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In Vitro Evaluation of the Anti-inflammatory and Antioxidant Properties of *Mesua ferrea Linn.* Stem Bark Extract

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ABSTRACT: Plants rich in antioxidants are also a potential source of and exhibit anti-inflammatory effects paving the path for the development of new drugs with minimal/no side effects. Hence, in this study, the antioxidant and anti-inflammatory properties of the ethanolic extract of *Mesua ferrea* Linn. stem bark were evaluated using various *in vitro* methods. The qualitative and quantitative analyses were conducted to determine the presence of secondary metabolites. The extract exhibited significant antioxidant activity, as demonstrated by its ability to scavenge 2,2-Diphenyl-1-picrylhydrazyl (DPPH), nitric oxide, hydroxyl radicals, and metal chelating ability. The extract's anti-inflammatory activity was also assessed by measuring the inhibition of pro-inflammatory cytokines, NO, PGE2 production, HRBC membrane stabilization, protein denaturation, inhibitory effects on proteinase, lipoxygenase and cyclooxygenase. The results showed that the ethanolic extract possessed substantial antioxidant and anti-inflammatory properties, making it a promising source for the development of novel drugs with minimal side effects.

Keywords: *Mesua Ferrea* Linn., Antioxidant, Anti-inflammatory, Macrophages, cytokines.

INTRODUCTION

Inflammation is a complex process caused by several factors like bacterial infection, and chemicals, which result in cell injury. The inflammatory conditions or diseases are treated with a variety of medication classes, including biologics, NSAIDs, and corticosteroids. All of these medications have several side effects *viz*., hypertension, cardiac complications, platelet dysfunction, osteoporosis, etc. For initiation, progression and eventual healing of inflammation's acute condition, macrophages and neutrophils secrete several mediators *viz.*, Tumor necrosis factor α(TNF-α), NFkB etc., which are involved in inflammatory responses. Additionally, cytokines cause the up-regulation of other pro-inflammatory cytokines and chemokines, immunoglobulins, and also boost the expression of numerous cellular adhesion molecules (Saklatvala *et al.,* 2003). Lipopolysaccharide (LPS) and proinflammatory cytokines are the endogenous and exogenous inflammatory inducers, which stimulate inflammatory macrophages to increase Prostagladin E2 (PGE2), leukotrienes and Nitric oxide (NO) (Chaitanya *et al.,* 2015).

Lipopolysaccharide (LPS) is the most abundant component within the cell wall of gram-negative bacteria and stimulates cells innate immune system like monocytes/macrophages to generate proinflammatory cytokines including TNF, IL-1β and IL-6. It is known that LPS is an effective activator of monocytes/macrophages (Ngkelo *et al.,* 2012; Huang and Kraus 2016). Small proteins generated by cells called cytokines have an impact on how cells communicate and interact with one another. Pro-inflammatory cytokines are those cytokines that promote inflammation and are primarily responsible for initiating an effective defence against exogenous pathogens (Ng *et al.,* 2003). Pro-inflammatory cytokines trigger inflammation by triggering a cascade of gene products that are generally not produced in healthy people. The three main pro-inflammatory cytokines *viz*., IL-1β, IL-6, and TNF-α play a critical role in immune system modulation. TNF-α was identified as a major regulator of inflammatory responses. Pro-inflammatory cytokines control and eradicate intracellular pathogens at the site of infection by regulating cell proliferation, differentiation and activation. Transforming growth factor (TGF-β), IL-4 and IL-10 are anti-inflammatory cytokines that reduce the strength of cascades. TNF-α and IL-1β stimulate both arachidonic acid-dependent and independent inflammatory pathways (Fadok *et al.,* 1998). A balance between pro-inflammatory and anti-inflammatory cytokines is necessary for the disorders to manifest (Dinarello, 2000). Natural products can be used as inflammatory agents to treat all of these issues because there is always a need to investigate improved anti-inflammatory therapeutic medicines.

Mesua ferrea Linn. (MF) a medicinal plant that has been widely used in the Indian medicine system belongs to family Clusiaceae, distributed in mid-hills of Eastern Himalayas and rain forest of Konkan and Karnataka in Western Ghats. MF contains various

bioactive chemical constituents *viz.*, flavonoids, terpenoids, coumarins, xanthones, pyranoxanthones, and steroids, and can be easily isolated. It can act as a precursor to many ailments (Chahar *et al.*, 2013). These bioactive constituents have many pharmacological activities such as antioxidant (Prasad *et al.*, 2012), anticancer (Teh *et al.*, 2013), antimicrobial (Mazumder *et al.*, 2004), antifungal (Kumar *et al.*, 2006), anti-inflammatory, anti-arthritis (Jalalpure *et al.*, 2011), analgesic (Hassan *et al.*, 2006), anti-hemorrhoid (Aggrawal *et al.*, 2014), anti-gastric and antivenom (Uawonggul *et al.*, 2006). Bark of the plant is used as tonic after childbirth and is also useful in anemia. Unripe fruit and Bark are sudorific. Stem bark and root of the plant are used as an astringent for gastritis and bronchitis in the form of tincture or decoction (Sharma *et al.*, 2017). Every part of the plant has been reported to possess medicinal properties. Traditionally, the plant is recommended for pain and inflammation-related diseases (Singh *et al.*, 2019). The xanthones from MF have promising anti-inflammatory activities in carrageenam-induced paw oedema rat models (Gopalakrishnan *et al.*, 1980). Petroleum ether and methanol extract of stem bark has potent antioxidant and anti-inflammatory activities (Manjunatha *et al.*, 2013). Mesuol from seed oil of MF showed potent antioxidant and immunomodulatory activity in the cyclophosphamide-induced myelosuppression model (Chahar *et al.*, 2012). Drawing inspiration from existing literature and ethnopharmacological knowledge, the objective of this study was to assess the in vitro antioxidant and anti-inflammatory properties of the ethanol extract derived from the stem bark of *Mesua ferrea* Linn.

