

Research Article

## Tyrosine Rich Fraction as an Immunomodulatory Agent from *Ficus Religiosa* Bark

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### Abstract

*Ficus religiosa* Linn (Moraceae) is being used in traditional medicine to improve immunity hence, petroleum ether and 70% ethanol extracts (50 and 100 mg/kg, i.p.) of *F. religiosa* bark were screened for immunomodulatory activity by delayed type hypersensitivity (DTH), neutrophil adhesion test and cyclophosphamide-induced neutropenia in Swiss albino mice. 70% ethanol extract showed significant immunostimulant activity hence subjected to column chromatography to find out active compounds. Tyrosine rich fraction (TRF) obtained was screened for immunomodulatory activity by above methods at the dose of 10 mg/kg, i.p. TRF showed potentiation of DTH response in terms of significant increase in the mean difference in foot-pad thickness and it significantly increased neutrophil adhesion to nylon fibres by 48.20%. Percentage reduction in total leukocyte count and neutrophil by TRF was found to be 43.85% and 18.72%, respectively. Immunostimulant activity of TRF was more pronounced and thus it has great potential as a source for natural health products.

**Keywords:** *Ficus religiosa*; Neutropenia; Delayed type hypersensitivity; Immunomodulator; Neutrophil; Tyrosine rich fraction.

### Introduction

*Ficus religiosa* (Moraceae) commonly known as 'Peepal tree' is a large widely branched tree with leathery, heart shaped long tipped leaves on long slender petioles and purple fruits growing in pairs. The tree is regarded as a sacred tree to both Hindus as well as Buddhists [1, 2]. The tree grows throughout India and widely cultivated in south-east Asia especially in vicinity of temples. Preliminary phytochemical screening of *F. religiosa* barks, showed the presence of tannins, saponins, flavonoids, steroids, terpenoids and cardiac glycosides [3, 4]. Plant showed anti-diabetic [5], anti-inflammatory, analgesic [6], anti-microbial [7] and anti-ulcer activities [8].

In the present study, an effort has been made to establish the scientific validity of the immunomodulating activity of *F. religiosa* bark and to find out the probable constituent responsible for this activity.

### Material and methods

#### Plant material

Fresh sample of bark of *F. religiosa* was collected from Ahmednagar district, Maharashtra and authenticated by Dr. T. Chakraborty,

Scientist D, Botanical Survey of India, Pune. A voucher specimen number KADSAGFICRE10 is being maintained.

#### Extraction and isolation

Fresh sample of bark of *F. religiosa* (180 g) was pulverized in form of moderately coarse powder (40 mesh size) and extracted using petroleum ether in Soxhlet extractor to obtain petroleum ether extract. The marc left was extracted using 70% aqueous ethanol in reflux condenser to obtain hydro-alcoholic extract. Both the extracts were vacuum dried to yield 3.2 and 12.3% w/w of extracts, respectively. 70% Ethanol extract (10 g) was found to be more active hence subjected to column chromatography over silica gel column by using a step gradient of ethyl acetate (1L, F1), ethyl acetate/methanol (8:2, 1L, F2), ethyl acetate/methanol (7:3, 1L, F3), ethyl acetate/methanol (1:1, 1L, F4), ethyl acetate/methanol (3:7, 1L, F5), ethyl acetate/methanol (1:9, 1L, F6), and methanol (1L, F7). Fraction F5 was in major amount and showed crystalline structure hence purified to yield a white solid (30 mg) which was identified as tyrosine by studying its melting point, UV, FTIR, and GC-MS spectroscopy.

#### Drugs and chemicals

Cyclophosphamide (cyc) was obtained from Endoxan<sup>®</sup>, Mumbai, Ethanol AR and EDTA solution AR from Merck, Mumbai. Levamisole was obtained from Bluck Pharma, Kolhapur.

#### Preliminary phytochemical screening

Preliminary phytochemical screening of *F. religiosa* extract of 70% ethanol extract was performed for various secondary metabolites as per described methods [9].

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### Sheep RBC (SRBC)

Sheep blood was collected from local slaughterhouse in sterile 0.49% EDTA solution in 1:1 proportion of EDTA in saline (freshly prepared). Blood was kept in the refrigerator and processed for the preparation of sheep RBC by centrifugation at 2000 rpm for 10 min and washing with physiological saline for 4-5 times.

### Animals

Swiss albino mice of either sex weighing 20-25 g were housed and were maintained at standard laboratory conditions. All mice were fed with synthetic pelleted diet (Amrut laboratory animal feed, Sangli, Maharashtra) and clean purified water *ad libitum*. Mice were maintained at  $22 \pm 1^\circ\text{C}$  with 60% relative humidity, and kept under 12 h light: dark cycle. The animals were allowed to acclimatize to laboratory conditions prior to experimentation. The experimental protocol was approved by institutional animal ethical committee (Approval No. CPCSEA/C/448/10-11-/09).

