimulation agent, i.e. release of TNFα, with LPS (1 µg/ml) [14, 15]. Each drug was preincubated for 30 min before stimulation. After incubation cells were pelleted, and TNFα in the supernatants (fresh or frozen at 80°C) were measured by ELISA according to the manufacturer's instructions.

Cytotoxicity assay

Erythrocytes present were lysed with red cell lysis buffer (0.5 M ammonium chloride, 0.1 mM disodium ethylene diamine tetraacetic acid and 10 mM potassium bicarbonate, pH 7.2) for 5 min. Lymphocytes obtained were then washed thrice with PBS. Cell number was counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95 %.

To evaluate the effect of variable doses of aqueous extract of *Mimusops elengi* in PBMC (2×10^6 cell/ml) was pipetted into 96 well plates (200 µl/well) cultured at 37 °C for 48 h, the plates were centrifuged at 1400 x g, 5 min and the supernatant was discarded and add fresh 100 µl fresh complete media in 96 well plate and again incubate for 24 h and then add 20 µl of MTT solution (5 mg/ml) were added to each well and incubated for 4 h. The plates were centrifuged (1400 × *g*, 5 min) and the untransformed MTT was removed carefully by pipetting. In each well, add 100 µl of a DMSO working solution was added and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min [16].

Preparation of erythrocytes suspension

Five ml of blood was collected from a healthy individual in a tube containing EDTA. The blood was centrifuged at 2000 rpm for ten minutes in a laboratory centrifuge. Plasma (supernatant) was discarded and the pellet was washed three times with phosphate buffer saline solution by centrifugation at 2000 rpm for 10 min. The cells were resuspended in phosphate buffered saline.

***In vitro* hemolytic activity**

For this experiment, 1% red-blood cell suspension in pH 7.4 phosphate buffer was used throughout in the preparation of experimental (test) and control tubes. Aqueous extracts prepared as described above were initially dissolved in phosphate buffered saline and transferred into test tubes containing a fixed volume of red-blood cell suspension. The extracts were tested or screened at different concentrations of 50 µl of 0.5, 1, 10 and 30 mg/ml. Negative controls (blanks) contained 1 % distilled water in red-blood cell suspension. The result for each test concentration of extract was interpreted qualitatively *in vitro* hemolytic action either being present or absent. Overall, the result was a semi-quantitative evaluation of hemolytic activity for each extract in accordance with international guidelines for the evaluation of this activity in medicinal plant materials [17].

Statistical Analysis

Data are reported as means ± standard deviation (SD).

3. Results and Discussion

Results

Effect of *Mimusops elengi* on human whole blood counts and estimation of monocyte marker CD14 using flow cytometry

The effect of the aqueous extract (leaf, stem and root) of *Mimusops elengi* on lymphocytes, monocytes and granulocytes count as shown in **Fig.1**. In *Mimusops elengi*, there is a dose dependent decrease in the monocytes

count and increased the lymphocytes count as compared to control. To confirm the decline of monocytes using marker CD14 in peripheral blood mononuclear cells. The results showed that the aqueous extract of leaf, stem and root at higher doses showed rapidly decline in the number of monocytes as compared to control (**Fig.2**).

Estimation of Th1 and Th2 cytokine profile

The effect of the aqueous extract (leaf, stem and root) of *Mimusops elengi* on Th1 (TNF alpha) in PBMC as shown in **Figure 3**. In *Mimusops elengi*, there is a dose dependent decrease in the TNF alpha as compared to control. The results showed that the aqueous extract of leaf, stem and root at higher doses showed rapidly decline in the TNF alpha as compared to control. These results showed that the aqueous extract showed anti-inflammatory activity.

Cytotoxicity assay

The effect of the aqueous extract (leaf, stem and root) of *Mimusops elengi* on peripheral blood mononuclear cells (PBMC) as shown in **Fig. 4**. In *Mimusops elengi*, the aqueous extract of leaf, stem and root showed cytotoxicity at higher doses as compared to control.

Hemolytic activity

The hemolytic activity of *Mimusops elengi* as shown in **Fig. 5.** In *Mimusops elengi*, hemolytic activity is observed at higher doses as compared to control. Distilled water and phosphate buffered used as positive and negative control. These results showed that the aqueous extract showed less hemolytic activity in human erythrocytes as compared to distilled water.

