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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

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Abstract

In the last three decades, aqueous extract have been isolated from the leaf, stem and root from medicinal plants and used as a source of therapeutic agents. The most promising immunopharmacological activities of these aqueous extract are their immunomodulatory as well as anti-inflammatory effects. The aqueous extract isolated from the leaf, stem and root of *Ficus religiosa* and evaluated the effect on human whole blood to observe the monocytes, lymphocytes and granulocytes count using flow cytometry, hemolytic activity, cytotoxicity assay and also observed the Th1 (TNF alpha) type of cytokine from human peripheral blood mononuclear cells. The results showed that the effect of variable doses of aqueous extract of root of *Ficus religiosa* at lower concentration showed immunomodulatory effect i.e. increase in the level of monocytes at a dose range in case of root (0.5 mg/ml) and also increased the pro-inflammatory cytokine i.e. TNF alpha in peripheral blood mononuclear cells but in case of leaf and stem showed rapidly decline in the level of monocytes and TNF apha at higher doses (30 mg/ml) as compared to control and these results showed anti-inflammatory activity. The presented data indicate that root of *Ficus religiosa* at lower concentration has broad immunomodulatory effects and in leaf and stem showed anti-inflammatory effect at higher doses in human whole blood and PBMC. Furthermore, at higher doses i.e. 30 mg/ml exhibited significant hemolytic activity as compared to control.

Keywords: *Ficus religiosa*, monocytes, immunomodulatory, anti-inflammatory

Contents

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1. Introduction

The immune system is a part of our body which is able to detect the pathogen by using a specific receptor to produce immediately response by the activation of immune components i.e. cytokines, chemokines and also release of inflammatory mediators [1, 2]. Modification of the immune response through pharmacological agents is most effective in treatment therapy is begun before exposure to the antigen has an opportunity to generate a primary response. Generally, the medicinal plants affecting the immune system are termed as immunomodulatory. One of the most promising recent alternatives in immunopharmacology i.e. use of immunomodulators for enhancing host defense responses. Several types of immunomodulators i.e. natural as well as synthetic plant based molecules have been identified, including microorganisms. Most of the medicinal plants which are generally used as immunostimulator and immunosuppressor effect to provide alternative potential to maintain the immunorestorative condition or giving conventional chemotherapy for number of diseases, especially with respect to host defense mechanism. The use of plant based product like biopolymeric fractions i.e. Polysaccharides, lectins, peptides, flavonoids and tannins have been the immune response or immune system in various *in-vitro* models. [3, 4, 5, 6, 7, 8] Some of the plants with established immunomodulatory activity *are Panax ginseng* [9], *Asparagus racemosus* [10], *Azadirachta indica* [11], *Withania somnifera* [12] among others.

Ficus religiosa commonly known as Peepal tree as which belongs to the family Moraceae and it is found throughout tropical and subtropical regions in India especially found in Indian temples [13]. According to Ayurveda, one of the medicinal plant i.e. *Ficus religiosa* belongs to one of the class of drugs called rasayana [14]. Presently, the researchers is interested as well as focused on herbal medicines accompanied by laboratory investigation of the bioactive molecules or ingredients isolated and purified from the medicinal plants e.g. *Ficus religiosa* to treat several types of diseases [15, 16]. It is well known that *Ficus religiosa* leaf is used to cure some of the diseases like asthma, diabetes, epilepsy, gastric problems, inflammatory disorders etc [15, 16]. On the basis of Ayurveda and Unani, there are numerous drugs which are already available in the international market through exploration of ethno pharmacology. Although number of scientific studies in India related to ethno pharmacology has been carried out on a large number of natural plant based molecules and number of marketable drugs or phytochemical entities have entered the evidence-based therapeutics. Therefore, efforts are needed to establish and validate evidence regarding safety and practices of Ayurvedic medicines. On the basis of this objective, the present paper which have shown the immunomodulatory as well as anti-inflammatory activity of aqueous extract of leaf, stem and root of *Ficus religiosa* in human whole blood and peripheral blood mononuclear cells.

