

# Anti-oxidant and Anti-inflammatory activity of Terminalia paniculata bark extract in LPS induced Raw 264.7 Macrophages

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## Abstract

**Background:** In traditional and folklore medicine *Terminalia paniculata* is used to treat cholera, fever, and hypertension. The present study was designed to explore the anti-inflammatory and anti-oxidant potential of ethanolic extract of T.Paniculatabark (EETP).

**Materials and Methods:** Anti-inflammatory activity of ethanolic extract of Terminalia paniculata (EETP) in the dose range of 50-250 µg/ml, was evaluated against Lipopolysaccharide (LPS) activated Nitric Oxide (NO) production in Raw 264.7macrophages as well as in human red blood cells (HRBC) by membrane stabilization method. Total phenolic content (TPC) and total flavonoid contents were determined by Folin–Ciocalteu’s phenol reagent and by aluminium chloride methods respectively. In vitro antioxidant activities were evaluated by DPPH and FRAP assays.

**Results:** The ethanolic extract of T.Paniculata bark (EETP) demonstrated strong anti-inflammatory activity at 250 µg/ml in terms of inhibition of nitric oxide production (77%) and membrane stabilizing activity (72 %).

**Conclusions:** EETP may be considered to develop a potential therapeutic candidate for the treatment of inflammation and oxidative stress.

**Key words—** Anti Inflammatory, antioxidants, HRBC, LPS, Macrophages, Nitric Oxide production and *Terminalia paniulata*.

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## 1 INTRODUCTION

Inflammation is considered as a part of body's immune system. Inflammation signifies a highly mediated group of events that allow tissues and organs to respond against infection/injury. Inflammation contributes to various types of cell expressions and reaction to diverse mediators along a very precise sequence of actions [1]. Typically, inflammation is instigated through the production of targeted cytokines or chemokines characterized by conscription of immune cells to the impairment site. Conversely, sustained or excessive inflammation can lead to numerous diseases including insulin resistance, rheumatoid arthritis, inflammatory bowel disease, psoriasis, diabetes and obesity [2,3], and chronic inflammation may cause hay fever, periodontitis and certain cancers [4].

Many studies have shown that extracts or isolated bio active compounds of plants possess anti-inflammatory, anti-allergic, anti-microbial, anti-diabetic, anti-tumor and anti-mutagenic activities to a larger or lesser extent [3, 5, 6, 7] and. Scientists stated that intake of dietary fibers, fruits, vegetables and other nutraceuticals with high antioxidant activity have been found to reduce risks of allergies, diabetes, cardiovascular disease, cancer and other diseases [8, 9]. In this paper we aimed to evaluate the antioxidant and anti-inflammatory activity of *Terminalia paniculata* bark ethanolic extract on Raw macrophages (264.7 cell types).

*T. Paniculata* is a flowering plant of Combretaceae family which includes 530 species. It is a tropical tree with a large natural distribution in Western Ghats, India. Conventionally, flower juice and bark of *Terminalia paniculata* have been used as a medication for cholera, for the treatment of inflamed parotid glands and in menstrual disorders [10, 11]. However, till date only a few investigations supporting the pharmacological properties like anti-inflammation, anti-diabetic and anti-obesity on this plant were carried out [11,12, 13, 14].

## 2 Materials and methods

### 2.1. Reagents and cell culture

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Himedia Bangalore, LPS from *Escherichia coli* (serotype 0111:B4) and Penstrep were purchased from Sigma-Aldrich (St. Louis, MO). A Griess reagent system kit was obtained from Promega (Madison, WI). Raw macrophage 264.7 cells were obtained from NCCS Pune.

### 2.2 Preparation of plant extract

*Terminalia Paniculata* (T.P) bark was obtained from Seshachalam hill ranges located around Tirupati, shade dried and made in to coarse powder. This was soxhlet extracted under reflux over a boiling water bath by 2 liters of an ethanol / distilled water (3: 1) mixture for 8 hours. The extract was filtered and the process was repeated 3 times. The solvent was removed under reduced pressure at 450C by a Rota evaporator. The process yielded finally sticky dark brown material which was stored at -200C for further use.

