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In vitro anti-inflammatory activity of root aqueous extract of *Mesua ferrea* in human whole blood and peripheral blood mononuclear cells using flow cytometry

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Abstract

The purpose of this study is to present the available *in vitro* evidence of the anti-inflammatory properties of *Mesua ferrea* and examine their potential usefulness in the human ailments or infections. In the last three decades, there are number of primary as well as secondary metabolites or aqueous extract isolated from the leaves, root and stem of medicinal plants and used as a source of therapeutic agents. The most promising anti-inflammatory activities of these aqueous extract isolated from the roots of *Mesua ferrea*. Human whole blood were treated with variable doses of aqueous extract (0.5 – 30 mg/ml) and evaluated the lymphocytes, monocytes and granulocytes count using flow cytometry and observed the monocyte marker i.e. CD14 marker and TNF alpha in peripheral blood mononuclear cells and also determined its hemolytic activity. The results showed that the aqueous extract showed increased in the lymphocytes count and reduction of granulocytes and monocytes level which is evidenced through the decline of CD14 surface marker and TNF alpha which are the indicators of inflammation in human peripheral blood mononuclear cells. At high doses of aqueous extract i.e. 30 mg/ml showed hemolytic activity. The results showed that the root aqueous extract of *Mesua ferrea* showed anti-inflammatory activity.

Key-Words: *Mesua ferrea*, Aqueous extract, Anti-inflammatory

Introduction

Inflammation is an important process in our body's immune system, which acts as to remove and repair the damaged tissue or to neutralize the harmful pathogens or agents (Cragg and Newman, 2013; Balunas and Kinghorn, 2005). Inflammation includes cascade of events like increased in the permeability of blood vessels, attachment of circulating cells to the blood vessels in the vicinity of injury site, migration of several cell types, growth of new tissue and blood vessels ((Provenza and Villalba, 2010). Inflammation may release or generate a diverse population of pro-inflammatory mediators like histamines, bradykinins, serotonin, prostaglandins and nitric oxide. Although number of anti-inflammatory drugs can control inflammation occurrence and development, it is not enough. Traditionally medicinal plants play an important anti-inflammatory role in number of ways to treat inflammatory diseases, based on their active primary as well as secondary metabolites (Provenza and Villalba, 2010).

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The medicinal plant based products especially roots, stem, leaves etc are getting more importance in the treatment of inflammation because of the toxic effects as compared to the current therapy which is generally used to treat inflammation using synthetic drugs. Natural products are less toxic and inexpensive when compared to the synthetic drugs which are available in the market. Now a day, number of pharmacologists throughout the world has been focused on finding out the safer, cost effective and more potent anti-inflammatory drug (Handa *et al*, 1992). Generally, these natural products today symbolize safety in comparison to the synthetic drugs that are regarded as unsafe to humans. So, people especially villagers in India are returning back to the natural products with the hope of safety and security. There are number of medicinal plants which showed anti-inflammatory activity like *Mimusops elengi* (Gupta *et al*, 2014), *Ficus religiosa* (Gupta *et al*, 2014), *Aegle marmelos* (Arul *et al*, 2005), *Azadirachta indica* (Manogaran *et al*, 1998) etc.

One of the medicinal plant i.e. *Mesua ferrea* (Nagkeshar, common name) belongs to the family Guttifere and is widely distributed in India, Pakistan, Malaya etc (Chahar *et al*, 2012, Chow and Quon, 1968; Ali *et al*, 2004). Traditionally, this plant is widely used for curing human as well as animal diseases (Adewale *et al*, 2012). In India especially tribal's of Assam use this plant for its antiseptic, purgative, blood purifier activity and also showed number of medicinal uses in different parts of the plant i.e. leaves and flowers of this plant are antidotes for snake bite and scorpion sting; tincture of bark and roots is a bitter tonic and useful in gastritis and bronchitis (Garg *et al*, 2009, Govindchari *et al*, 1967, Gopalakrishnan et al, 1980). Moreover, this plant showed number of activities likes antiasthmatic activity (Chahar *et al*, 1980), CNS dependent activities (Gopalakrishnan et al, 1980), antimicrobial activity (Adewale *et al*, 2012), immunomodulatory activity (Chahar et al, 2013), antioxidant activity (Garg *et al*, 2009) etc. The present study which deals with the aqueous extract of root for studying as well as examine its anti-inflammatory activity.