2. Materials and Method

2.1. Sample collection

The leaf, stem and roots of plant *Ficus religiosa* were collected from Vidya Pratishthan School of Biotechnology (VSBT), Baramati (Pune), Maharashtra. The leaf, stem and root of the plants were collected and washed thoroughly in tap water followed by distilled water and then shade dried at room temperature. Dried leaf, stem and root were uniformly grinded and finely to form the powder, this was then used for aqueous extract preparation and was taken for the immunological studies. The aqueous extraction of leaf, stem and root of *Ficus religiosa* in mortar and pestle and was grinded in phosphate buffered saline and the extract was centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was collected and was used within four hours for various immunological assays.

2.2. High performance thin layer chromatography (HPTLC) fingerprinting

The aqueous extract was purified from the leaf, stem and root of *Ficus religiosa* and detects the aqueous samples using HPTLC to determine the primary as well as secondary metabolites. The solvents and other purified reagents, HPTLC plates (10 x 10 cm) were purchased from Qualigens and Merck. The solvent system used in mobile phase and detect its wavelength at 366 nm. The stock solution of *Ficus religiosa* 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 100, 30, 10 and 0.5 mg/ml. The aqueous extract of leaf of *Ficus religiosa* showed the presence of terpenoids, flavonoids, phenolics and saponin in the phytochemical profile of *Ficus religiosa*.

2.3. Flow Cytometric analysis of whole blood and peripheral blood mononuclear cells (PBMC)

Flow cytometry analysis of whole blood for counting the cells i.e. lymphocytes, monocytes and granulocytes count suspended in a stream of fluid. To examine the variable doses of aqueous extract on whole blood using forward and side scatter gating applied for data acquisition on 10,000 events and fraction of cell populations representing different phenotypes analyzed using cell quest software. Briefly, 100 µl of whole blood was pipetted directly into a falcon tube containing 1 ml of phosphate buffered saline or with containing 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 red cell lysis buffer and incubated the sample for 30 minutes. After centrifugation at 1800 rpm for 10 minutes, the supernatant was aspirated and washed two times with phosphate buffered saline. After centrifugation, pellet dissolved in PBS and observed the cells through flow cytometer [17, 18].

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 1800 rpm with 2 ml of PBS and then samples were analyzed for 10000 cells on the flow cytometer [17, 18].

2.4. PBMC isolation and estimate the Th1 (TNF alpha) cytokine

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 TNF α, with LPS (1 µg/ml) [19, 20]. Before stimulation, the aqueous extract of stem, leaf and root was preincubated for 30 min. After incubation cells were pelleted, and TNFα in the supernatants (fresh or frozen at 80°C) were measured by ELISA reader (Victor model, Perkin Elmer) according to the manufacturer's instructions.

2.4. 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 *Ficus religiosa* 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 [21].

2.5. Hemolytic activity of human erythrocytes

To examine the variables doses of aqueous extract on human erythrocytes. EDTA blood was collected from the healthy individuals (age 21-30 years) in a tube. Then, blood was centrifuged at 1800 rpm for ten minutes. The supernatant (plasma) was discarded and the pellet was washed three times with sterile phosphate buffer saline solution by centrifugation at 1800 rpm for ten minutes. The cells were resuspended in normal saline to 0.5%. Briefly, 0.1 ml of the cell suspension was mixed with 0.1 ml of the aqueous extracts (0.5, 1, 10 and 30 mg/ml, 100 µl) dissolved in phosphate buffered saline. The cells were incubated for 30 min at 37°C in a carbon dioxide incubator and then centrifuged at 1800 rpm for 10 min. The free hemoglobin in the supernatant was measured in spectrophotometer at 540 nm. Phosphate buffer saline and distilled water were used as minimal and maximal hemolytic controls. Each experiment was performed in triplicates at each concentration.