MATERIALS AND METHODS

A. Plant collection and extraction

The stem bark of *Mesua ferrea* Linn., sourced from Thirthahalli, Shimoga District, Karnataka, was collected and verified for authenticity by the botanist, Prof. Y.L. Krishnamurthy, from the Department of Botany at Kuvempu University. A voucher specimen was preserved for reference. Following collection, the stem bark was air-dried in shade and subsequently crushed into coarse plant material. Approximately 500g of the powdered plant material was subjected to sequential hot soxhlet extraction using petroleum ether, chloroform, and ethanol solvents based on their respective polarities. The resulting extracts were filtered, concentrated under reduced pressure and controlled temperature (40ºC to 50ºC) using a rotary evaporator, and stored in a desiccator until further experimentation.

B. Qualitative phytochemical analysis

To determine the phytochemical composition of the ethanolic extract obtained from the stem bark of *M. ferrea*, standard procedures (Wagner *et al*., 1984) were employed. Established protocols for identifying steroids, alkaloids, tannins, flavonoids, glycosides, and other phytochemical constituents were utilized in this analysis.

Total phenol content assay. The quantification of total phenol content in the plant extracts was conducted using the Folin-Ciocalteu method, as described in a previously published study (Kumar *et al*., 2012). A gallic acid standard curve was employed for accurate determination, and the total phenol content was expressed as milligrams of gallic acid equivalent per gram of extract.

Total flavonoids content assay. The quantification of total flavonoids in the plant extracts followed a previously established approach, employing rutin as a reference standard. The measurement of total flavonoid content was expressed in milligrams per gram of extract as quercetin equivalent, as outlined by Kumar *et al*. (2012).

C. Determination of antioxidant activity

Total antioxidant capacity. The phosphomolybdenum method, as described by Prieto *et al.* (1999), was utilized to assess the total antioxidant capacity of *Mesua ferrea* stem bark ethanol extract (MSEE). Different concentrations (50, 100, 200 µg) of MSEE (0.3 ml) were mixed with a reagent mixture (3 ml) consisting of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The sample tubes were then subjected to an incubation period of 90 minutes at 95 °C. After cooling to room temperature, the solution's absorbance was measured at 695 nm against a blank. The antioxidant capacity of each extract was expressed in ascorbic acid equivalents (µg/mg of dry mass).

Total reductive capability. The determination of the total reductive capacity of *Mesua ferrea* stem bark ethanol extract (MSEE) followed the methodology established by Oyaizu (986). Different concentrations (50, 100, 200 µg) of MSEE (1 ml) were mixed with 2.5 ml of potassium ferricyanide (0.2 M) and phosphate buffer (0.2 M, pH = 6.6). The mixture was then incubated at 50 °C for 20 minutes. Following the incubation period, 2.5 ml of 0.6 M trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 minutes. Next, 2.5 ml of the supernatant was combined with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.6 mM), and the absorbance was measured at 700 nm against a blank. The total reducing capacity of the extract was expressed in quercetin equivalents.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The free radical scavenging activity of *Mesua ferrea* stem bark ethanol extract (MSEE) was evaluated using the DPPH method, as described by Braca *et al*. (2001). The assessment of the extract's free radical scavenging activity was based on its ability to scavenge stable DPPH radicals. Different concentrations of MSEE (100, 200 and 300 µg) were analyzed, with ascorbic acid serving as the standard. The calculation of radical scavenging activity involved the formula: % inhibition = $[(A_{control} - A_{test}) / A_{control}] \times 100$, where $A_{control}$ represents the absorbance of the control reaction and A_{test} represents the absorbance of the extract reaction. The IC_{50} value, indicative of the concentration at which the extract inhibits 50% of the radicals, was determined using the formula:

$$IC_{50} = \frac{\Sigma C}{\Sigma I} \times 50$$

Where ΣC represents the sum of extract concentrations and ΣI represents the sum of the percentage of inhibition at different concentrations.

Hydroxyl radical scavenging activity. The determination of hydroxyl radical scavenging activity followed the methodology established by Klein *et al*., (1981). *Mesua ferrea* stem bark ethanol extract (MSEE) was analyzed at different concentrations (100, 200, and 300 µg). The percentage of hydroxyl radical scavenging was calculated using the formula: % inhibition = 1 − [A_{sample} / A_{blank}] × 100, where A_{sample} represents the absorbance of the reaction mixture containing the extract and A_{blank} represents the absorbance of the blank. The IC_{50} value, which indicates the concentration at which 50% of hydroxyl radicals are inhibited, was calculated using the formula:

$$IC_{50} = \frac{\Sigma C}{\Sigma I} \times 50$$

Where ΣC represents the sum of the extract concentrations used for testing and ΣI represents the sum of the percentage of inhibition at different concentrations.

Metal chelating activity. The measurement of metal chelating activity in *Mesua ferrea* stem bark ethanol extract (MSEE) followed the procedure outlined by Dinis *et al*. (1994). MSEE was tested at various concentrations (100, 200, and 300 µg). For comparison, EDTA was used as the standard. The percentage of metal chelating activity was calculated using the formula: % inhibition = [($A_{control}$ − A_{test}) / $A_{control}$] × 100, where $A_{control}$ represents the absorbance of the control reaction and A_{test} represents the absorbance of the extract reaction. The IC_{50} value, indicating the concentration at which 50% of metal chelating activity is inhibited, was calculated using the formula:

$$IC_{50} = \frac{\Sigma C}{\Sigma I} \times 50$$

Where ΣC represents the sum of the extract concentrations used for testing and ΣI represents the sum of the percentage of inhibition at different concentrations.

