



Protection by *Terminalia Chebula* against Lipopolysachharide-reactivated Antigen-induced Arthritis via Modulation of Toll-like Receptors in Murine Model

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ABSTRACT

Reactivation of antigen induced arthritis by bacterial lipopolysachharide (LPS) in a murine model was used to determine whether *Terminalia chebula* exerts its protective activity via modulation of Toll-like receptors (TLR). Arthritis was induced in 8-9 weeks old female Swiss albino mice by intra-articular antigen challenge following immunization with antigen. Oral administration of LPS caused reactivation of arthritis. Treatment with hydroalcoholic extract of *Terminalia chebula* lowered the plasma level of tumor necrosis factor α (TNF α), a TLR4 downstream cytokine and increased the plasma levels of interleukin 2 (IL2), which is TLR2 cytokine, significantly. ($p < 0.001$) The results indicate that *Terminalia chebula* might be acting as TLR2 agonist or at some intermediary stage between TLR2 activation and IL-2 production. The role of TLR4 in mediating the protective action of TC cannot be ruled out.

Keywords: *Terminalia chebula*, Haritaki, Toll-like receptors, Antigen-induced arthritis, lipopolysachharide.

INTRODUCTION

Mammalian Toll-like receptors (TLRs) are important for innate immunity. Phagocytes like neutrophils, macrophages and dendritic cells discriminate between self and foreign with help of TLRs. TLRs recognize conserved molecular motifs of microbes which are absent or rare in vertebrates. Dendritic cell activation, which is important for acquired immune response, is also initiated by activation of TLRs expressed on cell surface. TLRs, therefore, are important for innate immune response as well as acquired immune response.¹⁻² TLRs are reported to recognize endogenous ligands produced during inflammatory reaction. LPS is a known ligand for TLR4.^{3,4}

Terminalia chebula Retz. (Fam. Combretaceae), commonly known as Haritaki, (Sanskrit: Abhaya, English: Myrobalan, Hindi: Harad) has many traditional uses like constipation, tastelessness, diseases of abdomen, piles, anaemia, inflammation, chronic and intermittent fever, bronchial asthma and heart disease. Haritaki is one of the three constituents of the famous Ayurvedic formulation *Triphalaa*. The major active constituent of the plant are polyphenols like chebulagic acid and chebulinic acid.⁵ The fruits of haritaki have been shown to be potential source of natural anti-Alzheimer compound, 1,2,3,4,6-penta-O-galloyl- β -D-glucose, having significant acetylcholinesterase and butyrylcholinesterase inhibitory activity and strong anti-oxidant property.⁶ *Terminalia chebula* extract was found to lower the tumor necrosis factor α (TNF α) levels in Complete Freund's adjuvant induced and formalin induced arthritis in rats.⁷ Water soluble fraction of haritaki was reported to decrease histamine release from rat peritoneal mast cells (RPMC), but it increased TNF α secretion from RPMC.⁸ Methanolic fruit extract of haritaki was reported to inhibit lipid peroxide formation and to scavenge hydroxyl and

superoxide radicals *in vitro*. Oral administration of the extract (100 mg/kg body weight) reduced the blood sugar level in normal and in alloxan (120 mg/kg) diabetic rats significantly within 4 h.⁹ Chebulagic acid, the major constituent of haritaki, suppressed onset and progression of collagen-induced arthritis in mice by immune suppression via induction of transforming growth factor β and CD4+, CD25+ T cells.¹⁰ The same study reported that chebulagic acid elevates phosphorylation of extracellular signal-regulated kinase (ERK) in Jurkat cells previously activated by PMA/ionomycin, ultimately leading to increased protein kinase C (PKC). However, in an *in vitro* enzyme based study, chebulagic acid was found to have PKC inhibitory action.¹¹