3. Results

3.1. Effect of Ficus religiosa on human whole blood counts and estimation of monocyte marker CD14 from PBMC using flow cytometry

The effect of the aqueous extract (leaf, stem and root) of *Ficus religiosa* on lymphocytes, monocytes and granulocytes count as shown in **Fig.1**. In leaf and stem, there is a dose dependent decrease in the monocytes count which is confirmed through CD14 marker from PBMC as compared to control where as in root, there is a dose dependent increase in the monocytes count which is also confirmed through CD14 marker from PBMC as compared to control (**Fig. 2**).

3.2. Estimation of Th1 (TNF alpha) cytokine profile

The effect of the aqueous extract (leaf, stem and root) of *Ficus religiosa* on Th1 (TNF alpha) in PBMC as shown in **Figure 3**. In *Ficus religiosa* that the aqueous extract of leaf and stem at higher doses showed dose dependent

decrease in TNF alpha as compared to control where as in root, at lower concentration i.e. 0.5 mg/ml showed increase in TNF alpha as compared to control. These results showed that the aqueous extract of leaf and stem showed anti-inflammatory activity where as root showed immunomodulatory activity at lower doses.

3.3. Cytotoxicity assay

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

3.4. Hemolytic activity

The hemolytic activity of leaf, stem and root of *Ficus religiosa* as shown in **Fig. 5.** In *Ficus religiosa*, 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 of leaf, stem and root showed less hemolytic activity in human erythrocytes as compared to distilled water.