Nitric oxide radical scavenging activity. The determination of nitric oxide radical scavenging activity in Mesua ferrea stem bark ethanol extract (MSEE) followed the methodology described by Marcocci *et al*. (1994). The percentage of nitric oxide radical scavenging was calculated using the formula: % inhibition = [($A_{control}$ − A_{test}) / $A_{control}$] × 100, where $A_{control}$ represents the absorbance of the control reaction and A_{test} represents the absorbance of the extract reaction. The IC50 value, indicating the concentration at which 50% of nitric oxide radical scavenging is achieved, was calculated using the formula:

$$IC_{50} = \frac{\Sigma C}{\Sigma I} \times 50$$

Where ΣC represents the sum of the extract concentrations used for testing and ΣI represents the sum of the percentage of inhibition at different concentrations.

D. In vitro anti-inflammatory activities

Cell culture of THP-1 monocyte. THP-1 cells, a type of human acute monocytic leukemia cells, were acquired from the National Centre for Cell Science (NCCS), located in Pune. These cells were cultivated in T_{25} flasks utilizing RPMI 1640 medium. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% 100 U/ml penicillin, 1 mM sodium pyruvate, 2 g/l sodium bicarbonate, and 0.05 mM mercaptoethanol. The cell lines were maintained in a humidified environment at 37 °C with a mixture of 5% carbon dioxide and 95% air. To maintain optimal cell concentration, the medium was refreshed three times per week, and cells were passaged at regular intervals. To transform THP-1 cells into the THP-1 macrophage-attached cell line, phorbol 13 myristate-12 acetate (PMA) was used. After transformation, 1×10^6 cells/ml of complete media were seeded into each well of the plates, after the cells had reached 80% confluency and the plates were then incubated at 37°C with 5% CO_2. Once in two days, the media was changed. Macrophage cells generated from THP-1 were grown up to full confluency.

Cell viability assay. The THP-1 cells were collected by trypsinization and transferred to a 15 ml centrifuge tube. The cells were then centrifuged at 300 xg to obtain a cell pellet. The cell count was adjusted using DMEM medium so that approximately 10,000 cells were present in 200 µl of suspension. Each well of a 96-well microtiter plate was filled with 200 µl of the cell suspension, and the plate was incubated at 37 ºC with a 5% CO_2 atmosphere for 24 hours. After 24 hours, the spent medium was removed, and different concentrations (100, 200, 300, 400, and 500 µg/ml) of MSEE along with LPS (1 µg/ml) were added to the respective wells. The control wells were treated with 0.1% DMSO. The plate was then incubated at 37 ºC with a 5% CO_2 atmosphere for an additional 24 hours. Subsequently, the drug-containing media was aspirated, and 200 µl of medium containing 10% MTT reagent was added to each well to achieve a final concentration of 0.5 mg/ml. The plate was incubated at 37ºC with a 5% CO_2 atmosphere for 3 hours. After carefully removing the culture medium without disturbing the formed crystals, 100 µl of DMSO was added to solubilize the formed formazan while gently shaking the plate on a gyratory shaker. The absorbance was measured at 570 nm and 630 nm wavelengths using a microplate reader. The percentage of growth inhibition was calculated by subtracting the background from the blank, and the concentration of the test drug required to inhibit cell growth by 50% (IC_{50}) was determined from the dose-response curve using the cell lines (Mosmann, 1983; Gerlier and Thomasset, 1986; Kiran *et al.*, 2018).

Determination of Pro-inflammatory cytokine production. The THP-1 cells were subjected to the activation of pro-inflammatory cytokines using an ELISA kit. The cells were initially seeded in a 6-well plate and incubated for 24 hours at 37 °C in a humidified atmosphere with 5% CO_2. Following the removal of the culture medium, the cells were supplemented with fresh medium at a concentration of 1,600 µM, along with MSEE ranging from 75 to 300

µg/ml, based on the viability assay results. After approximately 1-2 hours of extract addition, LPS from Sigma Aldrich (1 µg/ml) was introduced to each well. The plate was then incubated for 24 hours at 37 °C in a humidified atmosphere with 5% CO_2. The production of TNF-α, IL-1β, and IL-6 was evaluated using specific sandwich ELISA, and the results were expressed in pg/mL.

Determination of NO and PGE_2 production. To quantify the accumulation of nitric oxide (NO) in cell culture, the synthesis of NO was assessed using Griess reagent following the method outlined by Heiss *et al*, (2001). A mixture of Griess reagent, composed of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl, was combined with an equal volume (100 ml) of the cell culture medium. The mixture was then incubated at room temperature for 10 minutes. The absorbance of the samples was measured at 550 nm using a microplate reader, with fresh culture media serving as the blank. The quantity of nitrite in the samples was determined by referencing a standard curve of sodium nitrite. Additionally, the concentration of PGE_2 secreted in the culture media was measured using an ELISA kit, following the instructions provided by the manufacturer.

HRBC membrane stability assay. Fresh and healthy human blood was collected from an individual who had refrained from using NSAIDs for the past two weeks. The blood was promptly mixed with an equal volume of sterilized Alsever solution, which consists of 2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% sodium chloride. Following centrifugation at 3000 rpm, the packed cells were washed with isosaline solution (0.9% w/v NaCl). A 10% suspension was prepared using the same isosaline solution. Distilled water was used to create different concentrations of the extract (75, 150, and 300 µg/ml). To each concentration, 1 ml of phosphate buffer, 2 ml of hyposaline solution, and 0.5 ml of the HRBC (human red blood cell) solution were added. The mixtures were then incubated at 37 °C for 30 minutes, followed by centrifugation at 3000 rpm for 20 minutes. The concentration of hemoglobin in the supernatant solution was measured at 560 nm. Diclofenac at a concentration of 100 µg/ml served as the reference standard, and a control group was prepared by excluding the extracts. Assuming that the hemolysis rate in the control group was 100%, the percentage of hemolysis was determined. The percentage of HRBC membrane stabilization or protection was calculated using the formula: % protection = 100 x $[(OD_1 - OD_2)/OD_1]$, where OD_1 represents the optical density of the drug-treated sample and OD_2 represents the optical density of the control sample, as described by Gandhidasan *et al*. (1991).