These reports describing various and sometimes contradictory activities of Haritaki prompted us to investigate the mechanism of Haritaki at cellular level in whole animals. As TLRs have been found to be a link between cellular and humoral immune systems, we hypothesized that haritaki exerts its immunomodulatory action via TLRs. It is important to study whole animals in studies involving TLRs as the final immune response seen in clinical practice is the result of cross-talk between various components of immune system, which may be easily neglected while using cell lines. The study used a murine model of reactivation of antigen-induced arthritis (AIA) by lipopolysaccharide (LPS). AIA closely mimics human rheumatic arthritis.¹² Rheumatic arthritis is an auto-immune disorder; but its exact etiology remains poorly understood. It has been proposed that exposure to microorganisms like mycobacteria, mycoplasma, *Escherichia coli*, *Proteus mirabilis*, Epstein-Barr virus and human parvovirus B19¹³ and oral bacteria like *Porphyromonas gingivalis*¹⁴ may trigger auto-immune reaction, ultimately leading to this debilitating disease. The objective of present study is to investigate whether *Terminalia chebula* (TC) acts by modulation of TLR. We



report that simultaneous administration of TC significantly lowers tumor necrosis factor alpha (TNF α) levels and increases interleukin 2 (IL2) levels in plasma of treated animals suggesting the down regulation of TLR4 and up regulation of TLR2. TC also protects the joint tissues against reactivated AIA.

MATERIALS AND METHODS

Animals

Female Swiss albino mice of 8-9 weeks of age were used in experiments. The study protocol was approved by the Institutional Ethics committee. The animals were kept at a controlled temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with 12 h light/dark cycle. The animals had *ad libitum* access to food and water.

Preparation and quantification of extracts

Terminalia chebula fruit powder was procured from local suppliers. Powder was examined for microscopical characters to establish the purity and identity. For the preparation of hydroalcoholic extracts, the powdered drug was allowed to soak in water-alcohol (40:60) mixture overnight and then extracted using Soxhlet apparatus to a syrupy consistency. This liquid extract was dried in vacuum oven at a temperature not exceeding 55°C . The extract was quantified by HPTLC method (Camag TLC scanner 4^{*} and Camag Linomat 5^{*} semiautomatic sample applicator, Switzerland) using Ethyl Acetate: Toluene: Formic Acid: Methanol (6: 1: 1: 2, v / v) as mobile phase against chebulagic acid (Natural Remedies Private Limited, Bangalore, India) as external standard.

Induction of AIA

Mice were divided into three groups of six animals each. Group I was kept as phosphate buffer saline (PBS) control. Group II and III were subjected to AIA induction followed by LPS. Group III additionally received the treatment of TC (300 mg/kg, p.o.) daily, starting from day 0.

AIA was induced in female Swiss albino mice as described previously.¹⁵ Briefly, mice were immunized by administering subcutaneously 100 μg of Bovine Albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50 μl of PBS and emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Twenty-one days later, the animals were intra-articularly challenged by injection of 25 μl of PBS containing 50 μg of BSA into left ankle joints. The right ankle joints, which received 25 μl PBS served as control arm. LPS from *E. coli* (Sigma-Aldrich, St. Louis, MO, USA) in a dose of 25mg/kg in PBS was administered orally using a modified syringe 48 h after BSA challenge. The severity of arthritis was evaluated by measuring the thickness of the both ankles using a digital screw gauge 52 h post challenge. The net increase in joint thickness attributable to the LPS administration was calculated by subtracting the increase in thickness of the right ankle from that of the left ankle.

Measurement of cytokines

Blood samples were collected by retro-orbital method 4 h after LPS administration. Blood was centrifuged at 3000 rpm for 10 minute at 4°C to separate plasma. The plasma samples were stored at -40°C until analyzed for TNF α and IL-2. Analysis was done using commercially available ELISA kits (BD Biosciences, USA).

Histology

Animals were killed 4 h after LPS administration. Ankle joints were removed and stabilized in 10% formalin prepared in PBS. Decalcification of specimens was carried out before processing for paraffin blocks. The embedded tissue were stained with hematoxylin and eosin stain and observed under microscope (Carl Zeiss, Germany) at 45X magnification for determining the pathological changes in the joints.

RESULTS

Quantification of extracts

HPTLC of the extract showed that TC contained 0.591 ± 0.096 % bioactives calculated as chebulagic acid.