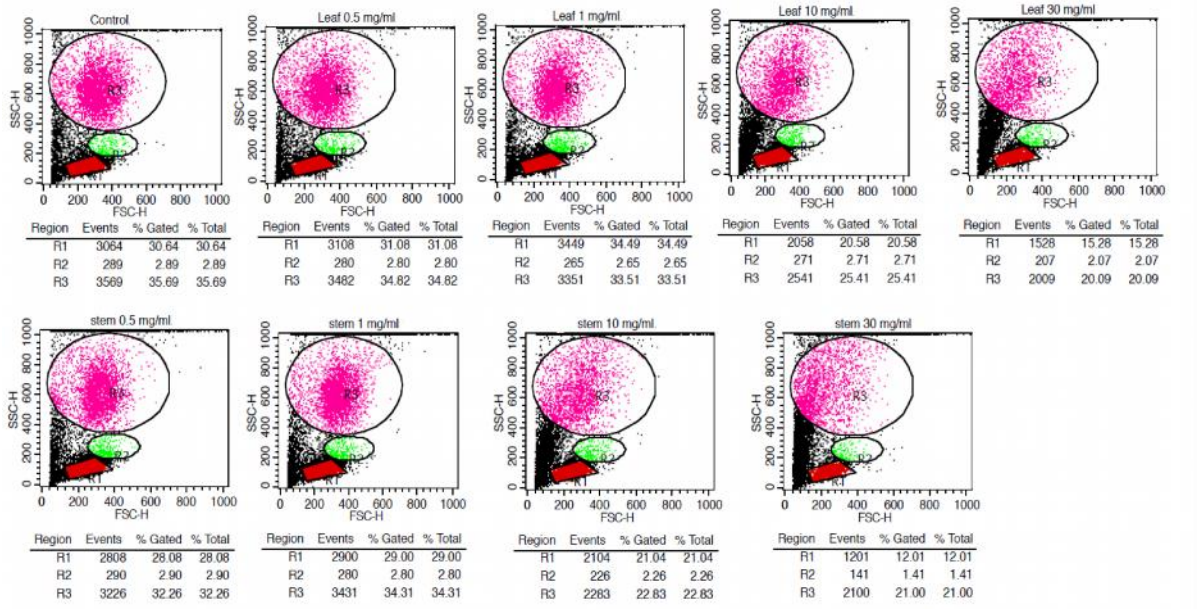

Figure 1: A

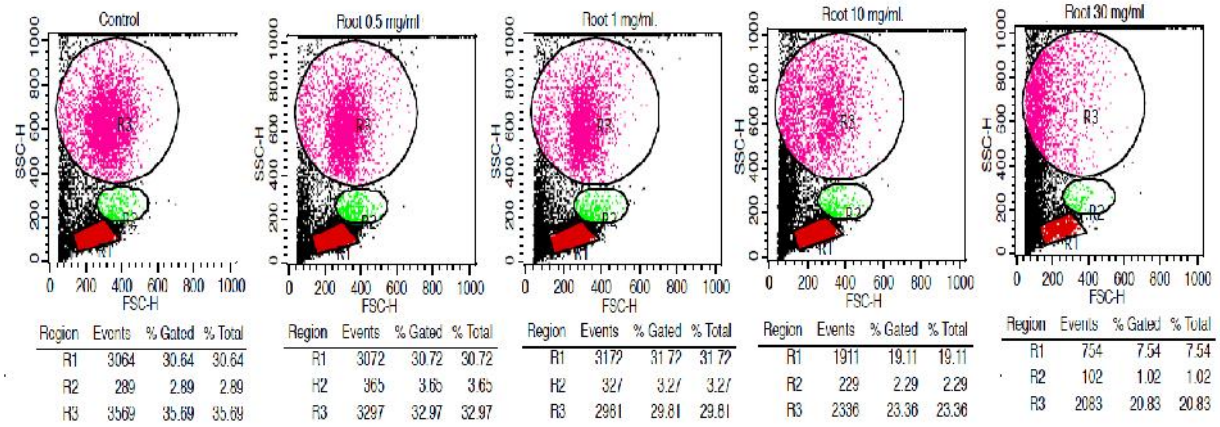

Figure 1: B

Figure 1: Effect of aqueous extract of leaf, stem and root of *Ficus religiosa* on lymphocytes, monocytes and granulocytes count using flow cytometry. Human whole blood was incubated with variable doses of aqueous extract (0.5, 1, 10 and 30 mg/ml). After incubation, cells were lysed using lysis buffer and washed with phosphate buffered saline. After centrifugation, pellet dissolved in PBS and observed the cells through flow cytometer.

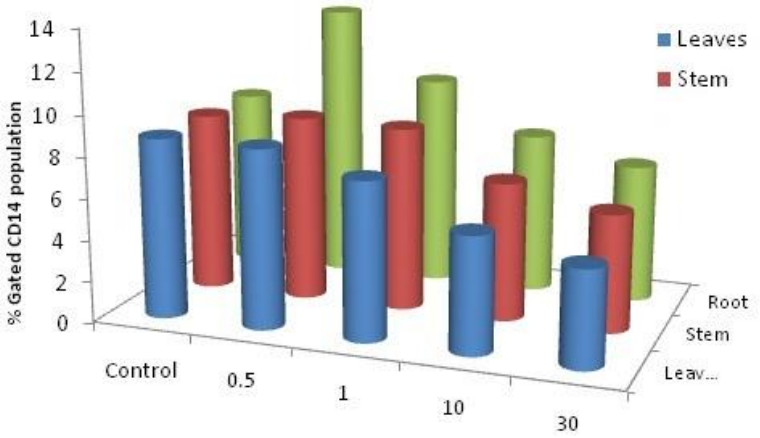

Figure 2: Effect of aqueous extract of leaf, stem and root of *Ficus religiosa* on monotype CD14 marker using flow cytometry. Values represents the mean ± S.E. PBMC were incubated with variable doses of aqueous extract and incubated at carbon dioxide incubator (37°C, 5% CO2) for 24 h. After incubation, staining of PBMC with T cell marker CD14 (FITC conjugated monoclonal antibody) and observed the cells through flow cytometer.