Protein denaturation Inhibitory assay. In each test tube, 2.0 ml of different concentrations of MSEE, 2.8 ml of phosphate buffer saline (PBS) with a pH of 6.4, and approximately 0.2 ml of egg albumin derived from fresh hen eggs were combined. The final concentrations of the extracts in the 5.0 ml reaction mixture were 75, 150, and 300 µg/ml. As a control, distilled water (5.0 ml) was used. The test tubes were placed in a BOD incubator and incubated at 37 °C for 15 minutes, followed by heating at 70 °C for 5 minutes. After the test tubes were cooled, the absorbance was measured at 660 nm using a UV Spectrophotometer. A standard drug, diclofenac at a concentration of 100 µg/ml, was treated in a similar manner to determine its absorbance. The percentage inhibition of protein denaturation was calculated using the formula: % inhibition = $100 \times (V_t / V_c - 1)$, where V_t represents the absorbance of the test sample and V_c represents the absorbance of the control sample, as described by Sen *et al.* (2015).

Proteinase Inhibitory assay. The reaction mixture (2 ml) consisted of 0.06 mg of trypsin, 1 ml of 20 mM Tris HCl buffer (pH 7.4), and 1 ml of the test sample at different concentrations. After incubating the reaction mixture at 37°C for 5 minutes, 1 ml of 0.8% w/v casein was added. Following an additional 20 minutes of incubation, 2 ml of 70% perchloric acid was added to the mixture to halt the process. The cloudy sample was then centrifuged, and the absorbance of the supernatant at 210 nm was measured using a buffer as a reference. Diclofenac, at a concentration of 100 µg/ml, was used as the standard. The percentage of inhibition of proteinase inhibitory activity was calculated based on the absorbance measurements, as described by Oyedapo and Famurewa (1995).

5-Lipoxygenase (5-LOX) Inhibitory assay. To perform the 5-LOX inhibition assay, linoleic acid was employed as the substrate and 5-Lipoxygenase was utilized as the enzyme. The assay mixture had a total volume of 200 µl, comprising 160 µl of sodium phosphate buffer (100mM, pH 8.0), 10 µl of test extracts (75-300 µg in 100mM Tris buffer, pH 7.4), and 20 µl of lipoxygenase enzyme. After incubating the contents at 25°C for 10 minutes, the reaction was initiated by adding 10 µl of linoleic acid solution. The change in absorbance was then monitored at 234 nm after 6 minutes, following Kumar *et al*. (2016). All reactions were conducted in triplicates using 96-well microplates, and diclofenac served as the reference standard. The percentage inhibition was calculated using the appropriate formula.

$$\text{Inhibition (\%)} = [A_{Control} - A_{Test} / A_{Test}] \times 100$$

Cyclooxygenase inhibitory assay. The evaluation of in vitro COX-2 inhibitory activities of MSEE was performed using a 96-well plate and a commercial kit following the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). Prior to use, all reagents were prepared freshly. Initially, separate tubes were prepared for COX-1 and COX-2 activities by combining 950 µl of reaction buffer, 10 µl of heme, and 10 µl of COX-1 and COX-2 enzymes in their respective tubes. Additionally, COX-1 and COX-2 inhibitor tubes were created by adding 20 µl of the extract at various doses (10-100 µg/ml) to each tube. To generate background tubes representing inactivated COX-1 and COX-2 enzymes, the tubes containing the enzymes were placed in boiling water for 3 minutes along with the vehicle control. The reaction was initiated by adding 10 µl of arachidonic acid to each tube, followed by quenching with 50 µl of 1M HCl and the subsequent addition of 100 µl of $SnCl_2$. The prostaglandin

produced in each well was quantified by using prostaglandin-specific antiserum that binds with major prostaglandins, and the absorbance was measured at 405 nm (Kumar *et al*., 2016).

E. Statistical analysis

The results are presented as the mean ± SD. Statistical comparisons between groups were conducted using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests with the assistance of GraphPad Prism statistical software.

RESULTS AND DISCUSSION

Treatment with medicinal plants is considered very safe as there are no or minimal side effects, hence they are used as complementary medicines for many of the diseases over time. Medicinal plants are recognized as a valuable repository of natural compounds that hold potential for drug development. Various species of these plants have demonstrated notable anti-inflammatory and immunomodulatory properties (Li *et al.*, 2020; Tasneem *et al.*, 2019; Daniyal and Wang 2021). It is well recognized that infectious organisms such as pathogenic bacteria can activate monocytes or macrophages directly, initiating a cytokine cascade that causes inflammation and immune response. To assess the anti-inflammatory effects of natural products, the interference with the production of TNF-α, IL-1β, IL-6, NO, and PGE_2 might be used as the criterion.

In this study, we investigated the antioxidant and anti-inflammatory properties of MSEE using *in vitro* methods. We assessed the impact of MSEE on the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6), as well as the expression of NO and PGE_2 in LPS-induced THP-1 cells. These measurements were conducted to understand the underlying mechanisms of action.