### 2.3 Determination of total phenolic content (TPC)

The concentration of phenolic compounds of *Terminalia paniculata* bark was estimated by using Folin-Ciocalteu method [15]. Briefly, the sample solution (0.6 ml) was mixed with 2.58 ml of Folin-Ciocalteu's phenol reagent. After 3 min, 0.3 ml of saturated sodium carbonate solution was added to the mixture. The reaction mixtures were incubated at room temperature (25 °C) for 20 min. The absorbance was measured at 760 nm with a spectrophotometer. Gallic acid in the concentrations ranging from 25 to 400 mg/ml was used for calibration. A dose response linear regression was generated by using the gallic acid standard absorbance and the phenolic content of the samples was expressed as gallic acid equivalent (mg of GAE/gm of extract). The estimation was per-

formed in triplicate, and the results were expressed as mean  $\pm$  SD.

#### **2.4 Determination of total flavonoid content (TFC)**

The total flavonoid content was estimated by aluminium chloride method [16]. EETP (0.5 ml) was mixed with 2.5 ml of distilled water and 150  $\mu$ l NaNO<sub>2</sub> (5 %) solution. The contents were vortexed for 10 s and left at room temperature for 5 min. Then, 300  $\mu$ l AlCl<sub>3</sub> (10 %), 1 ml NaOH (1 mM) and 550  $\mu$ l of distilled water were added. The solution was mixed well and kept for 15 min. The absorbance for sample was measured at 510 nm. Quercetin concentrations ranging from 25 to 400 mg/ml were prepared and the standard calibration curve was obtained. The total flavonoid content was calculated using standard quercetin calibration curve. The results were expressed as mg of quercetin equivalent (QE) per gram of sample.

#### **2.5 Free radicals scavenging activity by DPPH**

The free radicals scavenging activity of the EETP against 2, 2-di phenyl-1-picryl hydrazyl (DPPH) radical was determined spectrophotometrically at 517nm [17]. Different concentrations of EETP were prepared (50- 250mg/ml) in ethanol. To this, 4 ml of 0.4mM DPPH in methanol was added. Vitamin C (1-5mg/ml) was used as the standard. The free radical scavenging activity was calculated using the formula [Absorbance = control -test  $\times$ 100/control].

#### **2.6 Ferric Reducing Antioxidant Power (Frap) Assay**

The reducing power of EETP was determined according to the method of Oyaizu [18]. Different concentrations of EETP 1 ml were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of potassium ferric cyanide (1%) and incubated at 50°C for 20min. A few drops of Trichloroacetic acid (10%) was added and centrifuged at 3000rpm for 10min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and FeCl<sub>3</sub> (0.5ml, 0.1%) then taken the absorbance at 700nm. The reference standard was ascorbic acid and the blank solution contained phosphate buffer. Results were expressed as  $\mu$ mol of as-

corbic acid equivalents/g dry weight of defatted samples.

#### **2.7 Cell culturing and maintenance**

RAW 264.7 cells, a murine macrophage cell line, were obtained from NCCS Pune and cultured in Falcon plates (100 mm), grown at 37°C in 5% CO<sub>2</sub> in DMEM supplemented with 10% FBS, 2 mM glutamine, and 1,000 U/ml penicillin-streptomycin. The medium was changed on alternate days. The cells were passaged by trypsinization (TrypsinEDTA) to disrupt cell monolayer at confluence while splitting RAW264.7 cells for the continuation of cultures and plating the cells for the in vitro assays.