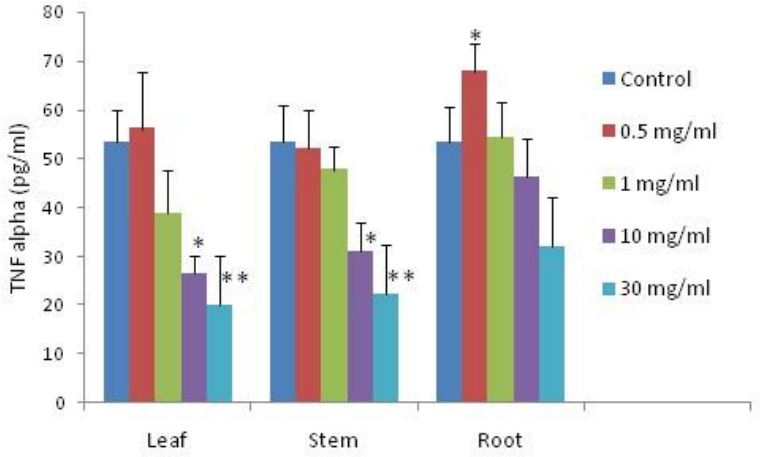

Figure 3: Cytokine (TNF-alpha) estimation from human peripheral blood mononuclear cells. 100 µl of PBMCs were cultured in 96-well tissue-culture plates (10^6 cells/ml for TNF α at 37°C in a humidified atmosphere with 5% CO_2 and 37 °C for 24 h. Aqueous extract of *Ficus religiosa* containing different 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). **V**alues represent the mean ± S.E.

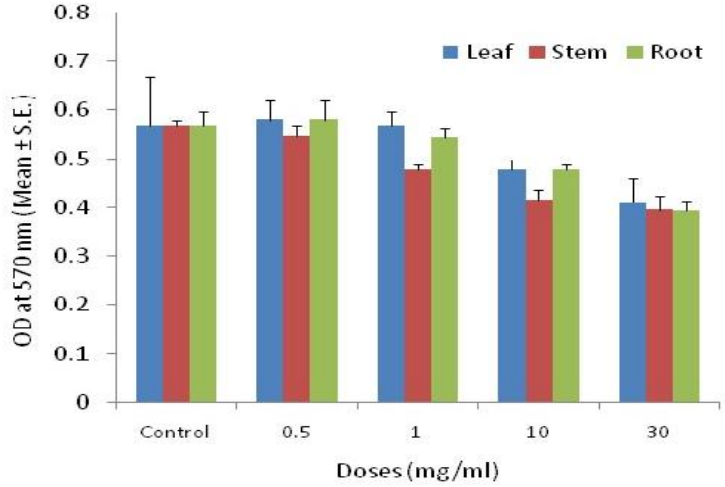

Figure4: Effect of aqueous extract of leaf, stem and root of *Ficus religiosa* on human peripheral blood mononuclear cells using MTT. PBMC were isolated and treated with different concentrations of aqueous extract (0.5- 30 mg/ml,

50 µl) respectively. Cells were incubated for 72 h and proliferation was measured by MTT assay. Data are Mean ± S.E. (n = 10).

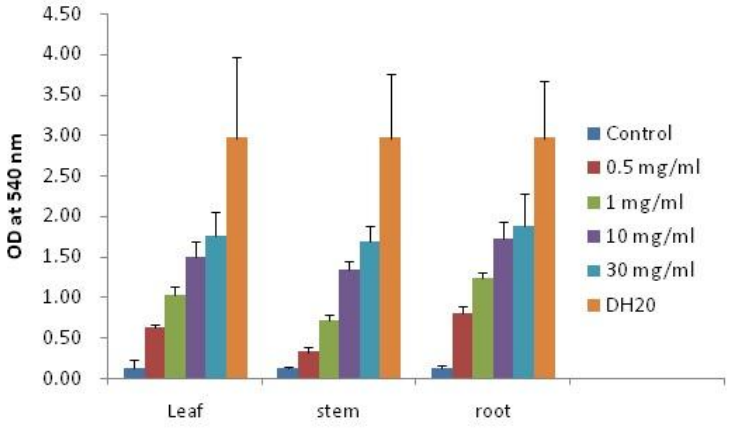

Figure 5: Effect of aqueous extract of leaf, stem and root of *Ficus religiosa* on hemolytic activity. Human red blood cells (plasma removed from the blood) was incubated with variable doses of aqueous extract (0.5, 1, 10 and 30 mg/ml). The cells were incubated for 30 min at 37°C in a carbon dioxide incubator and then centrifuged. The free hemoglobin in the supernatant was measured in spectrophotometer at 540 nm. Values are expressed as Mean ± S.E. of three set of experiments.