A. Qualitative and quantitative analysis of MSEE

Qualitative and quantitative analysis of MSEE revealed the presence of various antioxidant and anti-inflammatory phytochemicals such as alkaloids, flavonoids, saponins, phenolics, and tannins. The total alkaloid content in MSEE was measured to be 128.32±1.55 µg/mg. The total polyphenolic content in the extracts, expressed as equivalent to Gallic acid (EGA), was determined to be 436.28±1.55 µg/mg, while the total flavonoid content in the extracts was found to be 213.84±1.63 µg/mg of dry extract.

B. In vitro antioxidant activity

According to Prieto *et al.* (1999) description, the phosphomolybdenum technique was used to determine the extract's total antioxidant capacity. The antioxidant capacity of the extract was measured in terms of ascorbic acid equivalents, and MSEE exhibited significant activity, as shown in Fig. 1.

The extracts demonstrated remarkable antioxidant and reductive capabilities, indicating their potential as free radical inhibitors or scavengers. The antioxidant capacity of MSEE was compared to that of the standard ascorbic acid, and MSEE displayed significant antioxidant and reductive competence, as depicted in Fig. 1. Furthermore, the extract's ability to scavenge free radicals was evaluated using the DPPH radical scavenging method. This method assesses the hydrogen-donating ability of antioxidant molecules in the extract, which reduces the purple-colored DPPH. MSEE exhibited substantial DPPH radical scavenging activity, with an IC_{50} value of 127.48 µg/ml, compared to the standard with an IC_{50} value of 98.23 µg/ml. Additionally, MSEE was tested for its hydroxyl radical scavenging, nitric oxide radical scavenging, and metal chelating activities. The results of these assays, presented in Table 1, demonstrated that MSEE's performance in all the tested activities was dose-dependent. The extracts have shown remarkable antioxidant and reductive capability, hence it can serve as free radical inhibitors or scavengers. The antioxidant capacity of MSEE was compared with the standard ascorbic acid. MSEE shows significant antioxidant and reductive competency (Fig. 1) and hence can serve as the scavenger or free radical inhibitor. The extract was also screened for free radical scavenging ability by DPPH radical scavenging method. It is based on the measurement of hydrogen donating ability of antioxidant molecules present in the extracts to reduce purple colored DPPH. MSEE showed good DPPH radical scavenging activity (IC_{50}: 127.48 µg/ml) compared to standard (IC_{50}: 98.23 µg/ml). MSEE was also screened for hydroxyl radical scavenging, nitric oxide radical scavenging, and metal chelating activity. The results of above activity of MSEE was illustrated in Table 1, and all the assays are dose-dependent. The percentage of radical scavenging activity revealed that MSEE has relatively effective scavenging activity against OH radicals compared to ascorbic acid. The result of metal chelating activity suggested that the MSEE was the potent metal chelator. The result of Nitric oxide scavenging activity was expressed as percentage inhibition exhibited by MSEE and standard (Table 1).

IC_{50} was calculated and it was observed that the percentage inhibition was increased with an increase in concentration of extract. MSEE IC_{50} for scavenging nitric oxide was found to be 163.59 µg/ml. The results obtained for radical scavenging and metal chelating activities showed that MSEE was a potent antioxidant and can protect cells and tissues from oxidative damage. This shows that the extract contains more phenolic content. The results obtained for radical scavenging and metal chelating activities showed that MSEE was a potent antioxidant and can protect cells and tissues from oxidative damage.