#### **2.8 Cell viability assay (MTT Assay)**

Murine macrophage-like RAW 264.7 cells were cultured as described previously. Cells were seeded at a density of 104cells/well in 96-well plates, grown overnight followed by the pre-treatment with different concentrations of the EETP (50, 100, 150, 200 250 mg/ml) for 1 h before the addition of the LPS. RAW 264.7 cells viability was measured after 24 h of exposure to the extracts with a colorimetric assay, based on the ability of mitochondria in viable cells to reduce MTT [19]. A concentration of 0.5 mg/ml of MTT solution was added to each well and, after 4 h of incubation at 37°C, the medium was discarded and the formazan blue formed in the cells was dissolved in dimethyl sulfoxide (DMSO). Optical density at 570nm was determined with a microplate reader (Bio Rad). The optical density of the formazan formed in LPS treated cells was taken as 100% of viability.

#### **Anti-inflammatory activity**

##### **2.9 Quantification of NO in RAW 264.7 Cells.**

The levels of NO production in the culture medium of 264.7 macrophages were measured using a standard procedure using Griess reagent kit according to the manufacturer's instructions. Cells were seeded onto 96-well plate with 2X10<sup>5</sup>cells/well and allowed to adhere overnight. Then, medium was removed and replaced with 0.2 ml of fresh medium alone or containing 0.5 mg/ml of extracts. After 1 h of incubation, LPS stimulation was performed. LPS was added at a concentration of 1 mg/ml for 24 h. The cell-free culture

medium was collected, 50  $\mu$ l were used for NO determination and the remainder was stored at -200C for further use. The nitrite accumulated in culture medium was measured as an indicator of NO production, based on the Griess reaction [20]. Briefly, 50  $\mu$ l of culture supernatants were gently mixed with an equal volume of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) and incubated in the dark at room temperature (RT) for 10 min. After the incubation, 50  $\mu$ l of 0.1% n-naphthyl ethylenediamine dihydrochloride (NEDA) was added to the reaction and incubated in the dark at RT for another 10 min. The absorbance at 540 nm was measured in a microplate reader (Bio Rad max 100). Nitrite concentration, an indicator of NO production, was calculated from a NaNO<sub>2</sub> standard curve.

### 2.10 Membrane stabilization assays

Membrane stabilization method has been used for preparation of blood samples of human red blood cells (HRBC) to study in vitro anti-inflammatory activity [21]. The blood was collected from healthy volunteers who had not taken any Non-steroidal anti-inflammatory drug (NSAIDs) for 3 weeks prior to the experiment and mixed with equal volume of Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42 % NaCl). All the blood samples were stored at 4°C for 24h before use. They were centrifuged at 3000 rpm for 5 min and the supernatant was discarded. The cell suspension was washed with sterile saline solution (0.9 % w/v NaCl) and centrifuged at 2500 rpm for 5 min. Centrifugation was repeated till the supernatant was clear and colorless and the packed cell volume (PCV) was measured. The cellular component was reconstituted to a 40% suspension (v/v) with phosphate buffered saline (10 mM, pH 7.4) and was used in the assays.

### Hypotonicity-induced haemolysis

To 0.5 ml of HRBC suspension, 1 ml of EETP in phosphate buffer and 2 ml hyposaline was added, incubated at 37 °C for 30 min and centrifuged at 3000 rpm for 20 min. The haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Ibruofen (200  $\mu$ g/ml) was used as reference

standard. The percentage inhibition of haemolysis or membrane stabilization was calculated [22].

$$\% \text{ Inhibition of Haemolysis} = 100 \times (\text{OD1} - \text{OD2} / \text{OD1})$$

where: OD1 = Optical density of hypotonic-buffered saline solution alone.

OD2 = Optical density of test sample in hypotonic solution.

### 3. Statistics

Data are expressed as mean values  $\pm$  SD and standard errors of the mean (SEM). Comparisons between EETP-treated groups and untreated group were done with the Student's t-test. Differences were considered significant at  $p < 0.05$ . The experiments were performed in triplicate.

### 4. Results

#### 4.1. Total phenolic content (TPC)

Total phenolic content is expressed in terms of GAE of EETP used. The TPC of the tested samples are shown in table.1. The highest content of phenols noted is  $351.21 \pm 8.07$  mg of GAE/g. TPC was calculated using the following linear equation based on the calibration curve of gallic acid;  $y = 0.0068x + 0.2719$ ,  $R^2 = 0.9715$ .