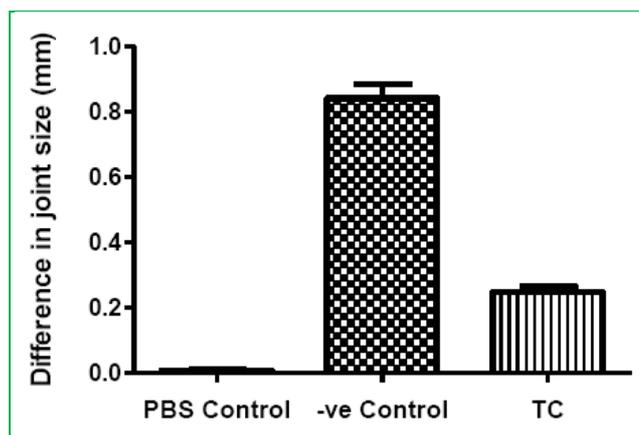


Figure 1: Effect of TC treatment on joint swelling. PBS Control received only phosphate buffer saline, -ve Control was immunized with Bovine Albumin (BSA) followed by intra-articular challenge of BSA on day 21. LPS was orally administered 48 h post challenge. TC additionally received *Terminalia chebulahydroalcoholic* extract (300mg/kg) daily from day 0. Joint size was measured using digital screw gauge. The net increase in joint thickness attributable to the LPS administration was calculated by subtracting the increase in thickness on the right ankle from that of the left ankle. Data shown are mean \pm SEM of six mice. ($p < 0.001$, One-Way ANOVA followed by Tukey's Test using GraphPad Prism[®])

Protection to reactivation of AIA

Marked swelling was observed in ankle joints of the animals at 48 h post LPS administration. Although TC did not show complete remission of inflammation, it afforded significant protection against LPS-induced reactivation of arthritis as shown in the graph. (Fig. 1)

Histology

Histological analysis of joints showed that LPS causes marked swelling, cartilage destruction and infiltration of leucocytes. TC treated mice showed less severe pathological changes in joints. (Fig. 2)



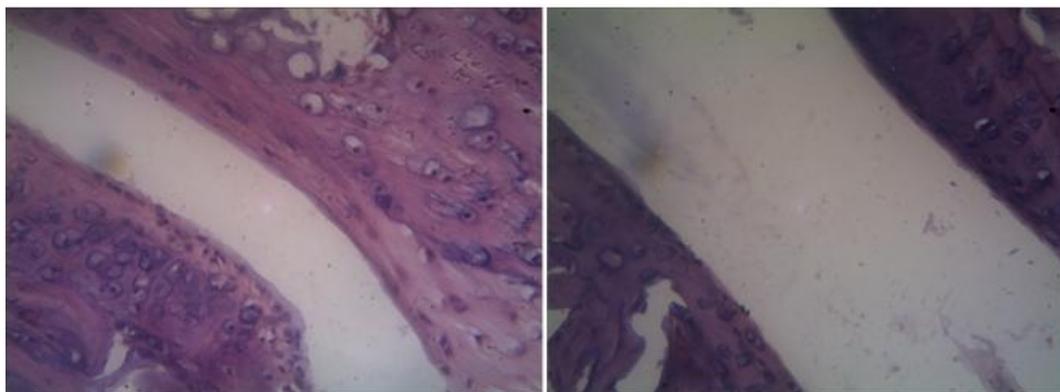


Figure 2: Histopathologic changes in reactivation of Antigen-induced arthritis by lipopolysaccharide. Left: Ankle joint of mouse from -ve Control group showing marked inflammation and cell infiltration. Right: Ankle joint of mouse treated with hydro-alcoholic extract of *Terminalia chebula* (300mg/kg) daily, shows moderate inflammation and cell infiltration.

Measurement of TNF- α and il-2

Reactivation of AIA following LPS administration was accompanied by increased TNF α levels and diminished IL-2 levels. Mice treated with TC showed significantly lower levels of TNF α and increased levels of IL-2 compared to negative control group. (Fig. 3 & 4)

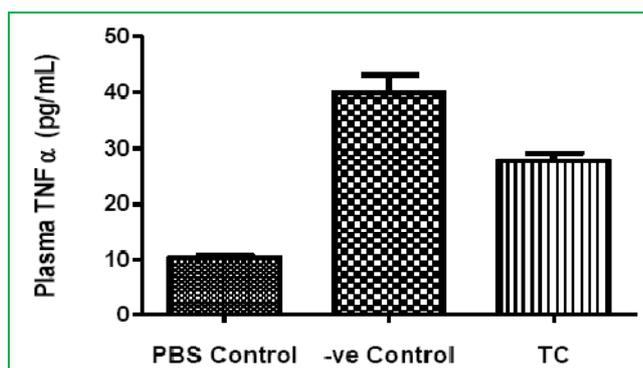


Figure 3: Effect of TC treatment on serum tumor necrosis factor alpha (TNF α). PBS Control received only phosphate buffer saline, -ve Control was immunized with Bovine Albumin (BSA) followed by intra-articular challenge of BSA on day 21. Lipopolysaccharide (LPS) was orally administered 48 h post challenge. TC additionally received *Terminalia chebulahydroalcoholic* extract (300mg/kg) daily from day 0. Blood was collected by retro-orbital method 4 h after LPS administration and TNF α was measured by ELISA. Data shown are mean \pm SEM of six mice. ($p < 0.001$, One-Way ANOVA followed by Tukey's Test using GraphPad Prism[®])

