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Anti-Inflammatory, Antioxidant and Phytochemical Analysis of *Mesua Ferrea* Bark Extracts

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A B S T R A C T

In the present study investigation was carried out to assess the *in vitro* anti-inflammatory, antioxidant activities and phytochemical analysis of *Mesua ferrea* bark ethyl acetate extract. Novel anti-inflammatory and antioxidants screened from medicinal plants used as a medication for treatment of diseases without having any side effects. The anti-inflammatory activities of *Mesua ferrea* bark extracts was evaluated by *in vitro* 5-LOX and HRBC membrane stabilization assay. Among all the extracts MFBEE exhibited significant 5-LOX inhibitory activity and shown protective effect on the HRBC membrane stabilisation. ROS and RNS mediates oxidative stress has been implicated in pathogenesis of several chronic diseases. MFBEE exhibited significant DPPH, ABTS, NBT and NO free radical scavenging activity. The phytochemical analysis of *Mesua ferrea* bark ethyl acetate extract revealed the presence of high amount of phenolic content, flavonoids, terpenoids and coumarins which may be responsible for anti-inflammatory and antioxidant activities of *Mesua ferrea* bark ethyl acetate extract.

Keywords: *Mesua ferrea*, anti-inflammation, antioxidant, membrane stabilization, 5-Lipoxygenase, phytochemicals, reactive oxygen species, reactive nitrogen species, oxidative stress, Total phenolic content.

A R T I C L E I N F O

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1. Introduction

The immune system has developed gradually as a unique complex network that defends the host body from both infectious and non-infectious foreign substances. Malfunctioning of immune network either innate or adaptive branches, is associated with chronic inflammatory diseases such as inflammatory bowel diseases, arthritis, asthma, neurodegenerative diseases and autoimmune diseases [1]. Inflammation is vital response against infectious and non infectious agents. Both exogenous and endogenous inflammatory inducers such as lipopolysacharides (LPS) and proinflammatory cytokines (TNF-α, IL-1β, IL-6) and IFN-γ stimulates inflammatory macrophages M1 which elevates inflammatory mediators such as prostaglandin E2 (PGE_2) and leukotrienes (LT-4) and nitric oxide (NO) by cyclooxygenase-2 (COX-2), 5-lipoxoygenase (5-LOX) and inducible nitric oxide synthase (iNOS). Nuclear factor kappa B (NF-κB) plays a key role in upregulation of inflammatory pathways. Proinflammatory cytokines such as TNF-α and IL-1β act synergistically induce arachidonic acid dependent and independent inflammatory pathways (2). Inflammatory response leads to a cascade activation of NF-κB and signal transducer activator of transcription 3 (STAT3) controls stress response. TNF-α, IL-1β play a vital role in ROS and RNS induced inflammation (3). Oxidative stress is an imbalance between reactive oxygen and nitrogen species (ROS/RNS) and antioxidant systems. Mitochondrial respiration is a major source of ROS such as hydrogen peroxide (H_2O_2), super oxide radicals (O_2^-) and hydroxyl radicals (OH^-) (4). Steroidal, non-steroidal, anti-inflammatory and synthetic antioxidant drugs are used for treatment of inflammatory and oxidative stress related diseases. Though these drugs have potent anti-inflammatory activity, long term administration is required for treatment of chronic diseases. Furthermore these anti-inflammatory drugs have various severe side-effects on organ functions. Therefore naturally occurring anti-inflammatory agents with high therapeutic index and few side-effects are required as substitutes for chemical anti-inflammatory drugs. *In vivo* carrageenan-induced rat paw edema is significantly inhibited by 200 and 400mg/kg body weight ethanolic extracts of *Mesua ferrea* flowers (5). *In vitro* antioxidant studies (DPPH, ABTS and NBT) of *Mesua ferrea* ethanolic leaf extracts have shown potent antioxidant activities (6). Hence the present study has been undertaken to evaluate in *vitro* anti-inflammatory, antioxidant and phytochemical screening of *Mesua ferrea* bark extracts.

2. Materials and Methods

2.1 Collection of plant material

Mesua ferrea, commonly known as nagkeshara, used in traditional medicinal practices like Ayurveda to cure various diseases such as arthritis, allergy, asthma, leprosy, cough, fever, wounds and rheumatism. It was collected from Uppa Village, Chintapalli Mandal, Visakhapatnam District, A.P., India, and authenticated by Dr. S.B. Padal, Associate Professor, Department of Botany, Andhra University. A voucher specimen was deposited in Botany Department Herbarium, Andhra University for future reference (AU BDH 21910).

2.2 Preparation of plant extracts

Mesua ferrea bark was properly washed with distilled water, shade dried and coarse powdered. Powder weighing 350 grams was first defatted with petroleum ether and then extracted with hexane, ethyl acetate and methanol by hot percolation method using Soxhlet apparatus. The successive plant extracts were filtered through a whatmann filter paper no. 1 and concentrated to dryness under reduced pressure to obtain organic solvent crude extracts. The obtained hexane (6.46g), ethyl acetate (20.06g) and methanol (15.2g) extracts were used for *in vitro* and *in vivo* anti-inflammatory studies and phytochemical analysis.