Discussion

In the present study, the plant extract of leaf, stem and root of *Mimusops elengi* are very effective against bacteria, fungi, viruses, harmful insects and vectors/pest and showed its antimicrobial, antiviral, antioxidant, hepatoprotective and cytotoxic activities and these studies are well accepted because of the scientific literature supporting these effects. Instead of several tests on animal model studies, *Mimusops elengi* is not yet widely used against human. On the basis of this concept, our group focused on the aqueous extract of leaf, stem and root of *Mimusops elengi* in human whole blood and peripheral blood mononuclear cells to determine its anti-inflammatory activity. Now a day, people are getting more aware towards natural sources of drugs because of the non-steroidal anti-inflammatory drugs (NSAIDS) present in the market induce several side effects like gastric ulcer [18] and hepatotoxicity [19].

To explore the wound healing of plants is an ancient concept for centuries people have been trying to reduce the side effects and develop effective anti-inflammatory agents using different plant extracts and formulations [20]. To determine the anti-inflammatory activity in human whole blood using flow cytometry which is a qualitative and quantitative assessment of biological and physical characteristics of prokaryotic and eukaryotic cells. Today in preclinical and clinical immunology, flow cytometry is routinely used in clinical laboratories for the assessment of the immune status of healthy animals as well as human [21]. However, the application of this method is so important in veterinary as well as clinical immunology and also in the fields such as hematology, transplant medicine, oncology, genetics, toxicology and transfusion medicine [21]. During flow cytometry the assessment of immunophenotype as well as the application of specific monoclonal antibodies for differentiating leukocyte antigens – markers (membrane and/or intracellular glycoproteines) provides the data for relative proportions of certain cell subsets from the entire leukocyte population [22]. Due to the complexity of the human immune system, the effect of the variable doses of aqueous extract of *Mimusops elengi* on human whole blood counts and it was confirmed in FACScan experiments, demonstrating that the aqueous extract significantly decreased the absolute counts of monocytes and increased the lymphocyte count at higher doses as compared to control and also confirmed the results through the human monocyte CD14 marker. CD14 (glycophosphatidylinositol-linked protein), which is part of the LPS receptor complex.

CD14 binds lipopolysaccharide and as such, acts as a pattern recognition receptor. CD14 surface marker which is involved in the endotoxin mediated release of tumor necrosis factor-alpha by monocytic cells [23, 24]. CD14 is present on most monocytic and macrophage likes cell types: Kupffer cells, macrophages, monocytes, pleural phagocytic cells and dendritic reticular cells. On the other hand, granulocytes, however, presented showed non-significant fluctuations. On the other hand, the aqueous extract of leaf, stem and root of *Mimusops elengi* was screened against cytoxicity assay and normal human erythrocytes. These studies suggest that the aqueous extract of whole plant of *Mimusops elengi* showed cytotoxicity at higher doses as compared to control and exhibited very low haemolytic effect toward human erythrocytes as compared to control. However, these aqueous extracts showed dose dependant increase in hemolytic activity.Inspite of the fact, number of immunological investigations on the cytokine profile of peripheral blood mononuclear cells with variable doses of aqueous extract of *Mimusops elengi* using concanavalin A and lipopolysaccharide. These studies suggest that the aqueous extract showed anti-inflammatory activity due to the inhibition of tumor necrosis factor alpha. Mononuclear cells are the first line of defense against various microbial pathogens due to phagocytic, cytotoxic and intracellular killing capacities [25]. Macrophage activation by lipopolysaccharide (LPS) results in the release of proinflammatory cytokines, tumor necrosis factor-α

[26]. In this study, we demonstrated that the aqueous extract suppressed tumor necrosis factor-α secretion at higher doses, both of which are crucial in the inflammatory and healing mechanism [27]. As seen in this experiment, the ability of this fraction to suppress inflammation when it is applied after the onset of inflammation is likely to be due to the genuine anti-inflammatory activity. In conclusion, these results provide evidence for the anti-inflammatory activities of aqueous extract of whole plant of *Mimusops elengi*. Further experimental and clinical trial studies are now warranted to investigate the benefit of aqueous extract treatment for cancer patients.