Material and Methods

Preparation of the extracts

Fresh plant roots were gathered from the Garden of Vidya Pratishthan School of Biotechnology (VSBT), Baramati (Pune), Maharashtra. The plant roots were washed properly with tap or distilled water three to four times and cut into small pieces and then dried in a shady area and then macerated with liquid nitrogen to prepare the finely powdered form and then used for aqueous extract preparation and was taken for the immunological studies. Plant root powder was grinded in phosphate buffered saline by continuously stirring. The aqueous extract of plants was centrifuged at 5000 rpm for 15 minutes. The supernatants were filtered through Whatman filter paper and the supernatant was collected and was used for various immunological assays.

High performance thin layer chromatography (HPTLC) fingerprinting

The aqueous extract was purified from the roots of *Mesua ferrea* and detects various metabolites using HPTLC. The solvents, reagents and HPTLC plates (10 x 10 cm) were purchased from Qualigens and Merck. Generally, the solvent system used in mobile phase and detect its wavelength at 366 nm. The stock solution of aqueous extract of roots of *Mesua ferrea* was prepared for HPTLC studies and dissolved the 5 g of weighed compound in phosphate buffered saline or with different solvents in a final volume of 50 ml. Further dilutions were made to obtain working standards. The aqueous extract of leaf of *Mesua ferrea* showed the presence of terpenoids, flavonoids, phenolics and glycosides in the phytochemical profile of *Mesua ferrea*. The retardation factor *(Rf)* values of terpenoids and glycosides are 0.96 and 1.8.

Human blood samples

Venous blood samples were collected from adults of different age groups between 20 – 30 years directly into Vacutainer tubes containing sodium heparin anticoagulant. Before donating the blood sample, none had ingested drugs such as aspirin or other anti-inflammatory agents for a period of at least 10 days prior to donation. 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 in human whole blood

Flow cytometry analysis of human whole blood for examine and counting the number of cells i.e. lymphocytes, monocytes and granulocytes count which are suspended in a stream of fluid. To examine the forward and side scatter gating of human whole blood with variable doses of aqueous extract ranging from 0.5 – 30 mg/ml for data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analyzed using cell quest software. Briefly, 50 µl of human whole blood was pipetted directly into a falcon tube containing 1 ml of phosphate buffered saline or with containing concentrations of aqueous extract and then incubated at carbon dioxide incubator (37 °C, 5 % CO_2) for 2 h. After incubation, RBCs were lysed using 2 ml of red cell lysis buffer and incubated the sample for 30 minutes. After centrifugation at 1800 rpm for 10 minutes, the supernatant was removed or aspirated and washed two- three times with phosphate buffered saline. After centrifugation, pellet dissolved in PBS and observed the cells through flow cytometer (Ramanathan, 1997; Fattaccioli *et al*, 2009).

Estimation of CD14 surface marker and TNF alpha from peripheral blood mononuclear cells (PBMC)

PBMCs were extracted from heparinized blood of healthy donors by means of density gradient centrifugation using Ficoll reagent (density 1.077 g/l).To evaluate the effect of variable doses of aqueous extract of roots of *Mesua ferrea* in PBMC, cell suspension (2×10^6 cell/ml) was pipetted into six well plate cultured at 37 °C and stimulated with LPS (1 µh/ml) for 48 h, the plates were centrifuged at 1400 x g, 5 min and the supernatant was collected for the estimation of TNF alpha and the cells settled at the

bottom analyzed for CD14 monocyte marker for flow cytometric analysis. 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 mononuclear cells, 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 (Ramanathan, 1997; Fattaccioli *et al*, 2009). On the other hand, cytokine concentrations in the cell culture supernatant were determined by ELISA kits that were specific against murine cytokines. Levels of TNF alpha were measured using ELISA.