### Immunomodulating study

#### Delayed type hypersensitivity (DTH)

The activity was performed as described by Bafna and Mishra (2004) and Devhare et al. (2011) [10, 11]. Animals were divided into seven groups consisting of six animals each. Control group received vehicle (1 ml/kg distilled water, i.p.) from first to seventh day of the study. Second groups received levamisole (50 mg/kg, i.p.) from first to seventh day of the study. Third group received cyclophosphamide as negative control (50 mg/kg, i.p.) from fourth to sixth days of the study. Fourth to seventh groups received petroleum ether and 70% ethanol extracts (50 and 100 mg/kg, i.p.) respectively from first to seventh days. Eighth group received TRF (10 mg/kg, i.p.) respectively from first to seventh days. On 0<sup>th</sup> day of the study, all the groups I to VIII were immunized with SRBC (20%, s.c.) in normal saline. On 7<sup>th</sup> day all the animals from all groups were challenged with 0.05 ml of 1% SRBC in sub plantar region of right hind paw. Foot pad edema in mice was used for detection of cellular immune response. On 7<sup>th</sup> day, the initial thickness of right hind footpad was measured using digital vernier calliper and 24 hrs after the injection of 0.05 ml of 1% SRBC. Foot pad reaction was assessed after 24 hrs i.e. on 8<sup>th</sup> and 9<sup>th</sup> day, in terms of increase in the thickness of footpad due to edema caused as a result of hypersensitivity reaction, with the help of a digital vernier calliper. The footpad reaction was expressed as the difference in the thickness (mm) between the pre and post right foot pad injected with SRBC.

#### Neutrophil Adhesion test

Neutrophil adhesion test was performed as per the method described by Fulzele et al. (2002) and Devhare et al. (2011) [11, 12]. The animals were divided into seven groups consisting six animals each. Control group received vehicle (1 ml/kg distilled water, i.p.) from first to fourteenth days. Second groups received levamisole (50 mg/kg, i.p.) from first to fourteenth days. Third to sixth groups received petroleum ether and 70% ethanol extract (50 and 100 mg/kg, i.p.) respectively from first to fourteen days.

Seventh group received TRF (10 mg/kg, i.p.) from first to fourteen days. On the 14<sup>th</sup> day of the treatment, blood samples from all the groups were collected by puncturing retro-orbital plexus under mild ether anaesthesia. Blood was collected in vials pre-treated by disodium EDTA and analysed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with nylon fiber (80 mg/ml of blood sample) for 15 min at  $37^\circ\text{C}$ . The incubated blood samples were again analysed for TLC and DLC. Neutrophil index and percent neutrophil adhesion was calculated as follows,

Neutrophil Index (NI) = TLC X Percentage neutrophils

$$\text{Neutrophil adhesion (\%)} = \frac{\text{NIu} - \text{NIi}}{\text{NIu}} \times 100$$

Where,

NIu: Neutrophil Index before incubation with nylon fibers.

NIi: Neutrophil Index after incubation with nylon fibers.

#### Cyclophosphamide induced neutropenia

Cyclophosphamide induced neutropenia method was as described by Thatte et al. (1987) and Sudha et al. (2010) [13, 14]. Control group received vehicle (1 ml/kg distilled water, i.p.) from first to tenth days. Second groups received levamisole (50 mg/kg, i.p.) from first to tenth days. Third to sixth groups received petroleum ether and 70% ethanol extract (50 and 100 mg/kg, i.p.) respectively from first to tenth days. Seventh group received TRF (10 mg/kg, i.p.) from first to tenth days. On 10<sup>th</sup> day neutropenic dose of cyclophosphamide (200 mg/kg, i.p.) was injected and this day was labelled as day zero. Blood was collected, TLC and DLC count were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and neutrophil counts (%) in treated groups were compared with the values of the control.

