Immunomodulatory Activity of Terminalia Bellirica Extract in MICE

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Abstract: Terminalia bellirica is one of the oldest medicinal herb of India, is an ingredient of Indian Ayurvedic formulation ‘triphala’ used for the treatment of digestive and respiratory disorders. Present study is aimed to evaluate the Immunomodulatory activity of ethanolic extract of T. bellirica in mice. Immunomodulatory activity of ethanolic extract of T. bellirica (150 and 350 mg/kg, p.o.) was carried out by testing delayed type hypersensitivity (DTH) reaction, phagocytic index, cyclophosphamide induced neutropenia and relative organ weight. Pretreatment with both the doses of ethanolic extract of T. bellirica showed significantly (p<0.01) potentiated the DTH reaction by facilitating the footpad thickness response to SRBC’s in sensitized mice. Moreover, pretreatment with ethanolic extract of T. bellirica (350 mg/kg, p.o.) showed significant (p<0.01) increase in phagocytic index and significant (p<0.05) protection against cyclophosphamide induced neutropenia. Furthermore, significant (p<0.01) increase in relative weight of spleen at 350mg/kg was observed but there was no remarkable change in thymus index was observed in tested doses of plant extract. So, the study demonstrated that T. bellirica triggers both non-specific and specific cellular immunity.

Key words: Terminalia bellirica, Immunomodulatory, delayed type hypersensitivity

Introduction
According to the estimates of the WHO, more than 80% of people in developing countries rely on traditional medicine for their primary health needs. [1] The immune system is involved in the etiology and pathophysioligic mechanisms of various diseases. [2] Hence, the regulation of immune response, whether to stimulate when required or to suppress when unwanted is a major subject of research. [3] Because of the various adverse effects of conventional medicines, the use of natural products as an alternative to conventional treatment in healing and treatment of numerous diseases has been on the rise in the last few decades. [4]

Terminalia bellirica Roxb. (Combretaceae) is a large deciduous tree which occurs widely in the moist valleys of India and its fruits are most commonly used in Indian traditional systems of medicine. The fruit rind is used in different preparation for example, as an ingredient in the popular Ayurvedic formula known as Triphala, used for the treatment of fever, cough, diarrhea, dysentery, skin diseases and liver disorders. This plant exhibits several pharmacological effects including anti-microbial, anti-asthmatic, anti-tussive, anti-spasmodic, anti-mutagenic effects and anti-HIV. [5] Furthermore, some of Terminalia species like Terminalia chebula [6] and Terminalia arjuna [7] have been reported to possess immunomodulatory activity. However, there is no scientific data on the in-vivo immunomodulatory activity of the fruits of this plant. Hence, the present study was designed to evaluate the said activity of the ethanolic extract of the T. bellirica.

Materials and Methods

Collection of Materials
The dried powder of Terminalia bellirica fruits were collected from local market of Bangalore and...
authentified by Dr. Jawahar raveendra, Botanist from IAIM, Bangalore. Cyclophosphamide was received as a gift sample by Biochem pharmaceutical industries Ltd., Daman and other chemicals of analytical grade were used and obtained from the institute’s central store.

**Extract Preparation**

Dried powder of *Terminalia bellirica* fruits was extracted with ethanol (90%) at a temperature of 77-79 °C for 48 h by using soxhlet extractor. After completion of extraction, remaining solvent was removed by evaporation till the solid mass was obtained. Final obtained ethanolic extract was weighed; percentage yield was calculated and stored in airtight container. The freshly prepared solution of extract (dissolved in distilled water) was utilized for the further treatment of animals.

**Phytochemical Analysis**[^8]^[9]

The ethanolic extract of *T. bellirica* fruits was subjected to analysis for the various phytoconstituents like alkaloids, cardiac glycosides, saponins, tannins, flavanoids, proteins, steroids and carbohydrates. Tests for common phytochemicals were carried out with standard methods mentioned in the above references.

**Experimental Animals**

Healthy Swiss albino mice (24-30 g) were procured from Indian Institute of Science, Bangalore. Animals were housed in polypropylene cages and maintained under standard conditions (12 h light/dark cycle, 22 ± 2 °C and 55 ± % relative humidity). They were fed with standard diet and water *ad libitum*. The animals were maintained in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experimental Animals) guidelines for the care and use of laboratory animals. The study protocol was approved by Institutional Animal Ethics Committee (IAEC), Acharya & B.M. Reddy College of Pharmacy, Bangalore (Approval No. IAEC /Ph.cology /03/ 2009-10).