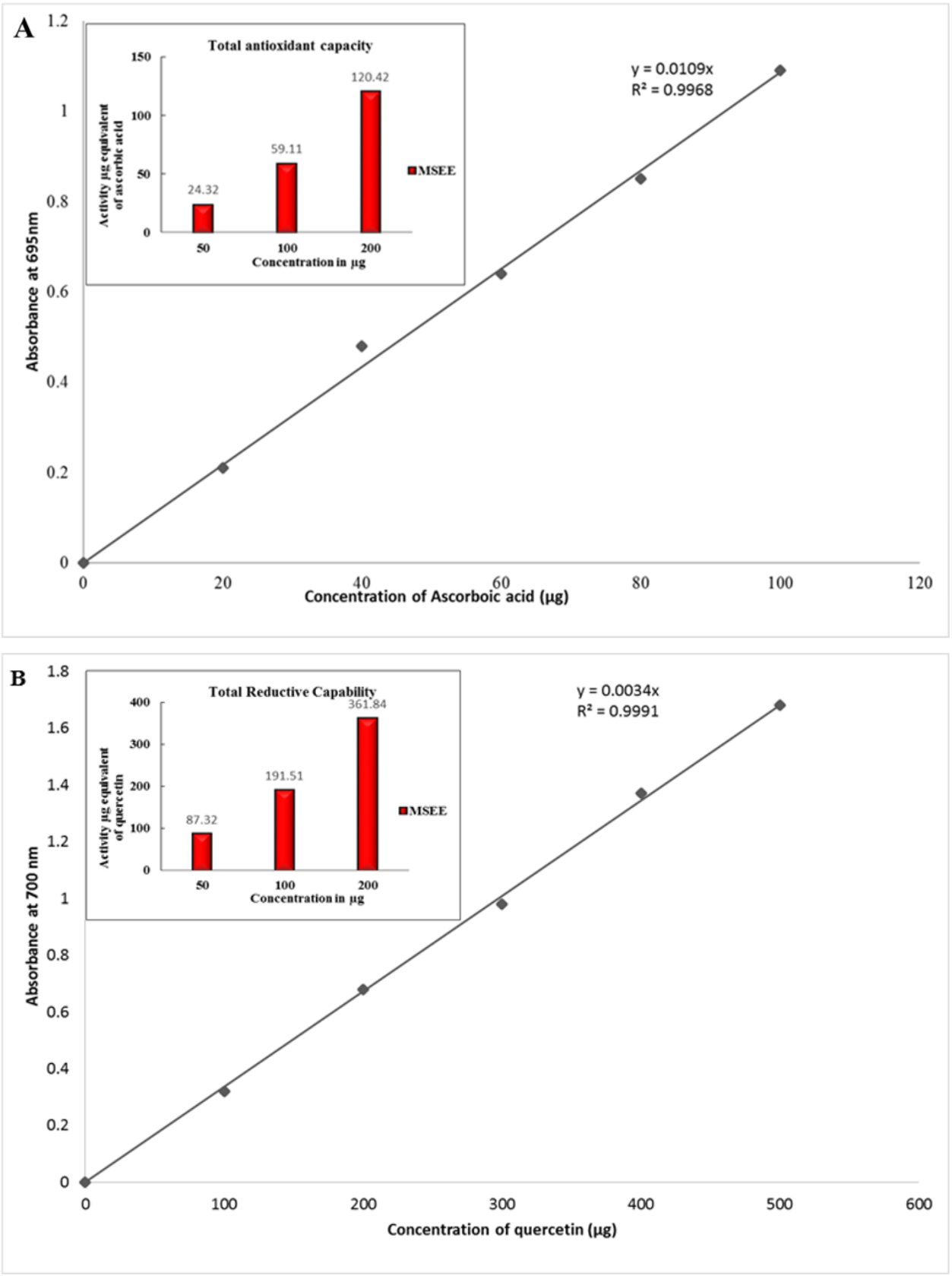

Fig. 1. Plots of (A) total antioxidant activity and (B) total reductive capability. The results shown are averages of three independent experiments.

Table 1: *In vitro* antioxidant activity of MSEE. The results shown are averages of three independent experiments, values are mean ± SD.

Sr. No.	Activity	Concentration of MSEE in µg.	% of inhibition MSEE	IC_{50} of MSEE(µg)	IC_{50} of Standard (µg)
1.	DPPH radical scavenging activity	100	67.51 ± 0.81	127.48	98.23
		200	79.33 ± 1.38		
		300	88.49 ± 1.14		
2.	Hydroxyl radical scavenging assay	100	18.94 ± 1.14	336.09	147.69
		200	31.11 ± 1.27		
		300	39.21 ± 1.55		
3.	Nitric oxide radical scavenging activity	100	37.52 ± 0.89	163.59	123.84
		200	60.87 ± 1.77		
		300	84.99 ± 1.42		
4.	Metal chelating	100	18.61 ± 1.55	259.56	26.45
		200	41.19 ±0.97		
		300	55.78 ± 1.51		

C. In vitro anti-inflammatory activity

Before investigating the anti-inflammatory activity of *M. ferrea*, we conducted an MTT assay to assess the potential toxicity of MSEE on THP-1 cells.

Assessment of cytotoxicity and cell viability of MSEE. The *in vitro* evaluation of cytotoxicity and viability of cells was carried out by MTT assay for different concentrations of MSEE (100-500µg/ml) for 24 hr to determine the optimum concentration. Since higher concentration of the extract might be toxic to cells. The results showed that MSEE doesn't have effects on cell survival from 100-300 µg/ml and there was slight effect above the range of 300 µg/ml (Fig. 2). Therefore, the optimum concentration of MSEE was determined at 300 µg/ml and at this concentration 81.19% of cells are viable, and it was adopted in the subsequent experiments. Based on this the IC_{50} value is calculated.

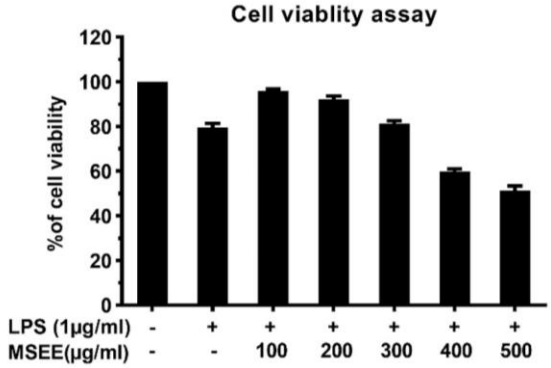

Fig. 2. Cytotoxicity and cell viability of MSEE in THP-1 cell. The cell treated with MSEE concentration range from 0-500µg/ml in LPS (1µg/ml) treated cells. As shown, MSEE didn't affect cell survival from 0-300µg/ml. the result represented as Mean±SD of three independent experiments.

Effect of MSEE on Pro-inflammatory Cytokines. Pro-inflammatory cytokines induced by LPS such as TNF-α, IL-1β, and IL-6 mediate both acute and chronic inflammation, and hence pharmacological inhibition/suppression of these inflammatory mediators is an important thing in the treatment of inflammation-related diseases.

To determine the anti-inflammatory effect of MSEE (75, 150 and 300 µg/ml), pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were measured in 1 µg/ml LPS-stimulated THP-1 cells with standard Diclofenac. The data demonstrated that at a concentration of 300µg/ml MSEE exhibited the greater inhibitory effect on TNF-α (127.61±11.54pg/mL), IL-1β (39.94±5.9731pg/mL) and as well as IL-6 (192.55±62.31pg/mL) and are shown in Fig. 3. This indicated that MSEE was actively involved in inhibiting the expression level of pro-inflammatory cytokines in differentiated THP-1 cells.

