

# Inhibitory effect of ethanolic leaf extract of *Acacia etbaica schweinf* on production of pro-inflammatory cytokines in lipopolysaccharide-stimulated RAW 264.7 macrophage cells

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## ABSTRACT

**Introduction:** *Acacia etbaica* Schweinf is a medicinal plant belonging to family Fabaceae that has long been used in traditionally to treat stomach pain, swelling, livestock eye infection, itching, and fire burn. **Objective:** The objective of the study was to investigate the *in vitro* anti-inflammatory effect of ethanolic leaf extract of *A. etbaica* Schweinf in lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cell lines. **Materials and Methods:** The ethanolic leaf extract of *A. etbaica* Schweinf was prepared and followed by to investigate the inhibitory effect on LPS -induced production of pro-inflammatory cytokines (interleukin-1 $\beta$  [IL]; IL-6; and tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ]) in RAW 264.7 cells were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) kit. **Results:** The ethanolic leaf extract of *A. etbaica* Schweinf was significantly decreased the production of inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in a dose depended manner in LPS-stimulated RAW 264.7 cells. **Conclusion:** It can be concluded that the ethanolic leaf extract of *A. etbaica* Schweinf is a potential candidate for the development of anti-inflammatory agents for the treatment of inflammatory-related diseases.

**KEY WORDS:** *Acacia etbaica* Schweinf, Anti-inflammatory, Interleukin-1 $\beta$ , Interleukin-6, Tumor necrosis factor- $\alpha$

## INTRODUCTION

Inflammation is the body's protective immune response to pathogens, chemicals, radiation, and mechanical injury. During the acute inflammation, macrophages accumulated at the site of infection then recognize, engulf, and digests cellular debris.<sup>[1]</sup> During the upregulation of acute inflammation has been implicated in chronic inflammatory-related disease, such as inflammatory bowel diseases, asthma, rheumatoid arthritis, multiple sclerosis, Parkinson's, and Alzheimer's diseases and cancer.<sup>[2]</sup> An endotoxin lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria, activates the macrophages and result in the secretion of a number of different pro-inflammatory mediators and cytokines, including nitric oxide (NO), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>[3]</sup>

IL-1 $\beta$  is a pro-inflammatory cytokine trigger fever by enhancing the production of prostaglandin E<sub>2</sub> and enhances the release of histamine from mast cells at the site of infection site and induces the production of different arachidonic metabolites and enhances inflammation and inflammatory diseases.<sup>[4]</sup> TNF- $\alpha$  is a pro-inflammatory molecule released from LPS-stimulated macrophage and is responsible to induce acute and chronic inflammation.<sup>[5]</sup> IL-6, in combination with its soluble receptors IL-6 Ra, leads the transition from acute to chronic inflammation by changing the nature of leucocytes infiltrate (from polymorphonuclear neutrophil to monocyte/macrophage).<sup>[6]</sup>

Non-steroidal anti-inflammatory drugs are clinically used for the treatment of inflammatory disorders; however, there are severe side-effects associated with current anti-inflammatory drugs and it is of the utmost importance to develop new anti-inflammatory compounds with fewer side-effects and different mechanisms of action.<sup>[7]</sup> For many years,

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plant-derived medicines have been used with apparent safety and efficacy for alleviating and treating various diseases in many countries. In Ethiopia, about 80% of the human population and 90% of livestock depends on the medicinal plant for curing their ailment.<sup>[8]</sup>

The genus *Acacia* can provide the nutrients and therapeutic ingredients to prevent, mitigate, or treat many diseases. This species *etbaica* contains a variety of bioactive components such as phenolic acids, alkaloids, terpenes, tannins, and flavonoids,<sup>[9]</sup> which are responsible for numerous biological and pharmacological properties such as hypoglycemic, anti-inflammatory, antibacterial and antiplatelet aggregatory, antihypertensive, analgesic, anticancer, and antiatherosclerotic activities due to their strong antioxidant and free radical scavenging activities.<sup>[10]</sup> The medicinal plant *Acacia etbaica* Schweinf commonly used traditionally in Ethiopia as for the treatment of inflammatory-related diseases.

Therefore, the present study was carried out to evaluate the inhibitory effect of ethanolic leaf extract of *A. etbaica* Schweinf on the production of LPS-stimulated RAW 264.7 macrophage cells.

## MATERIALS AND METHODS

The plant material was collected from Aksum Sofho district which is located in Central Zone of Tigray, Ethiopia. The plant specimen was authenticated by Mr. Melaku Wendafrash and Mr. Wegye Abebe, Department of Biology, Addis Ababa University, Addis Ababa. The voucher specimen number TT001 was deposited in the laboratory for future reference. The leaves were properly washed with distilled water, dried in the shade at room temperature for 10 days, crushed in a mill, and stored in an airtight container for further use.