#### 4.2. Total flavonoids content (TFC)

TFC was expressed as mg of quercetin equivalents /gm of dry extract and the highest flavonoid content observed was  $138.41 \pm 2.51$  mg of QE/gm of extract (Table.1). TFC in the samples were determined with reference standard curve of quercetin ( $y = 0.0062x + 0.0039$ ,  $R^2 = 0.932$ ).

Table 1. Total Phenolic and flavonoid contents present in EETP.

Results were expressed as mean of triplicate (n=3) mean and SD.

Based on the above results we selected ethanolic extract for further experiments.

### 4.3. Anti-Oxidant activity

DPPH assay: Antioxidant efficacy was increased with increase in concentration of EETP. The maximum antioxidant activity (70%) in terms of DPPH radical scavenging activity was noted with 250 mg/ml when compared with the standard (Ascorbic acid) (Figure 1). In reducing power assay, EETP showed 50 to 60% activity in comparison to ascorbic acid (Figure 2). The presence or absence of particular component(s) plays a major role in deciding the antioxidant property of medicinal plant.

Figure 1.

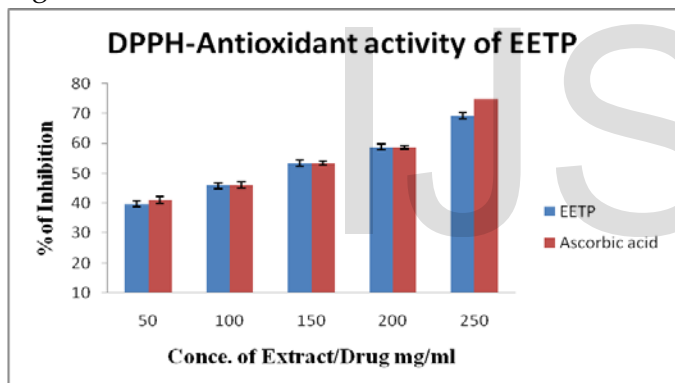
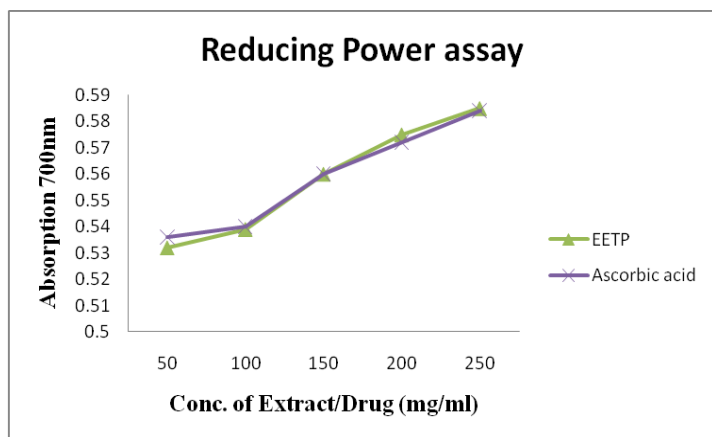


Figure 2.



Figures 1&2: DPPH and Reducing power activity of EETP. Values are mean of triplicate determination ±

| Sample        | Phenolic content | Flavonoid |
|---------------|------------------|-----------|
| Hexane        | 56.5±4.3         | 26±6.4    |
| Ethyl acetate | 155±8.4          | 85±4.1    |
| Ethanol       | 351±15.7         | 138.21±15 |
| Aqueous       | 210±21           | 98±32     |

SD (N = 3). The values are significantly different at P < 0.05, when compared with 50 mg/ml.

### 4.4. Effect of EETP on cell viability

To assess the anti-inflammatory activity of EETP, RAW 264.7 cells were used. Results are shown in Fig. 3 and expressed as percentage (%) of viability with respect to the LPS-treated cells which were taken as 100% viability. Different concentrations of EETP were tested and cell viability noted. Vehicle (DMSO) was also tested for its effect on cell viability. Up to a concentration of 250 (µg/ml), EETP did not affect cell viability. Therefore, this concentration of EETP was used for further experiments.