Effect of MSEE on NO Production. To explore the impact of MSEE on nitric oxide (NO) production, we investigated the accumulation of nitrite, a stable end product of NO, in the cell culture using Griess reagent. NO, produced by mammalian cells through the metabolism of L-arginine, plays a crucial role in regulating inflammation and immune responses. However, excessive NO production is associated with inflammatory diseases as it serves as a key pro-inflammatory mediator (Chen *et al.,* 2014; Shi *et al.,* 2019). In this study, THP-1 cells were stimulated with LPS (1 µg/ml) for 24 hours to induce NO synthesis. We observed that co-incubation of the cells with MSEE (75, 150, and 300 µg/ml) and LPS resulted in a dose-dependent decrease in NO formation. The reduction in NO production was significant with 300 µg/ml MSEE in LPS-induced cells, compared to the control (Table 2). **Effect of MSEE on PGE2 Synthesis.** PGE2 release is a crucial indicator of the inflammatory response in cells, generated by COX-2 at inflammatory sites, and contributes to the development of chronic inflammatory diseases (Ricciotti & FitzGerald 2011; Ahmad *et al.,* 2002). Following a 24-hour treatment with LPS (1 µg/ml), there was an increase in PGE2 production (43.08 ± 1.45 ng/ml). However, co-treatment of the cells with LPS and different concentrations of MSEE (75, 150, and 300 µg/ml) significantly inhibited the LPS-induced PGE2 production (Table 2). Notably, the highest inhibition of LPS-induced PGE2 production was observed in the group treated with 300 µg/ml of MSEE.

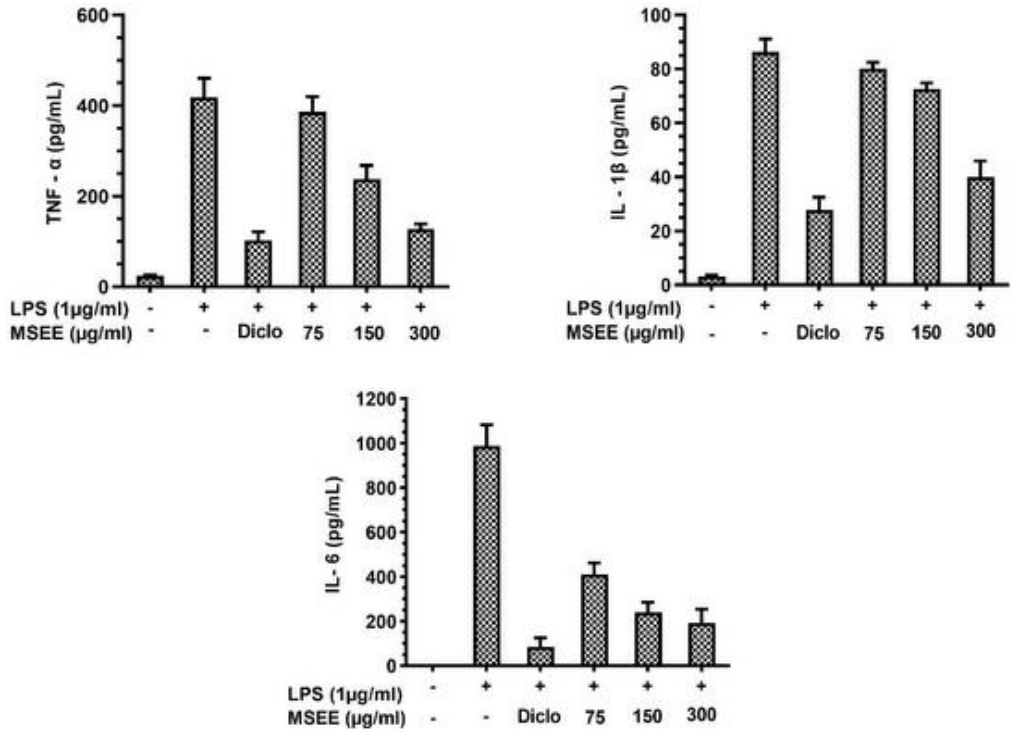

Fig. 3. Effects of MSEE on TNF-α, IL-1β and IL6release in THP-1 cell. The cell treated with MSEE concentration range from 75-300 µg/ml in LPS (1µg/ml) treated cells. The result represented as Mean±SD of three independent experiments.

Table 2: Effects of *M. feerra* stem bark ethanol extract (MSEE) on LPS (1 µg/ml)-Induced Nitrite Oxide (NO) and PGE2 Production.

Treatments	Nitric Oxide (µM)	Nitric Oxide (µM)	PGE2 (ng/ml)
Control	11.09 ± 0.15	11.09 ± 0.15	4.77 ± 0.49
LPS	44.94 ± 1.73	44.94 ± 1.73	43.08 ± 1.45
LPS+75 µg/ml MSEE	30.41 ± 1.77	30.41 ± 1.77	36.11 ± 0.81
LPS+150 µg/ml MSEE	23.59 ± 0.15	23.59 ± 0.15	27.97 ± 0.55
LPS+300 µg/ml MSEE	17.37 ± 0.55	17.37 ± 0.55	8.98 ± 0.25
Diclofenac	12.61 ± 0.35	12.61 ± 0.35	6.97 ± 0.53

Each data represents the mean ± SD of three independent experiments.

HRBC membrane stabilization. The stabilization of the erythrocyte (HRBC) membrane has been utilized as a method to investigate the anti-inflammatory activity, as it shares similarities with the lysosomal membrane (Vadivu and Lakshmi 2008; Shenoy *et al.,* 2010). The stabilization of the HRBC membrane implies that the extract could potentially stabilize the lysosomal membrane as well. This, in turn, can reduce tissue damage at the site of inflammation by preventing the release of activated neutrophil lysosomal components (Rahman *et al.,* 2012). In this study, we investigated the HRBC membrane stabilization to understand the mechanism of MSEE's anti-inflammatory activity, and the results are presented in Table 3. The findings demonstrate that both MSEE and Diclofenac effectively inhibit haemolysis at different concentrations. MSEE exhibits the highest inhibition percentage of 79.11 ± 2.42 at a concentration of 300 µg/ml, while Diclofenac shows an inhibition of 84.06 ± 2.94 at a concentration of 100 µg/ml. These results suggest that the anti-inflammatory activity of MSEE may be attributed to its ability to inhibit the release of inflammatory mediators responsible for inflammation through its membrane stabilizing effect.