4. Discussion

In the present study, we were looking for *Ficus religiosa*, medicinal plant that grow in our Vidya Pratishthan, Baramati where large number of people relies on them for health care, to investigate their immunomodulatory and anti-inflammatory activities of leaf, stem and root. Medicinal plants have been used for a long time in traditional system of medicine for several thousand years and these plants constitute an important and basic resource for ethnobotanical research in many ways [22]. In India, the knowledge of Ayurveda, Unani and Siddha has been accumulated for so many years ago and it is reported that traditional healers use 2500 herbal plant species and among them 100 species serve as regular sources of medicine [23]. Some of the medicinal plants are believed to enhance the natural resistance of the body to infections [23].

In this study, PBMC resembling primary culture is considered to be the best *in vitro* model for studying the immunomodulatory as well as anti-inflammatory properties of aqueous extract. In the current study, we demonstrate that aqueous extract of leaf and stem of *Ficus religiosa* inhibited the secretion of the pro-inflammatory cytokine TNF alpha and also decline in the monocyte level in human whole blood and it is confirmed through CD14 marker as compared to control. CD14 is a glycophosphatidylinositol-linked protein, which is part of the LPS receptor complex. CD14 binds lipopolysaccharide and as such it acts as a pattern recognition receptor. CD14 monocyte marker has been suggested to mediate phagocytosis of bacteria an apoptotic cells. CD14 monocyte marker which is generally involved in the endotoxin mediated release of TNF-alpha by monocytic cells [24, 25]. Comparing the three cell preparations i.e. lymphocytes, monocytes and granulocytes to assess anti-inflammatory potential, it can be concluded that human whole blood and PBMCs are well suited for the evaluation of the anti-inflammatory potential of aqueous extract of leaf and stem. In summary, aqueous extract of leaf and stem of *Ficus religiosa* was shown to suppress the release of inflammatory mediators at higher doses. The result obtained from the experiment it is concluded that the aqueous extract of leaf and stem of *Ficus religiosa* (30 mg/ml) having good anti-inflammatory activities. The results support the orthodox doctrines of this plant in inflammatory conditions and suggest the presence of biologically active components which may be worth further investigation and elucidation.

The study of the immunomodulatory effects of aqueous extract of root of *Ficus religiosa* on both cell mediated and humoral immune response is a matter of interest for many researchers. Several studies have previously demonstrated the immunomodulating effects of medicinal plants on lymphocyte proliferation in the presence of mitogen, allogenic cells and specific antigens [26, 27]. In our study, aqueous extract of root at lower concentration with human whole blood an PBMC showed rapidly increase in the monocyte profile and also confirmed through CD14 marker as well as increase in the pro-inflammatory cytokine TNF alpha from PBMC as compared to control. Our data are, however, the first to specify that aqueous extract purified from the leaf, stem and root of *Ficus religiosa* has a direct anti-inflammatory as well as immunomodulatory effect on cytokine production by monocytes and PBMC. The results showed that aqueous extract of root up-regulated the expression of TNF-alpha in monocytes and PBMC in a dose-

dependent manner. In summary, this study demonstrated the various effects of leaf, stem and root of *Ficus religiosa* on human whole blood and peripheral blood mononuclear cells. The difference in the way these extracts affected lymphocyte stimulation or inhibition perhaps indicated various modes of action. Further investigation should be considered in the effect of extracts on other immune parameters such as macrophage activity, NK cell activity including cell signaling and cytokine production.

5. Conclusion

These results, in assemblage with the potent activity of aqueous extract of leaf, stem and root in human whole blood and the good separation between its immunomodulatory and anti-inflammatory activities, especially after variable doses of aqueous extract administration, make aqueous extract an interesting candidate for several diseases.

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