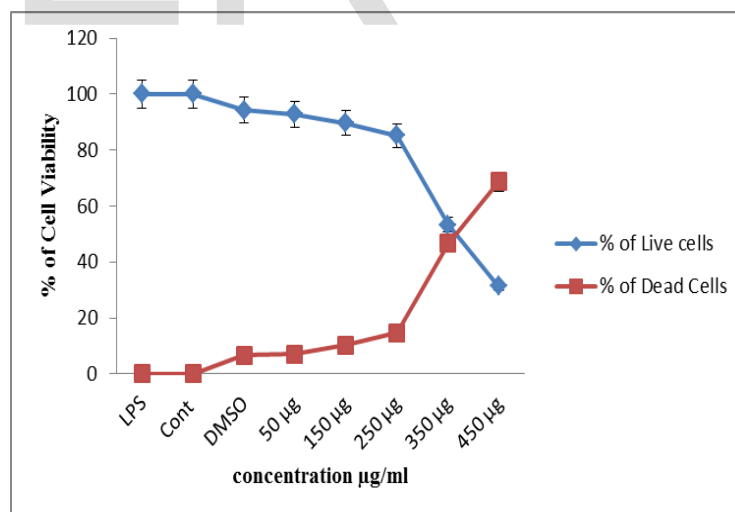


Figure 3. Effect of EETP on RAW 264.7 macrophages viability. Viability of cells treated with LPS alone has been taken as reference (100%). Experiments were performed in triplicate (n=3).

### Anti-inflammatory activity

#### 4.5. Inhibition of LPS induced NO production.

To evaluate the effects of Terminalia paniculata bark extract on NO production, NO release was measured as the accumulation of the stable metabolite, nitrite, in the culture supernatants. Little production of NO occurs in un-stimulated cells when compared with treated, but treatment with LPS induced a high release of NO in to the culture medium. Treatment with high concentration of EETP resulted in considerable inhibition of the NO production compared with low concentration of EETP (Figure.4).

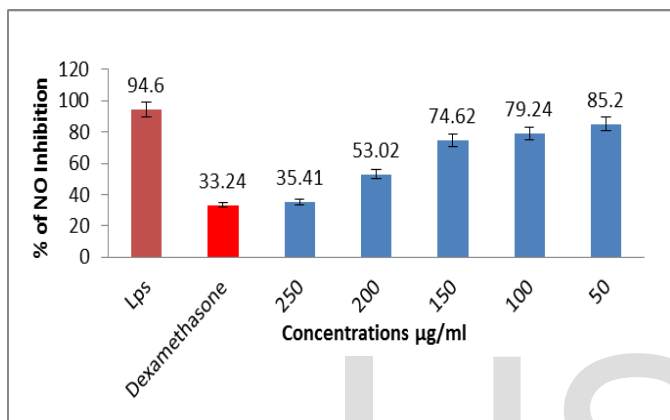


Figure 4. Effect of EETP on LPS-induced NO production in RAW 264.7 macrophages. Values shown are the mean  $\pm$  SD (n=3),  $p < 0.05$  significantly different from the LPS group.

#### 4.6. Membrane stability test

The anti-inflammatory activity was determined by membrane stability test of the human red blood cells as shown in Figure. 5. The EETP showed a concentration dependent activity, and the membrane protection percent was increased with increase in the concentration of the test samples. At concentration of 250µg/ml, EETP showed maximum 72.645 $\pm$  3.37% inhibition of RBC hemolysis, compared with standard Ibuprofen drug which showed 75.80  $\pm$  4.04 %.