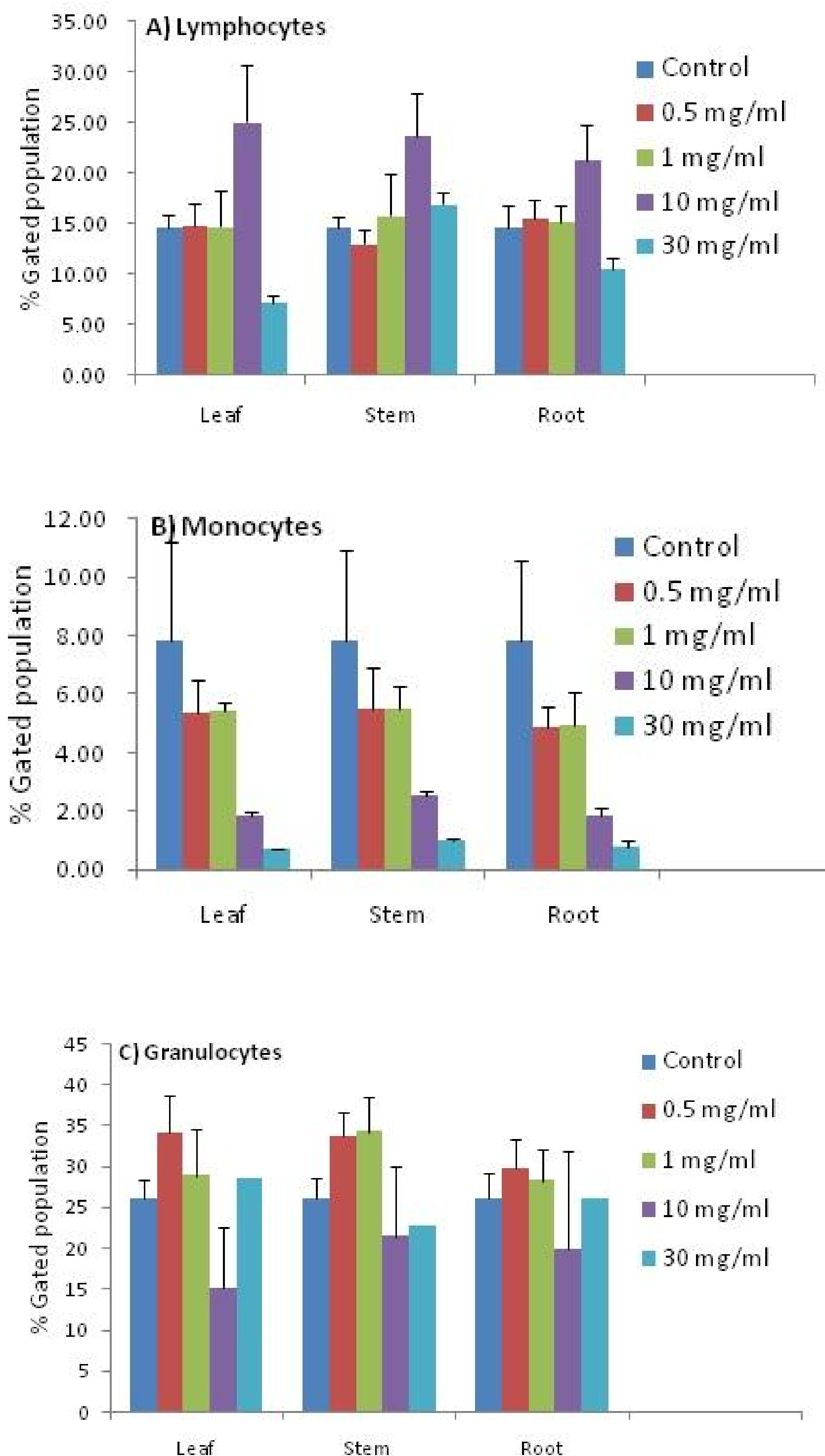

Figure 1: Flow cytometric analysis of leaves, stem, root and mixed proportion of leaf, stem and root of Mimusops elengi on lymphocytes, monocytes and granulocytes count. Values are expressed in Mean ± S.D of ten human whole blood samples.

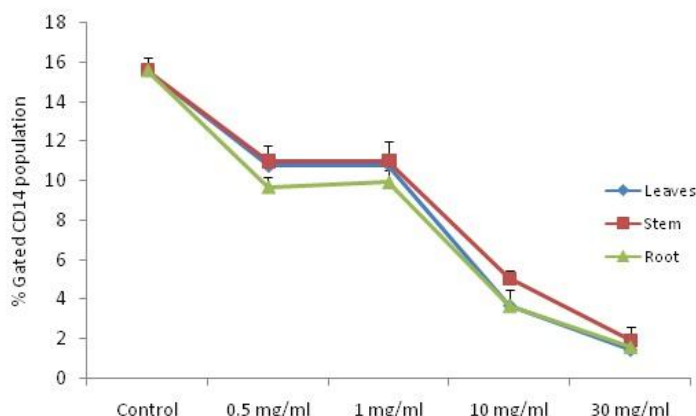

Figure 2: Effect of *Mimusops elengi* on monocyte marker CD14 on human peripheral blood mononuclear cells. Values represents the mean ± S.E. Staining of peripheral blood cells with T cell marker CD14 (FITC conjugated monoclonal antibody)