**Antigen**

Fresh blood was collected from sheep’s sacrificed in the local slaughter house in a sterile bottle containing Alsever’s solution (2.05% dextrose, 0.8% sodium citrate, 0.4% sodium chloride and 0.05% citric acid). Alsever’s solution was used in the proportion of 1:2 (Sheep blood: Alsever’s solution) for collection of sheep blood. Blood was centrifuged at 5000 rpm for 10 min and washed six times with normal saline to remove plasma. The number of Sheep red blood cells (SRBC) was then adjusted to a concentration of 1×10⁸ cells after the RBC count. This stock of RBC suspension was used for immunization and challenge.^[10]^[11]

**Dose selection of ethanolic extract of T. bellirica**

Dose of *T. bellirica* was selected from acute toxicity studies performed by Trivedi V P *et al.* According to result of the reference study, maximum tolerable dose of *T. bellirica* fruit extract was found to be 1000 mg/kg, i.p. in mice.^[12] Hence, in the present study we have selected 1/7th and 1/3rd (150 mg/kg and 350 mg/kg) dose of *T. bellirica* as a low dose and high dose respectively.

**Determination of Delayed Type Hypersensitivity Reaction (DTH) using SRBC as an antigen.^[10]^[11]**

In order to determine delayed type hypersensitivity reaction, Mice were divided into three groups of five each. Group I: received normal saline and served as control. Group II and III received ethanolic extract of *T. bellirica* at a dose of 150 and 350 mg/kg, respectively. On the day 0, all the animals were immunized by injecting 1×10⁸ sheep red blood cells (SRBC’s) i.p. The test extract were administered to all the animals from day 0 to day 7. On 7th day the thickness of the right hind foot pad was measured by using vernier caliper. The animals were then challenged by injecting 1×10⁸ SRBC’s in right hind foot pad. Foot pad thickness was measured again 24 h after the challenge. The difference in paw thickness was taken as a measure of delayed hypersensitivity (DTH) and the mean value obtained for treatment groups were compared with control group.

**Determination of Phagocytic Index by Carbon Clearance test:^[13]**

Mice were divided into three different groups of five in each. Group I: received normal saline for
five days and served as control. Group II and III received ethanolic extract of *T. bellirica* at a dose of 150 and 350 mg/kg respectively for five days. After 48 h of five days treatment, mice were injected with Indian ink [which was diluted with PBS (pH 7.4) to 8 times before use] at a dose of 10µl/g of body weight via tail vein. Blood sample were drawn from retroorbital plexus method at the interval of 0, 5, 10 and 15 min. 40 µl of blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and subjected for determination of absorbance at 660 nm by using spectrophotometer.

The Phagocytic index K was calculated by using following equation:

$$K = \frac{(\ln A_2 - \ln A_1)}{(t_2-t_1)}$$

Where, $A_1$ and $A_2$ are the absorbance at times $t_1$ and $t_2$, respectively.

**Cyclophosphamide Induced Neutropenia:**

Here, mice were grouped into three each consisting of five mice. Group I served as control and received the normal saline for 10 days whereas as Group II and III received the ethanolic extract of *T. bellirica* orally at dose of 150 and 350 mg/kg respectively for 10 days. On 10th day, neutropenic dose of cyclophosphamide (200 mg/kg, s.c.) was injected and this day was marked as day 0. Blood was collected by retro-orbital plexus method; total leucocytes count and differential leucocytes count were performed prior to and on day 3 after the injection of cyclophosphamide. The TLC and neutrophil counts (%) in treated groups were compared with the values of control group.

**Effect of *T. bellirica* on lymphoid organ weight:**

After evaluation of total leucocytes count and differential leucocytes counts in mice, on the 11th day all the groups were sacrificed, and organs like thymus gland and spleen were isolated, cleansed with normal saline, blotted and relative organ weight (organ weight/ 100 g of body weight) were recorded for each animals.

**Statistical Analysis**

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

**Results**

The percentage yield of ethanolic extract of *T. bellirica* fruit was found as 15 percent w/w and physical appearance was brown in colour. The result of preliminary phytochemical analysis of ethanolic extract of *T. bellirica* fruit revealed that it contain Carbohydrates, steroids, Tannins, Alkaloids, Saponins and Flavonoids.

**Effect of Ethanolic Extract of *T. bellirica* on DTH Reaction**

Ethanolic extract of *T. bellirica* showed significant ($p < 0.01$) increased in Delayed Type Hypersensitivity (DTH) in all treated groups when compared with control group. The DTH response for the control group was found to be 0.86 ± 0.06 mm, whereas pretreatment groups of ethanolic extract of *T. bellirica* at dose of 150 and 350 mg kg⁻¹ showed 1.25 ± 0.04 and 1.85 ± 0.07 mm respectively. The percentage increased in DTH response in pretreated mice with *T. bellirica* at dose of 150 and 350 mg kg⁻¹ were found to be 62.5% and 91.08% respectively as compared to control group. (Table 1)

<table>
<thead>
<tr>
<th>Group</th>
<th>Increase in DTH response (mm)</th>
<th>%increase in response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.86 ± 0.06</td>
<td>43.93</td>
</tr>
<tr>
<td>T. bellirica (150 mg/kg)</td>
<td>1.25 ± 0.04**</td>
<td>62.5</td>
</tr>
<tr>
<td>T. bellirica (350 mg/kg)</td>
<td>1.85 ± 0.07**</td>
<td>91.08</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=5), **P<0.01 when compared with control group.