DISCUSSION

A number of studies have linked the role of systemic administration of LPS with autoimmune disorders. Systemic LPS has been shown to enhance autoimmune nephritis in mice¹⁶, experimental autoimmune encephalomyelitis¹⁷ and collagen-induced arthritis (CIA)¹⁸. In addition, Yoshino et al¹⁵ have demonstrated the role of oral LPS in reactivation of AIA. Crude LPS is a known ligand for TLR2 and TLR-4, both of which are involved in inflammation. However, purified LPS, used in the present investigation, is recognized by TLR4 and not by TLR2. The reactivation of AIA by LPS indicates involvement of TLR4. This is confirmed by increased levels of TNF α . TC treatment effectively lowered TNF α levels, indicating the down-regulation of TLR4 (Fig. 3). These results are

consistent with those reported by Nair *et al* for CFA and formalin induced arthritis.⁷

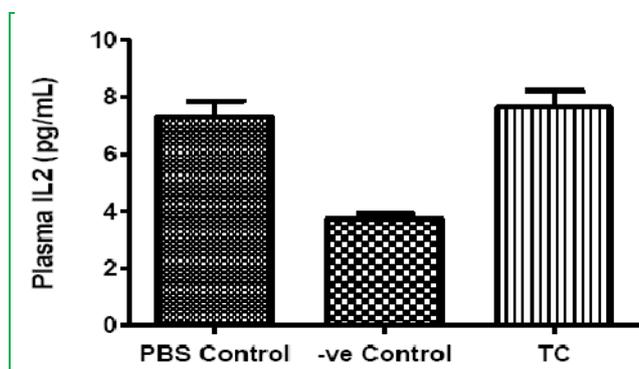


Figure 4: Effect of TC treatment on plasma interleukin-2 (IL2). PBS Control received only phosphate buffer saline, -ve Control was immunized with Bovine Albumin (BSA) followed by intra-articular challenge of BSA on day 21. Lipopolysaccharide (LPS) was orally administered 48 h post challenge. TC additionally received *Terminalia chebulahydroalcoholic* extract (300mg/kg) daily from day 0. Blood was collected by retro-orbital method 4 h after LPS administration and IL2 was measured by ELISA. Data shown are mean \pm SEM of six mice. ($p < 0.001$, One-Way ANOVA followed by Tukey's Test using GraphPad Prism[®])

The role of TLRs in various rheumatic disorders has been reviewed by Santegoets et al.¹⁹ Different TLRs have been known to have contrasting actions. It has been shown that TLR4 plays a role in disease aggravation, while TLR2 helps in suppressive function of regulatory T cells.²⁰ TLR2 has been shown to trigger direct Th1 effector functions²¹, which are important counter-actions to Th2 effects in maintaining the balance of immune system. One of the important Th1 cytokine IL2 is increased as a result of treatment with TC. (Fig. 4) This clearly shows that TC not only down-regulates TLR4, but up-regulates TLR2 also; thus correcting the skewed immunological balance in murine model of AIA reactivation. TLR2 agonists have been reported to increase the tolerance to LPS, a TLR4 agonist, and consequently, reduce the TNF α levels by MyD88-dependent pathway.²² In our study, TC-treated animals had significantly lower levels of TNF α compared to untreated animals (Fig. 3), clearly suggesting that TC acts by TLR2 upregulation.

CONCLUSION

It can be concluded that hydroalcoholic extract of TC acts either as a TLR2 agonist or acts on some intermediary pathway between TLR2 activation and IL-2 production, thus countering the effects of LPS administration. The role of TLR4 in mediating the protective action of TC cannot be ruled out. Further studies are required to determine the exact stage at which TC acts in TLR4 and TLR2 pathways.

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