#### Statistical analysis

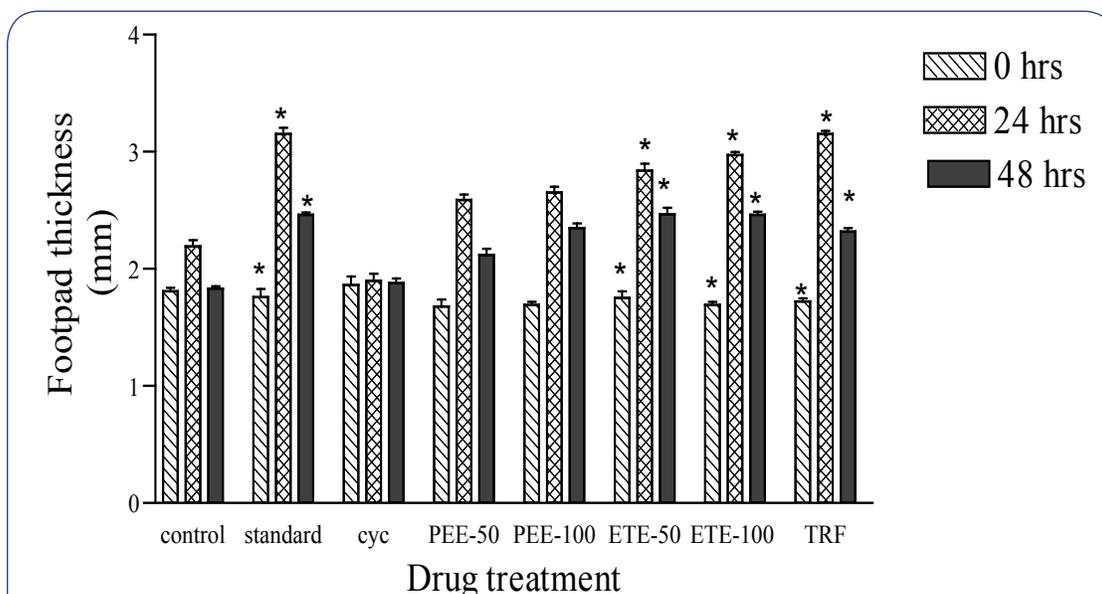
The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test. The values are expressed as mean  $\pm$  SEM and  $P < 0.05$  was considered significant.

### Results and discussion

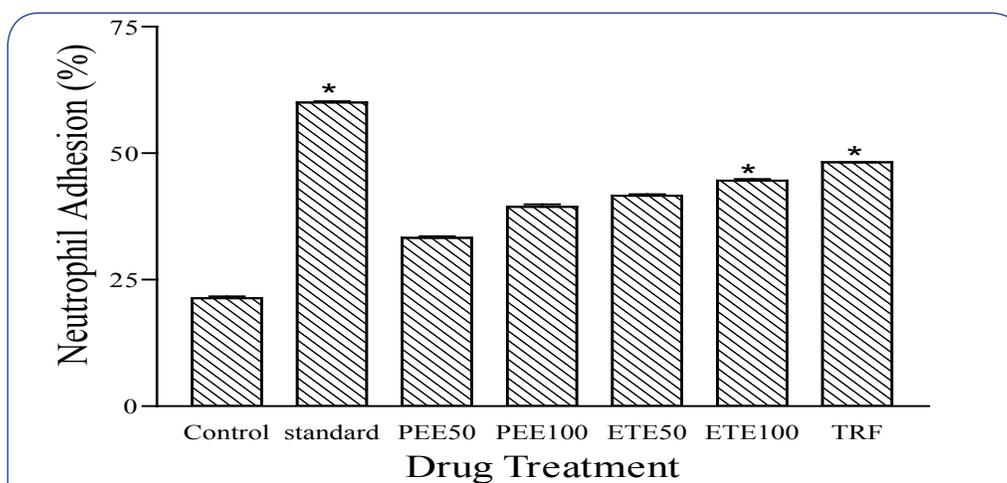
With the immune suppressed groups, where the immunity was suppressed by administration of cyclophosphamide, 70% ethanol extract (50 and 100 mg/kg, i.p) and tyrosine rich fraction (TRF) (10 mg/kg, i.p.) administration showed potentiation of DTH response in terms of significant increase in the mean difference in foot-pad thickness. Result was found to be dose dependant (Figure 1). The DTH response, which is a direct correlate of cell mediated immunity (CMI), was found to be significantly increased in TRF isolated from 70% ethanol extract. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are thus

immobilized to promote defensive (inflammatory) reaction. In our studies, foot volume was enhanced after TRF treatment suggesting cell mediated immune enhancement. Increase in the DTH response indicates that TRF has a stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction [15]. Incubation of neutrophils with nylon fibres produced a decrease in the neutrophil counts due to adhesion of neutrophil to the fibres. 70% Ethanol extract and TRF significantly increased neutrophil

adhesion compared to control (Figure 2). Cytokines are secreted by activated immune cells for margination and extravasations of the phagocytes mainly polymorph nuclear neutrophils. Significantly evoked increase in the adhesion of neutrophils to nylon fibres which correlates to the process of margination of cells in blood vessels [16]. Administration of cyclophosphamide reduced the TLC in control animals by 69.6%. Pre-treatment of animals with TRF showed 43.85% reduction in TLC and 18.72% reduction in neutrophil