### Extraction of the Plant Material

A hundred grams of the powdered plant material was taken and subjected to sequential extraction by the Soxhlet apparatus using 500 ml of ethanol. The extracted ethanolic extract was concentrated under reduced pressure and vacuum using a rotary evaporator. The ethanolic concentrate was kept in an airtight container and stored at 4°C further use.

### RAW 264.7 Macrophage Cell line Culture and Maintenance

The RAW 264.7 mouse macrophage cell line was obtained from American Type Culture Collection, the USA and cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin used as an antibiotic and maintained at 37°C in 5% CO<sub>2</sub> incubator.

### Effect of Ethanolic Leaf Extract of *Acacia etbaica* Schweinf on TNF-α, IL-1β, and IL-6 Produced by LPS-stimulated RAW 264.7 Macrophages Cells

Based on our previous reports of cytotoxicity and inhibitory effect on NO production on LPS-stimulated RAW264.7 cells, then two different concentrations (50 and 100 µg/ml) of ethanolic leaf extract of *A. etbaica* Schweinf were selected for further analysis.<sup>[11]</sup> The inhibitory effect on the production of TNF-α, IL-1β, and IL-6 was measured using a commercial Sandwich ELISA kit (e-biosciences, USA). The RAW 264.7 (5×10<sup>3</sup>) cells were cultured in 96 well plates for overnight and pre-treated with two concentrations (50 and 100 µg/ml of AE-ET) for 2 h then stimulated with 1 µg/ml of LPS for another 24 h. Supernatants were collected by centrifugation at 2500 g for 15 min and TNF-α, IL-1β, and IL-6 were quantified using enzyme-linked immunosorbent assay kit (eBioscience USA) according to manufacturer's instruction protocol.

$$\begin{aligned} & \% \text{ of Inhibition of } (TNF-\alpha, IL-1\beta \text{ and } IL-6) \\ & = \left( \frac{O.D. \text{ of control} - O.D. \text{ of test}}{O.D. \text{ of control}} \right) \times 100 \end{aligned}$$

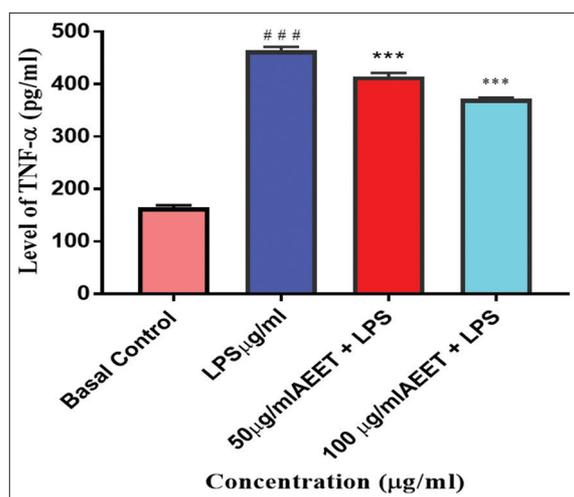
### Statistical Analysis

The data were expressed as the mean ± SD of triplicate analysis ( $n = 3$ ). The statistical analysis was carried out by one-way ANOVA, followed by Turkey's test using GraphPad Prism software version 7.0. The IC<sub>50</sub> values were calculated from the concentration-effect curves of linear regression using MS Excel software. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , and \*\*\* $P \leq 0.001$  represent a significant difference compared with the control group.

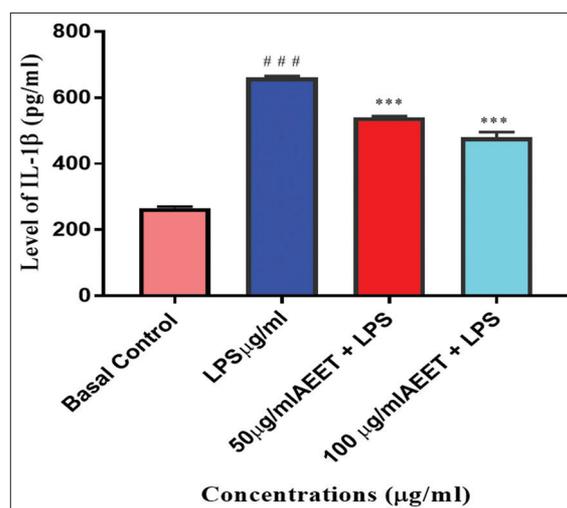
## RESULTS AND DISCUSSION

### The Inhibitory Effect of Ethanolic Leaf Extract of *A. etbaica* Schweinf on TNF-α Production by LPS-stimulated RAW 264.7 Macrophage Cells

In the present study, the LPS-stimulated RAW 264.7 macrophage cells, the TNF-α levels were 463.87 ± 7.09 pg/ml in which, it was significantly increased when compared to basal control (164.81 ± 3.97). Treatment of LPS-stimulated macrophage cells with ethanolic leaf extract of *A. etbaica* Schweinf significantly decreased the production of TNF-α in a concentration-dependent manner at  $P < 0.001$ , as shown in Figure 1. The results of the present study correlated with the same cell line reported by Chaithanya *et al.*,<sup>[12]</sup> the mesuaferriin-A belongs to bioactive flavonoid present in ethyl acetate bark extract of *Mesua ferrea* L. significantly reduced the TNF-α production in a dose-dependent manner on LPS-stimulated RAW.264.7 macrophage cells.