Preparation of erythrocytes suspension and determined its hemolytic activity

Blood was collected from a healthy person in a tube containing EDTA. The blood was centrifuged at 1800 rpm for five minutes in a refrigerated centrifuge. Plasma in the form of supernatant was discarded and the pellet was washed continuously two to three times with phosphate buffer saline solution by centrifugation at 1500 rpm for 10 minutes. The human red blood cells were resuspended in phosphate buffered saline. For this experiment, 1% human red-blood cell suspension dissolved in pH 7.4 phosphate buffer saline was used throughout the experiment. Aqueous extracts of root containing different concentrations transferred into test tubes containing a fixed volume of human red-blood cell suspension. The extracts were tested or screened at different concentrations. 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 (Sun *et al*, 2003).

Statistical analysis

Data are reported as means ± standard deviation (SD). The difference between the control and treated samples is determined by One way ANOVA test (Bonferroni multiple comparison test).

Results and Discussion

Effect of root aqueous extract of *Mesua ferrea* on blood counts

The effect of the aqueous extract of roots of *Mesua ferrea* on lymphocytes, monocytes and granulocytes count as shown in Fig. 1. In *Mesua ferrea*, there is a dose dependent decrease on monocytes and granulocytes count as compared to control. At a dose range of 1 mg/ml, there is increase in the number of lymphocytes as compared to control.

Effect of root aqueous extract of *Mesua ferrea* on CD14 surface marker and hemolytic activity

These studies suggest that the root aqueous extract of *Mesua ferrea* showed decline in CD14 monocyte marker at higher doses as compared to control and also showed the decline in the level of TNF alpha at higher doses as compared to control (Fig. 2 and 3). However, these results showed that the aqueous extract showed anti-inflammatory activity at higher doses.

Effect of root aqueous extract of *Mesua ferrea* on hemolytic activity

The hemolytic activity of *Mesua ferrea* as shown in Fig. 4. In *Mesua ferrea*, hemolytic activity is observed at higher doses (30 mg/ml) as compared to control. In this study, we used distilled water and phosphate buffered as positive and negative control. In Fig.4, the results showed that the aqueous extract showed less hemolytic activity in human whole blood erythrocytes as compared to distilled water.

According to World Health Organization (WHO), medicinal plant products especially leaves, stem and root may be the best source for a variety of drugs. More than 80 % of the population in India and abroad relies on traditional medicines based on plant products (Mathur and Velpandian, 2009). India is one of the rich in the variety of medicinal plants that could help to fight against number of disease like malaria (Kager, 2002) dengue (Guha and Schimer, 2005) and tuberculosis (Siripong, 2006). These diseases are generally seen or common in African countries where the health care access is so difficult. The use of the number of medicinal plants could constitute a reservoir of new molecules important for anti-fungal; anti-bacterial; anti-viral; antioxidant and anti-inflammatory substances therapies and research on composition and mechanism of action will create better treatment standards and improve the value of traditional plants as sources of new medications.

In this paper, we studied on the complexity of immunopharmacological activities present in traditional plant used in India and help to further our understanding of mechanisms for action and why most of the medicinal plants are used to treat individual diseases. On the basis of this objective, we evaluated the anti-inflammatory activity of root aqueous extract of *Mesua ferrea* using flow cytometer against human whole blood containing lymphocytes, monocytes and granulocytes count, CD14 monocyte marker from

peripheral blood mononuclear cells and also determined its hemolytic activity. Flow cytometer is a technique of quantitative single cell analysis and became an essential or important instrument in the field of biological sciences. In human whole blood, the decline in the number of monocytes in human whole blood treated with variable doses of aqueous extract showed that the root aqueous extract of *Mesua ferrea* showed anti-inflammatory activity.