**Evaluation of Phagocytic Index by using Carbon Clearance Test**

*In vivo* phagocytic activity of ethanolic extract of *T. bellirica* was determined by the carbon clearance assay in mice. The results of this assay are presented in (Table 2). The phagocytic index (K) for the control group was 0.0563 ± 0.001, whereas for the treated with ethanolic extract of *T. bellirica*
at dose of 150 and 350 mg kg\(^{-1}\) were 0.0445 ± 0.0008 and 0.1124 ± 0.008 respectively. The phagocytic index (K) was significantly (p<0.01) higher for the group treated with ethanolic extract of T. bellirica at a dose of 350 mg/kg, as compared to control group.

### Table 2

Effect of Ethanolic Extract of T. bellirica on Carbon Clearance Test

<table>
<thead>
<tr>
<th>Group</th>
<th>Phagocytic index K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0563 ± 0.001</td>
</tr>
<tr>
<td>T. bellirica</td>
<td>0.0445 ± 0.0008**</td>
</tr>
<tr>
<td>T. bellirica</td>
<td>0.1124 ± 0.008**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=5), **P<0.01 when compared with control group, ns= non significant.

### Effect of Ethanolic Extract of T. bellirica on Cyclophosphamide Induced Neutropenia

The neutropenic dose of cyclophosphamide reduced the TLC in control animals by 41.15% as compared to its initial value. Pretreatment of ethanolic extract of T. bellirica at a dose of 150 mg/kg and 350 mg/kg for 10 days before cyclophosphamide administration produced 38.64% and 33.46% reduction in TLC respectively as compared to its initial value. The percentage reduction in neutrophil count was found to be 29.26% in control group, whereas pretreatment with ethanolic extract of T. bellirica at a dose of 150 and 350 mg/kg showed 23.43% and 13.19% reduction in neutrophil count as compared to initial value. However, the treated groups did not produce any significant effect in TLC reduction when compared to control group (Table 3).

### Table 3

Effect of Ethanolic Extract of T. bellirica on Cyclophosphamide Induced Neutropenia

<table>
<thead>
<tr>
<th>Group</th>
<th>TLC 10^3 cells/mm^3 Before</th>
<th>TLC 10^3 cells/mm^3 After</th>
<th>% reduction</th>
<th>% neutrophils Before</th>
<th>% neutrophils After</th>
<th>% reduction in neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.51 ± 0.17</td>
<td>3.23 ± 0.10</td>
<td>41.15 ± 2.61</td>
<td>26.2 ± 1.02</td>
<td>18 ± 0.89</td>
<td>29.64 ± 2.88</td>
</tr>
<tr>
<td>T. bellirica</td>
<td>5.99 ± 0.16</td>
<td>3.65 ± 0.14</td>
<td>38.65 ± 4.01</td>
<td>24.4 ± 1.57</td>
<td>18.6 ± 1.21</td>
<td>23.43 ± 3.83**</td>
</tr>
<tr>
<td>T. bellirica</td>
<td>6.80 ± 0.16</td>
<td>4.52 ± 0.17</td>
<td>33.47 ± 1.54*</td>
<td>28 ± 1.61</td>
<td>24.4 ± 2.09</td>
<td>13.20 ± 3.76*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=5), *P<0.05 when compared with control group, ns= non significant.

### Effect of Ethanolic Extract of T. bellirica on Lymphoid Organ Weight

The pretreatment with ethanolic extract of T. bellirica did not produce any significant alteration in the relative weight of thymus as compared to control group. However, significant (p<0.01) increase was observed in the relative weight of spleen in the group of animals treated with 350 mg/kg dose of ethanolic extract of T. bellirica. (Table 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Organ weight relative to 100 g of body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymus (weight in g)</td>
</tr>
<tr>
<td>Control [0.1 ml / 10 g of (0.9 % normal saline)]</td>
<td>0.1205 ± 0.009</td>
</tr>
<tr>
<td>T. bellirica (150 mg/kg)</td>
<td>0.1260 ± 0.005*</td>
</tr>
<tr>
<td>T. bellirica (350 mg/kg)</td>
<td>0.1395 ± 0.005*</td>
</tr>
<tr>
<td></td>
<td>Spleen (weight in g)</td>
</tr>
<tr>
<td>Control [0.1 ml / 10 g of (0.9 % normal saline)]</td>
<td>0.3351 ± 0.015</td>
</tr>
<tr>
<td>T. bellirica (150 mg/kg)</td>
<td>0.3662 ± 0.008*</td>
</tr>
<tr>
<td>T. bellirica (350 mg/kg)</td>
<td>0.4316 ± 0.019**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=5), **P<0.01 when compared with Control group, ns= non significant.