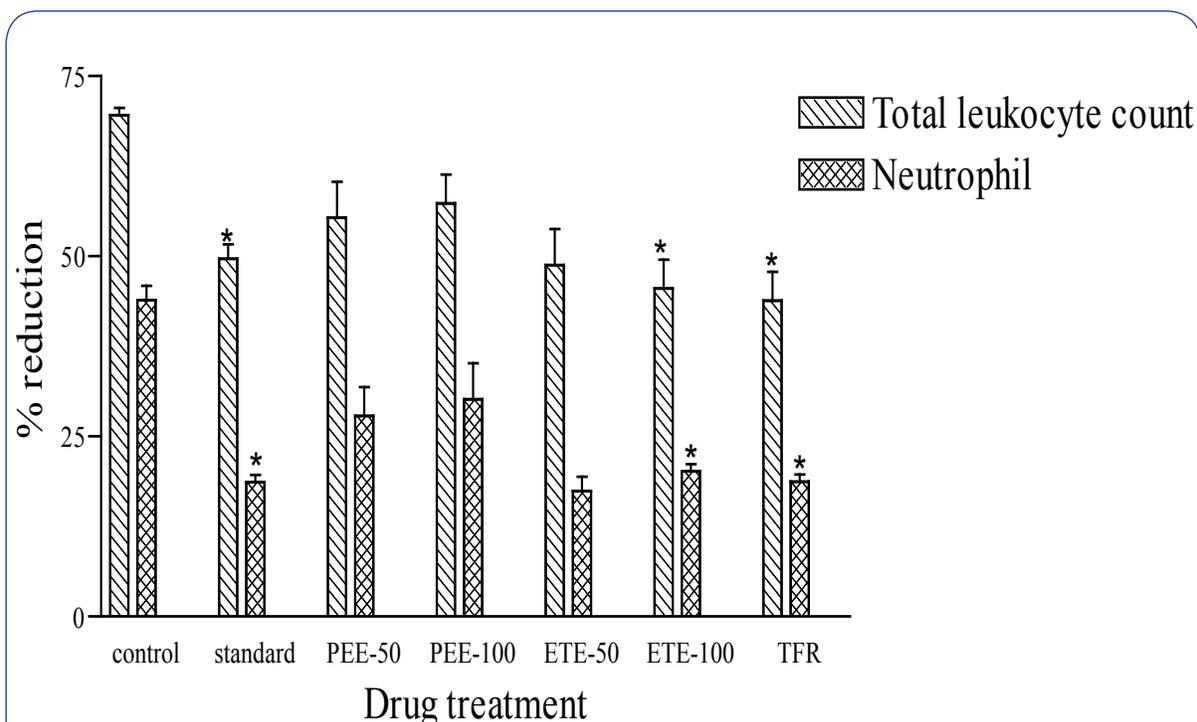
**Figure 1:** Effect of various extracts of *F. religiosa* on cell-mediated immune response by delayed type hypersensitivity induced footpad edema. Results are expressed as Mean  $\pm$  S. E. M. (n=6); \* $p$ <0.05 compared with control. 'cyc' is negative control group, PEE is petroleum ether extract and ETE is 70% ethanol extract. TRF is fraction isolated from 70% ethanol extract.



**Figure 2:** Effect of various extracts of *F. religiosa* L on neutrophil adhesion test. Results are expressed as Mean  $\pm$  S. E. M. (n=6); \* $p$ <0.05 compared with control group, PEE is petroleum ether extract and ETE is 70% ethanol extract. TRF is fraction isolated from 70% ethanol extract.

count at 10 mg/kg (Figure 3). TRF isolated from 70% ethanol extract evoked a significant increase in percent of neutrophils.

This may help in increasing immunity of body against microbial infections. The cyclophosphamide induced neutropenia model



**Figure 3:** Effect of various extracts of *F. religiosa* on cyclophosphamide induced neutropenia. Results are expressed as Mean  $\pm$  S. E. M. (n=6); \* $p < 0.05$  compared with control. PEE is petroleum ether extract and ETE is 70% ethanol extract. TRF is fraction isolated from 70% ethanol extract.

concentrates on the protective effects against cyclophosphamide induced myelosuppression in the experimental animals [17]. 70% ethanol extract and TRF caused decrease in the cyclophosphamide induced neutropenia suggesting that it attenuates the effect of cyclophosphamide on the haemopoetic system.

#### Spectral data of tyrosine

$C_9H_{11}NO_3$ , crystalline powder, Mp 295°C, UV  $\lambda_{max}$  (nm): 285, 265, 257 (MeOH), ESI-MS m/z: 181, 165, 150, 139, 120, 107, 91, 77, 65, 58, and 42, IR (KBr): 3640, 3508, 3410, 3014, 1715, 1656, 1605, 1554, and 1470.

It can be concluded that immunostimulant activity of TRF isolated from 70% ethanol extract was more pronounced in comparison with crude extract and thus it has great potential as a source for natural health products as an immunomodulatory.

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