**Figure 1:** Effect of two concentrations (50 and 100 µg/ml) of ethanolic leaf extract of *Acacia etbaica* Schweinf on tumor necrosis factor-α (TNF-α) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. The results are means of triplicate values ± SD <sup>###</sup> $P < 0.001$  LPS is significantly different compare to basal control and <sup>\*\*\*</sup> $P < 0.001$  represents significance difference TNF-α level compared to LPS-treated control group



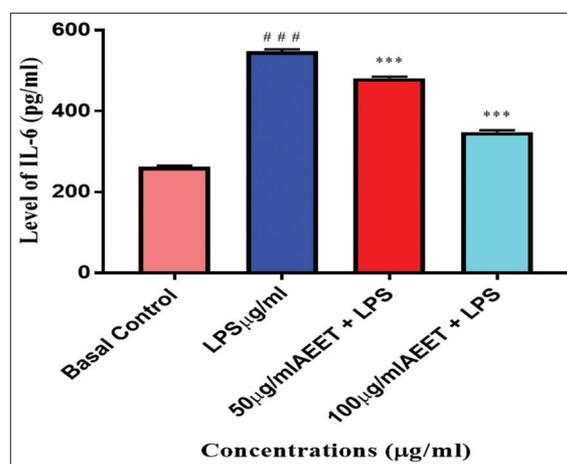
**Figure 2:** Effect of two concentrations (50 and 100 µg/ml) ethanolic extract of *Acacia etbaica* Schweinf on interleukin-1β produced by lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells. The results are means of triplicate values ± SD <sup>###</sup> $P < 0.001$  LPS is significantly different compare to basal control and <sup>\*\*\*</sup> $P < 0.001$  represents significance difference compared to LPS-treated control group

### The Effect of Ethanolic Leaf Extract of *A. etbaica* Schweinf on IL-1β Produced by LPS-stimulated RAW 264.7 Macrophage Cells

As shown in Figure 2, the treatment of RAW 264.7 macrophage cells with LPS significantly increased IL-1β ( $661.23 \pm 4.16$  pg/ml) compared to untreated RAW 264.7 macrophage cells ( $265.52 \pm 4.53$  pg/ml) (<sup>\*\*\*</sup> $P < 0.001$ ) and the treatment of LPS-stimulated RAW 264.7 macrophage cells with the ethanolic leaf extract of *A. etbaica* Schweinf significantly decreased the production of IL-1β in a concentration-dependent manner. This could be due to the presence of secondary metabolites present in the ethanolic leaf extract of *A. etbaica* Schweinf. *M. ferrea* bark ethyl acetate extract inhibits the synthesis of IL-1β concentration-dependently in LPS-induced RAW 264.7 cells reported by Chaithanya *et al.*<sup>[13]</sup> Pycnogenol is a phenolic compound purified from the bark of *Pinus maritime* Mill, highly capable of reducing the synthesis of IL-1β in LPS-stimulated RAW 264.7 cells Scarpioni *et al.*<sup>[14]</sup> The results of the present study correlated with the same cell line.

### The Effect of Ethanolic Leaf Extract of *A. etbaica* Schweinf on IL-6 Produced by LPS-stimulated RAW 264.7 Macrophage Cells

In the present study, the ethanolic leaf extract of *A. etbaica* Schweinf significantly decreased the production of IL-6 in LPS-stimulated RAW 264.7 macrophage cells, as shown in Figure 3. The result of the current study is consistent with the methanolic extract of *Acacia ferruginea* significantly reduced IL-6 in a Dalton lymphoma ascites bearing solid tumor



**Figure 3:** Effect of two concentrations (50 and 100 µg/ml) ethanolic extract of *Acacia etbaica* Schweinf on IL-6 produced by lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells. The results are means of triplicate values ± SD <sup>###</sup> $P < 0.001$  LPS is significantly different compare to basal control and <sup>\*\*\*</sup> $P < 0.001$  represents significance difference compared to LPS-treated control group

model.<sup>[15]</sup> The results of the present study correlated with the same genus.

## CONCLUSION

The ethanolic leaf extract of *A. etbaica* Schweinf showed the dose-dependent inhibitory effect on pro-inflammatory cytokines such as TNF-α ( $IC_{50} - 251.29 \pm 1.54$ ), IL-1β ( $IC_{50} - 177.67 \pm 2.82$ ), and IL-6 ( $IC_{50} - 133.22 \pm 0.86$ ) production in LPS-stimulated RAW 264.7 macrophage cells. The result of this study

supports that the traditional uses of the *A. etbaica* Schweinf by traditional medicine practitioners for the treatment of inflammatory diseases.

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