In another set of study, our group focused on the influence of *Mesua ferrea* that have shown anti-inflammatory activity on CD14 monocyte marker in PBMC population. CD14 was first identified on the surface of monocytes and macrophages (Zuckermann and Husmann, 1996). Lot of evidence collected from human studies has suggested that monocytosis can be an indicator of various inflammatory diseases. In human blood, monocytes can differentiate into inflammatory or anti-inflammatory subsets. During tissue damage or infection, monocytes are rapidly migrated into the tissue and transformed into tissue macrophages or dendritic cells. Given the rapid progress in the monocyte research from number of inflammatory diseases, there is a need to share our knowledge in monocyte heterogeneity and its impact in human disease. The results obtained from this study indicated that the root aqueous extract of *Mesua ferrea* exerted an anti-inflammatory effect on CD14 monocyte marker in human PBMCs with a dosage-dependent relationship. Macrophage activation through B cell mitogen i.e. lipopolysaccharide (LPS, agonist of TLR 4 receptor) results in the release of proinflammatory cytokine (TNF alpha) (Schimmer and Parker, 2001). In this study, we demonstrated that the aqueous extract suppressed TNF alpha secretion at higher doses, both of which are crucial in the inflammatory and healing mechanism (Shen *et al*, 1994). As seen in this experiment, the ability of this aqueous extract to suppress inflammation when it is applied after the onset of inflammation is likely to be due to the genuine anti-inflammatory activity.

Conclusion

The result obtained from the experiment it is concluded that the aqueous extract of *Mesua ferrea* having good anti-inflammatory activities and it shown dose dependent activities. The results of root aqueous extract of *Mesua ferrea* support the traditional use of this plant in inflammatory conditions and suggest the presence of biologically active components which may be worth further investigation and elucidation.

References

1. Cragg GM, Newman DJ (2013) Natural products: A continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA) - General Subjects.* **1830** (6): 3670 – 3695.
2. Balunas MJ, Kinghorn AD (2005) Drug discovery from medicinal plants. *Life Sciences.* **78** (5): 431 – 441.
3. Provenza FD, Villalba JJ (2010) The role of natural plant products in modulating the immune system: An adaptable approach for combating disease in grazing animals. *Small Ruminant Research*. **89** (2-3): 131 – 139.
4. Handa SS, Chawla AS, *et al.* (1992). Plants with anti-inflammatory activity. *Fitoterapia*. **63**: 3-23.
5. Gupta A, Khamkar PR, *et al.* (2014) Anti-inflammatory activity of aqueous extract of *Mimusops elengi* on human whole blood and peripheral blood mononuclear cells. *International Journal of Current trends in Pharmaceutical research.* **2**(5): 494 – 501**.**
6. Gupta A, Khamkar PR, *et al.* (2014) Immunomodulatory and anti-inflammatory activity of aqueous extract of leaf, stem and root of *Ficus religiosa* on human whole blood and peripheral blood mononuclear cells. *International Journal of Medicine and Pharmaceutical research.* **2**(4): 732 - 739.
7. Arul V, Miyazaki S, *et al.* (2005) Studies on the anti-inflammatory, antipyretic and analgesic properties of the leaves of Aegle marmelos Corr. *J Ethnopharmacol*. **96**:159–63.
8. Manogaran S, Sulochana N, *et al.* (1998) Anti-inflammatory and anti-microbial activities of the root, bark and leaves of Azadirachta indica. *Anc Sci Life*. **18**(1): 29–34.
9. Chahar MK, Sanjaya Kumar DS, *et al.* (2012). *In-vivo* antioxidant and immunomodulatory activity of mesuol isolated from *Mesua ferrea* L. seed oil. *Int Immunopharmacol.***13**: 386-391.
10. Chow YL, Quon HH (1968) Chemical constituents of the heartwood of *Mesua ferrea*. *Phytochemistry*. **7**:1871.
11. Ali M, Sayeed MA, *et al.* (2004). Antibacterial screening of *Cassia fistula* and *Mesua ferrea. J Med Sci*. **4**: 24-29.
12. Adewale AI, Mirghani MES, *et al.* (2012). Anti-Bacterial and Cytotoxicity Properties of the Leaves Extract of Nahar (*Mesua ferrea*) Plant. *Adv Natur Appl Sci*. **6**:583-587.
13. Garg S, Kameshwar S, *et al*. (2009). *In-vivo* Antioxidant activity and hepatoprotective effects of methanolic extracts of *Mesua ferrea* L. *Int J Pharmatechnol Res.* **1**:1692-1696.