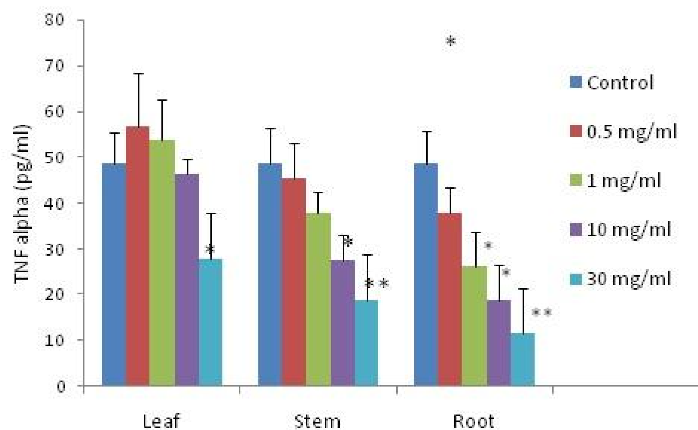

Figure 3: Reduction of cytokines (TNF-alpha) from human peripheral blood mononuclear cells. 100 µl of PBMCs were cultured in 96-well tissue-culture plates (10^6 cells/ml for TNF α at 37°C in a humidified atmosphere with 5% CO_2 and 37 °C for 24 h. Aqueous extract of *Mimusops elengi* containing different concentrations were diluted in RPMI 1640 medium, and added in triplicates to wells at a range of concentrations (0.5 – 100 mg/ml, 50 µl). Values represent the mean ± S.E.

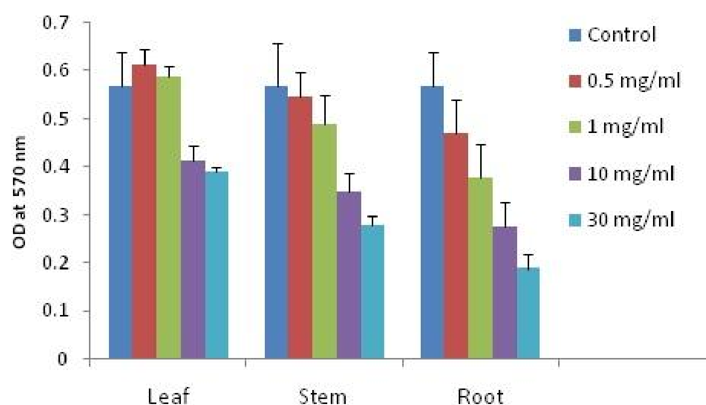

Figure 4: Effect of *Mimusops elengi* on human peripheral blood mononuclear cells using MTT. PBMC were isolated and treated with different concentrations of aqueous extract (0.5- 30 mg/ml, 50 µl) respectively. Cells were incubated for 72 h and proliferation was measured by MTT assay. Data are Mean ± S.E. (n = 10).

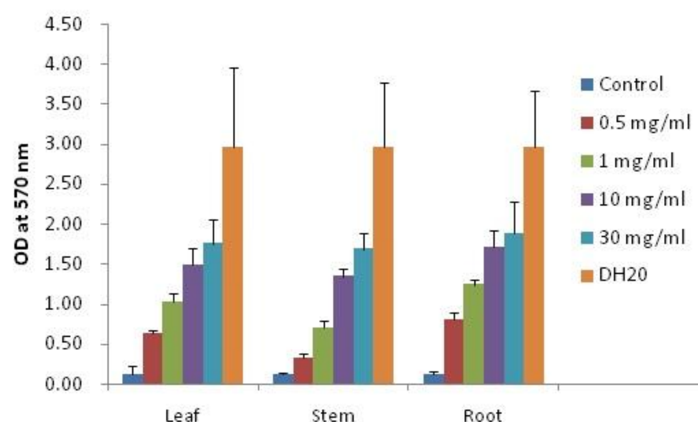

Figure 5: Hemolytic activity of *Mimusops elengi* on human erythrocytes. Data are represented as Mean ± S.D. of ten human whole blood samples. Distilled water and phosphate buffered saline used as positive and negative control.

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