Inhibition of Protein denaturation. As part of investigating the mechanism of the anti-inflammatory activity, the study examined the extracts' ability to inhibit protein denaturation, which is considered one of the causes of inflammation. The results indicated that the activity of the extracts in inhibiting protein denaturation was dependent on their concentration. As shown in Table 3, maximum inhibition of 84.99 ± 3.76 was observed at 300 µg/ml concentration of MSEE, and standard Diclofenac showed inhibition of 89.22 ± 3.17 at 100µg/ml. This result showed that MSEE has an effective inhibitory action on protein denaturation (Sen *et al.,* 2015) (Table 3).

Proteinase Inhibitory assay. Previous research has highlighted the crucial involvement of leukocytes in the tissue damage caused by proteinase during inflammation. Inhibitors have been shown to provide a notable level of protection against this process

(Govindappa *et al.*, 2011). In the present investigation, MSEE significantly exhibited antiproteinase activity at different concentrations as shown in Table 3. The result showed that maximum inhibition of 90.01 ± 2.52 was observed at 300 µg/ml. Diclofenac showed an inhibition of 91.59 ± 2.44 at 100 µg/ml.

Table 3: HRBC membrane stability, Inhibition of protein denaturation, and Proteinase inhibitory activities of stem bark ethanol and extract of *M. ferrea.*

Drug / Standard	Conc. (µg/ml)	% of Inhibition		
		HRBC membrane stabilization	Protein denaturation	Proteinase inhibition
MSEE	75	27.32 ± 0.88	26.69 ± 1.19	30.44 ± 0.97
	150	51.74 ± 1.21	54.77 ± 2.15	64.79 ± 3.13
	300	79.11 ± 2.42	84.99 ± 3.76	90.01 ± 2.52
Diclofenac	100	84.06 ± 2.94	89.22 ± 3.17	91.59 ± 2.44

Each data represents the mean ± SD of three independent experiments.

5-Lipoxygenase inhibitory assay. Lipoxygenases have been identified as significant contributors to various disorders such as asthma, inflammation, and angiogenesis. The assessment of 5-Lipoxygenase activity has been widely employed to investigate the impact of extracts on leukotriene production (Senthil Kumar *et al.*, 2018). Thus, different concentrations of MSEE were analysed for 5- lipoxygenase inhibitory activity. The extract potentially blocks the activity of the enzyme as observed in Table 4, MSEE was able to inhibit the activity of 5-LOX at the range of concentrations tested. The stem bark extract demonstrated higher activity, exhibiting a 90.07 ± 1.97% inhibition at a concentration of 300 µg/ml. Both the extract and the standard Diclofenac exhibited dose-dependent inhibition of 5-lipoxygenase, indicating the potential anti-inflammatory properties of the extracts.

Cyclooxygenase inhibitory assay. The evaluation of cyclooxygenase activity determines the effect of extracts on the production of prostaglandin. Therefore, in the present investigation, different concentration of MSEE was analysed for the inhibitory efficacy of cyclo-oxygenase. The potential inhibitory effect of extract and standard drug Diclofenac was observed in Table 4. The result showed that 49.73± 2.75 and 93.07 ± 2.83% of inhibition COX-1 and COX-2 respectively was observed at 300µg/ml and was comparable with standard Diclofenac (37.49 ± 1.91 and 96.51 ± 3.79). The observed activities may be due to the secondary metabolites such as alkaloids, tannins, phenolics, and flavonoids in the qualitative phytochemical screening of extract (Kumar *et al.*, 2016).

Table 4: 5-Lipoxygenase inhibitory and cyclooxygenase inhibitory activities of stem bark ethanol extract of *M. ferrea* Linn (MSEE).

Drug / Standard	Conc. (µg/ml)	% of Inhibition		
		5-Lipoxygenase	Cyclooxygenase	
			COX-1	COX-2
MSEE	75	38.22 ± 0.55	12.59 ± 1.05	41.93 ± 2.41
	150	64.59 ± 1.19	26.31 ± 1.97	76.18 ± 3.08
	300	90.07 ± 1.97	49.73 ± 2.75	93.07 ± 2.83
Diclofenac	100	92.76 ± 3.54	37.49 ± 1.91	96.51 ± 3.79

Each data represents the mean ± SD of three independent experiments.

CONCLUSIONS

The current study demonstrates that the ethanol extract of MF stem bark exhibits significant antioxidant and anti-inflammatory effects. The presence of alkaloids, flavonoids, and other phenolic compounds in the extract contribute to these observed activities, as they exhibit dose-dependent scavenging of free radicals. Moreover, our findings indicate that the extract effectively inhibits the production of TNF-α, IL-1β, IL-6, NO, and PGE2 in LPS-stimulated THP-1 cells. Further investigations are required to identify the active constituents of the extract and elucidate their mechanisms of action.

FUTURE SCOPE

Mesua ferrea Linn. a plant indigenous to the Western Ghats, has been substantiated for its anti-inflammatory properties in this study. Similar to other plants within the Mesua genus, such as *Mesua daphnifolia, Mesua eugeniifolia*, and *Mesua floribunda, Mesua ferrea* holds promise for exploring diverse medicinal effects owing to its rich assortment of secondary metabolites. Additionally, the isolation and characterization of bioactive compounds from this plant have the potential to pave the way for novel drug discoveries and pharmacological interventions.

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Author contributions. Jyothsna K- Conceived and designed the analysis; Collected the data; Contributed data or analysis tools; Performed the analysis; Wrote

the paper. Manjunatha H- Conceived and designed the analysis. Ravi Kumar S- Performed the analysis.

Conflict of interest. None.

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