14. Govindchari TR, Pai BR, *et al.* (1967) Constituents of *Mesua ferrea* L. 11-Ferruol A, A New 4-Alkylcoumarin. *Tetrahedron* **23**: 4161-4165.
15. Gopalakrishnan C, Shankarnarayanan D, *et al* (1980) Anti-inflammatory and CNS depressant activities of xanthones from *Calophyllum inophyllum* and *Mesua ferrea*. *Ind J Pharmacol*. **12**:181-191.
16. Chahar MK, Kumar SDS, *et al.* (2013) *Mesua ferrea* L.: A review of the medical evidence for its phytochemistry and pharmacological actions. *African Journal of Pharmacy and Pharmacology*. **7**(6), 211-219.
17. Ramanathan M (1997) Flow Cytometry Applications in Pharmacodynamics and Drug Delivery. *Pharm Res.* **14**(9):1106-1114.
18. Fattaccioli J, Baudry J, *et al.* (2009) Size and fluorescence measurements of individual droplets by flow cytometry. Soft Matter **5**: 2232–2238.
19. Sun HX, Pan HJ, Pan YJ (2003) Hemolytic activities and immunologic adjuvant effect of *Panax notoginseng* saponins. *Acta Pharmacol Sin*. **24** (11): 1150-1154.
20. Mathur R, Velpandian T (2009) Medicinal plant based health products: where is the medicinal constituent? *Indian J Pharmacol*. **41**(4): 205–206.
21. Kager PA (2002) Malaria control: constraints and opportunities. *Trop Med Int Health*. **7**:1042–1046.
22. Guha SD, Schimmer B (2005) Dengue fever: new paradigms for a changing epidemiology. *Emerg Themes Epidemiol.* **2**(1):1.
23. Siripong P (2006) Induction of apoptosis in tumor cells by three naphthoquinone esters isolated from Thai medicinal plant: Rhinacanthus nasutus Kurz. *Biol Pharm Bull*. **29** (10): 2070–2076.
24. Zuckermann FA, Husmann RJ (1996) Functional and phenotypic analysis of porcine peripheral blood CD4+/CD8+ double positive T-cells. *Immunol*. **87**: 500-512.
25. Schimmer BP, Parker KL (2001) Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In: Hardman, J.G., Limbird, L.E., Goodman Gilman, A. (Eds.). *Pharmacological Basis of Therapeutics tenth ed McGraw-Hill New York.*1649 – 1677.
26. Shen HM, Kenned JL, et al. (1994) Inhibition of cytokine release by cocaine. International *Journal of Immunopharmacology.***16**: 295–300.