### Discussion

Modulation of the immune response in the course of stimulation or suppression may help in maintaining a disease-free state. Drugs or agents which improve host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy. [2] Immunomodulatory activity of ethanolic extract of T. bellirica was explored by evaluating its effect on DTH response, phagocytic index, cyclophosphamide induced neutropenia and lymphoid organ weight in mice.
The Delayed type hypersensitivity (DTH) test is an antigen specific response, when small quantities of antigen injected dermally, a hallmark response was elicited which includes indurations, swelling and mononcytic infiltration into the site of the lesion within 24 to 72 h. Further, DTH reaction requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn enhanced vascular permeability, induce vasodilatation, macrophage accumulation and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. When activated TH1 cells come across certain antigens, viz. SRBCs. They secrete cytokines that induce a localized inflammatory reaction called delayed type hypersensitivity. DTH comprises of two phases, an initial sensitization phase after the primary contact with SRBC antigen. During this phase TH1 cells are activated and clonally expanded by APC with class II MHC molecule. A resultant exposure to the SRBCs antigen induces the effector phase of the DTH response, in which TH1 cells secrete a variety of cytokines that involve in recruitment and activation of macrophages and other non-specific inflammatory mediators. The delay in the onset of the response reflects the times required for the cytokines to recruits and activate macrophages. In the present study, DTH reaction was measured by foot-pad thickness, after 24 h of antigenic challenge and subsequent immunization with SRBC, the pretreated group animals showed significant \( p<0.01 \) increase in the DTH response indicates that both the dose of ethanolic extract of \( T. bellirica \) has stimulatory effects on lymphocytes and necessary cell types required for the expression reaction as compared to control. There are some plants like \( Aesculus indica, Argyreia speciosa, Randia dumetorum \) which are reported for acting on DTH has supported our observations.

Phagocytic index commonly increases whenever there is an increase in immune response and its effects are associated with varied pathologic conditions in humans. In addition, phagocytosis represents a crucial innate defense mechanism against ingested particulates including whole pathogenic microorganisms. The specialized cells that are involved in process of phagocytosis include blood monocytes, neutrophils and tissue macrophages. In a scrutiny of the key role played by the macrophages in coordinating the processing and presentation of antigen to B-cells hence in the present study ethanolic extract of \( T. bellirica \) was evaluated for its effect on macrophage phagocytic activity.

Hence the carbon clearance test was performed to evaluate the effect of extract on the reticulo endothelial system (RES). The RES is a diffuse system comprising of phagocytic cells. Cells of the RES play a dynamic role in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of carbon clearance from the blood by macrophage is governed by an exponential equation. This seems to be the ordinary way in which inert particulate matter is cleared from the blood. In the present study, pretreatment with ethanolic extract of \( T. bellirica \) at a dose of 350 mg/kg and 150 mg/kg showed remarkable augmentation in the Phagocytic ability. Ethanolic extract of \( T. bellirica \) at a dose of 350 mg/kg was found to increase the activity of reticuloendothelial system as evidenced by a significant \( p<0.01 \) increase in the rate of carbon clearance. While, pre-treatment with ethanolic extract of \( T. bellirica \) at a dose of 150 mg/kg did not produce any significant effect.

Cyclophosphamide induced neutropenia model concentrates on the protective effects of extract on the haemopoeitic system. Cyclophosphamide acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis, function and causes myelosuppression in the experimental animals. In the present study, pretreatment with ethanolic extract of \( T. bellirica \) at a dose of 150 and 350 mg/kg caused 23.43% and 13.19% reduction in the cyclophosphamide induced neutropenia suggesting that it attenuates the effect of cyclophosphamide on the haemopoeitic system. The prevention of neutropenia induced by cyclophosphamide may be through activation of macrophages, which secrete a large number of substances including colony stimulating factor and interleukin 1.
Moreover, relative organ weight of Thymus and Spleen, which are the two major lymphoid organs were evaluated. Pretreatment with ethanolic extract of *T. bellirica* considerably increased the relative organ weight of the spleen as compared to control group, indicating its potentiating effect on the restoration of production of immune cells which was decreased by the cyclophosphamide. While, there was no significant alteration in thymus index.

**Conclusion**

The studies have demonstrated non-specific and specific immunostimulatory properties of the ethanolic extract of *T. bellirica* fruits. This suggests its therapeutic usefulness in immunocompromised conditions.

**Acknowledgement**

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**References**