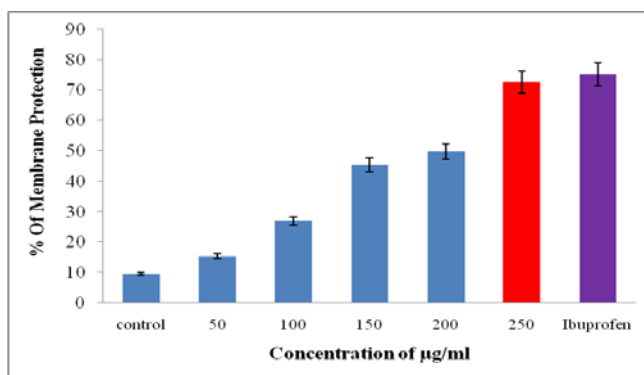


Figure 5. Represents the percentage of membrane protection of EETP with reference to Ibuprofen 200 µg/mL. The results were expressed as mean  $\pm$  SD (n=3).

#### 4.7. Discussion

Macrophages play major role in both, host-defense mechanisms and inflammation. Activated macrophages secrete a number of different inflammatory mediators, including NO, TNF $\alpha$ , IL-1b and IL-6. The overproduction of these mediators has been implicated in several inflammatory diseases such as rheumatoid arthrities, hay fever and cancer [23]. Thus, inhibition of these type of cells seem to be an important target for the treatment of inflammatory related diseases. NO is one ubiquitous cellular mediator of physiological and pathological processes, being largely released at inflammatory sites [24]. Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria that is known to induce the activation of monocytes and macrophages and production of pro-inflammatory mediators and cytokines [25]. Stimulation of macrophages with LPS induces a high production of NO by the inducible isoform of the enzyme, nitric oxide synthase (iNOS) [26]. In the present work, ethanolic extract from Terminalia paniculata effectively inhibited NO production in activated macrophages without affecting cell viability. As shown in figure. 4, LPS treatment resulted in a sharp increase in the nitrite level in the macrophages. EETP was able to alleviate the LPS-induced NO overproduction.

Antioxidant activity is evaluated by widely used methods that generate free radical species which are then counteracted by antioxidant compounds. In this study the antioxidant activity was found to be affirmative for EETP which can be attributed to the presence of phenols and flavonoids as shown in the quantification methods. In general high content of phenols and flavonoids were correlated with more potent scavenging activity [27]. The comparative study shows the higher content of phenols and flavonoids of EETP as compared to Hexane, Ethylacetate and water extracts. For Scavenging activity, the EETP showed more than 65% of efficacy in dose dependent manner which is almost equal to ascorbic acid. The ethanolic extract showed the highest percentage of inhibition at a concentration of 250mg/ml whereas the lowest inhibition percentage of 39.8% as shown in (figure 1) was observed at a concentration of 50mg/ml.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. It was observed that the reducing ability of the extract was dose dependent and all the three extracts showed an increase in absorbance with the increase in dose. The ethanolic extract showed an absorbance of 0.603 which was the maximum. Reducing power assay results reveal that ethanolic extract has higher antioxidant potential. The results strongly suggest that polyphenolic compounds are important components of this plant, and its other pharmacological effects like anti diabetic, anti obesity and hepato protective activities [12, 13, 11, 14] could be attributed to the presence of these valuable constituents.

The EETP at a concentration of 250 $\mu$ g/mL significantly protects the human erythrocyte membranes against lysis induced by hypotonic solution. At concentration of 250 $\mu$ g/mL, the extract inhibited 72.645% of RBC haemolysis as compared with 75.84% produced by Ibuprofen at 200 $\mu$ g/mL (Figure 5). This in vitro me-

thod was more time saving, flexible, and convenient as a measure of anti-inflammatory activity of drugs in HRBC. The results obtained demonstrated that Ethanolic extract of *Terminalia paniculata* could significantly and dose dependently inhibits HRBC haemolysis.

### Conclusion

The results demonstrate that, *Terminalia paniculata* bark extract showed effective and dose-dependent free radical scavenging activity, inhibition of Nitric oxide production activity and protects HRBC from haemolysis. Based on the above results *Terminalia paniculata* can be well considered as a potent anti-inflammatory and anti-oxidant agent.

### Conflict of interest statement

The authors state no conflict of interest.

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