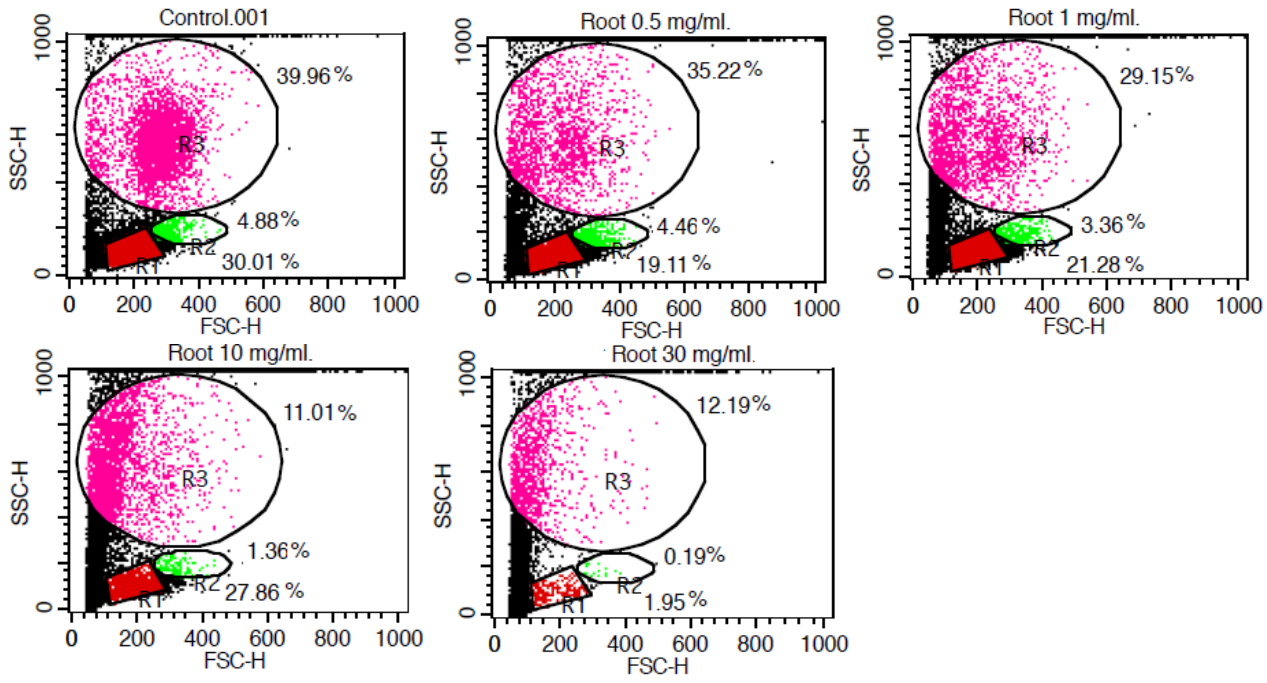

Fig. 1: Flow cytometric analysis of root aqueous extract on lymphocytes, monocytes and granulocytes count. Human whole blood samples were incubated with serial dilutions of root aqueous extract and incubated the samples at 37°C, 5% carbon dioxide incubator for 2 h. After 2h, lysed the blood samples and wash the samples two times with phosphate buffered saline and then observed the cells in flow cytometer (FACS Calibur). Data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analyzed using cell quest software

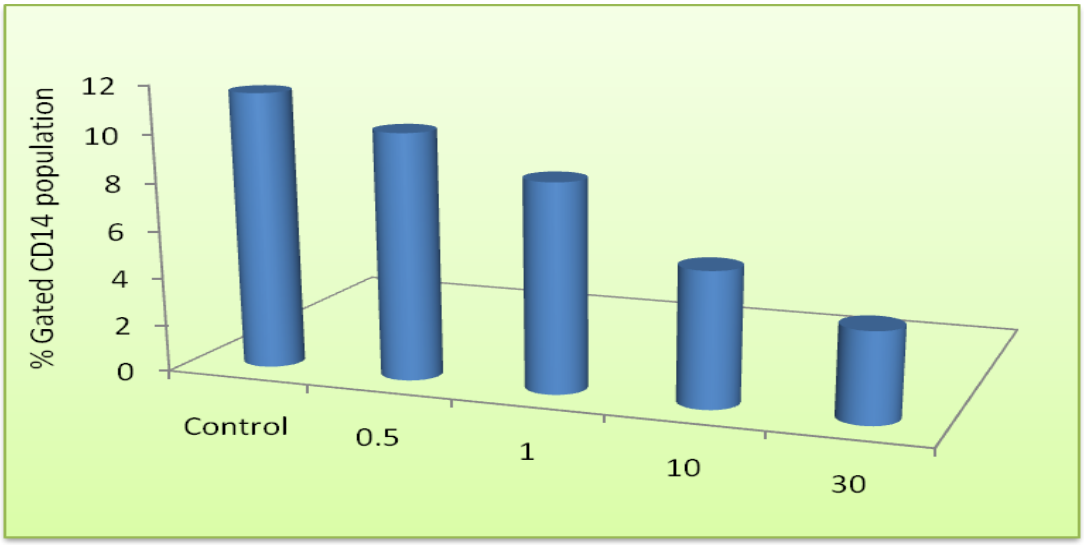

Fig. 2: Effect of root aqueous extract of *Mesua ferrea* on monocyte marker CD14 on human peripheral blood mononuclear cells. Values represents the mean ± S.E. Staining of peripheral blood cells with T cell marker CD14 (FITC conjugated monoclonal antibody)

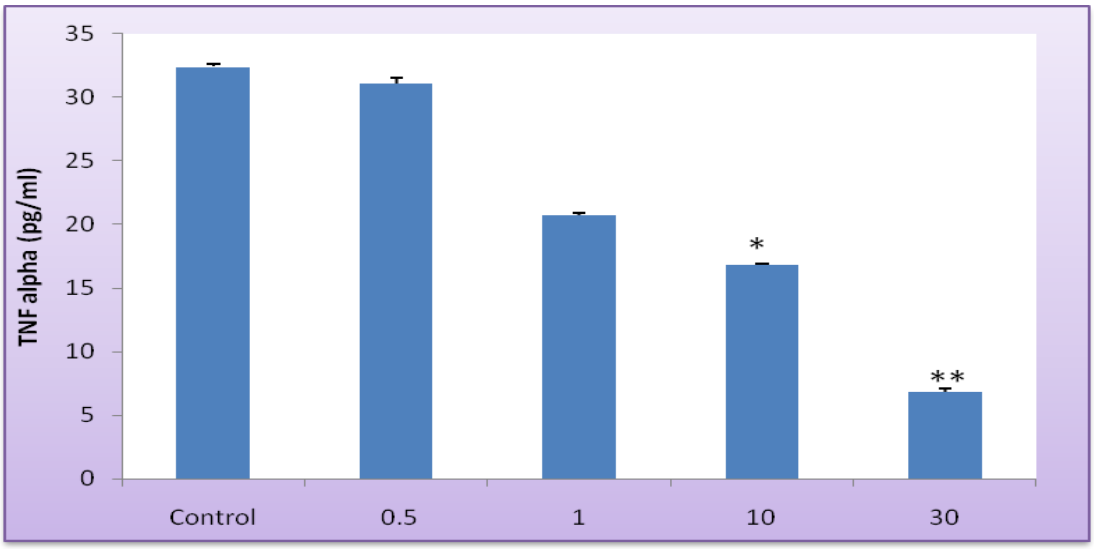

Fig. 3: Effect of root aqueous extract on TNF-alpha production from human peripheral blood mononuclear cells. 100 µl of PBMCs were cultured in 96-well tissue-culture plates (10^6 cells/ml) for TNF alpha at 37°C in a humidified atmosphere with 5 % CO_2 and 37 °C for 24 h. Aqueous extract of *Mesua ferrea* containing different concentrations were diluted in RPMI 1640 medium, and added in triplicates to wells at a range of concentrations (0.5 – 30 mg/ml, 50 µl). Values represent the mean ± S.E.

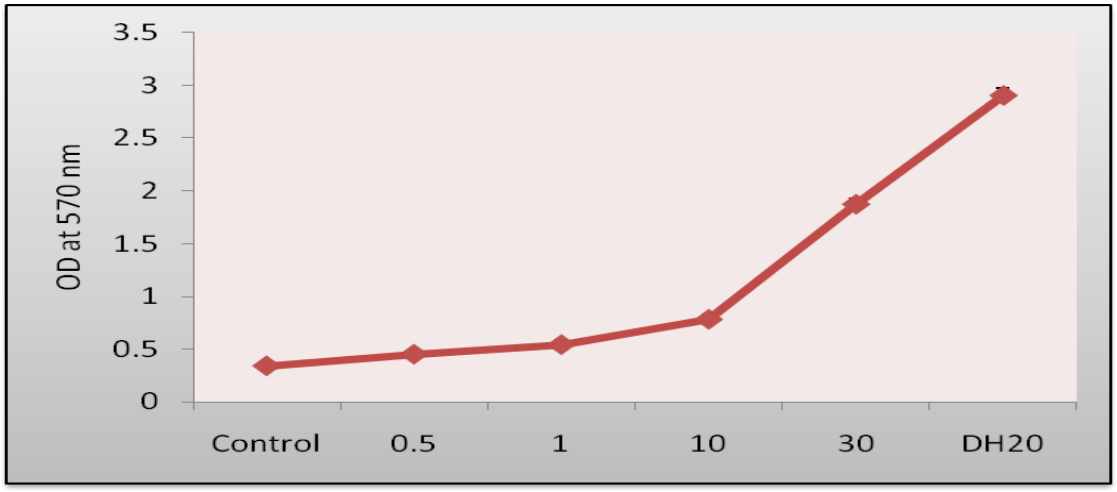

Fig. 4: Hemolytic activity of *Mesua ferrea* on human erythrocytes
Data are represented as Mean ± S.D. of human whole blood samples. Distilled water and phosphate buffered saline used as positive and negative control.

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