2.3 Reagents

Zileuton (1-(1-Benzo[b]thien-2-ylethyl)-l-hydroxyurea), linoleic acid, potato 5-LOX enzyme (Cayman Chemicals USA). Alsever's solution, hypotonic saline, isotonic saline, phosphate buffer (pH 7.4, 0.15M), Diclofenac sodium (Sigma Aldrich). 1,2 Diphenyl-1-picrylhydrazyl (DPPH), Nitrobluetetrazolium (NBT) Riboflavin, EDTA purchased from Sigma, USA. Quercetin, ascorbic acid, ferric chloride, potassium ferrocyanate ($K_3Fe(CN)_6$), sodium carbonate, sodium azide, DMSO, sodium hydroxide, EDTA-N-(1-1-Napthyl) ethylediamine dihydrochloride obtained from Himedia laboratory Ltd., Mumbai, India. All reagents used are of analytical grade.

2.4 *In vitro* anti-inflammatory assays

2.4.1 5-lipoxygenase inhibition assay (7)

The anti-inflammatory activity of *Mesua ferrea* bark extracts (MFBHE, MFBEE and MFBME) was determined using *in vitro* 5-LOX inhibition assay. This assay analyses the inhibitory activity against the 5-LOX enzyme, which is involved in synthesis of inflammatory mediators known as leukotrienes. This assay was first developed by Sircar et al., 1983(8), later it is altered by Reddana et al., 1988(7). Lipoxygenases are group of dioxygenases which are involved in insertion of molecular oxygen into proinflammatory ω-6 fatty acids such as arachidonic acid and linoleic acid. Leukotrienes are formed from the initial attack on arachidonic acid by 5-lipoxygenase which adds molecular oxygen to carbon 5, leading to the formation of hydroperoxyeicosa tetra enoic acid (5-HPETE). Dehydration of 5-HPETE gives the epoxide, the epoxides then undergo isomerisation of their double bonds and gives leukotriene A4 (LTA4). Hydrolysis of LTA4 leads to formation of stable LTB4.

Linoleic acid is used as a substrate for determination of 5-LOX enzyme inhibition assay because it shares structural resemblance with arachidonic acid (Baylac and Racine 2003). The increase in absorbance at 234 nm is due to formation of 1, 3-diene from 1, 4-diene in linoleic acid hydroperoxide which is used in determination of 5-lipoxygenase inhibitory assay.

Procedure

5-LOX inhibitory assay was performed by using UV kinetic method Reddanna et al., 1988(7). This method was performed by using assay mixture consisting 3ml of 50mM phosphate buffer pH 6.3, along with 10µl of 80mM of linoleic acid and potato 5-LOX enzyme. This assay solution was kept in ice and measured the enzyme activity throughout the experiment for every two minutes at 234 nm in UV visible spectrophotometer. The 5-LOX inhibitory activity of *Mesua ferrea* different crude solvent extracts was tested at different concentrations viz., 25, 50 and 100µg/ml. The activity of 5-Lipoxygenase was compared with the standard positive control Zileuton.

The percent inhibition of 5-lipoxygenase inhibitory activity of plant extracts was calculated by using formula.

$$Percentage\ of\ inhibition = \left(\frac{OD.\ of\ control - O.D\ of\ test}{O.D.of\ control}\right) \times 100$$

2.4.2 HRBC membrane stabilization method

The human red blood cell membrane stabilization method (HRBC) has been used as a method to evaluate the *in vitro* anti-inflammatory activity (9). The principle involved here is stabilization of HRBC membrane by hypotonicity induced membrane lysis.

2.4.2.1 Preparation of (10% V/V) of human red blood cells (HRBC)

The red blood cells were taken from human volunteer who had not taken any NSAID's for two week prior to the experiment and was mixed with equal volume of sterilized Alsever's solution. This blood solution was centrifuged at 3000rpm and the packed red blood cells were separated and washed with isosaline solution. A suspension of 10% V/V was made with isosaline and this HRBC suspension was used for study.

2.4.2.2 Assay of membrane stabilizing activity (Hypotonic induced hemolysis)

The assay mixtures contain 1ml of phosphate buffer (pH 7.4, 0.15M), 2ml of hyposaline (0.36%), 0.5ml of HRBC suspension (10% V/V) with 0.5ml of plant extracts of various concentrations (50, 100, 250, 500 and 1000µg/0.5ml), standard drug Diclofenac sodium (50, 100, 250, 500 and 1000µg/0.5ml) and control saline sample consists of 0.5ml of HRBC suspension mixed with hypotonic saline to produce 100% hemolysis were incubated at 37^0C for 30 minutes and centrifuged for 15 minutes respectively. The hemoglobin content in the suspension was estimated using spectrophotometer at 560nm. The membrane stabilizing effect of *Mesua ferrea* plant extracts was evaluated on HRBC by taking different concentrations of extracts viz., 50, 100, 250 and 500µg/ml and tested for % protection rendered by the plant extracts.

The percentage of hemolysis of HRBC produced in the presence or absence of plant extracts was calculated as a measure of anti-inflammatory activity using the formula

$$Percentage\ protection = \left\{100 - \left(\frac{O.D.of\ treated\ samples}{O.D.of\ control}\right)\right\} \times 100$$

2.5 *In Vitro* antioxidant assays

2.5.1 Determination of total antioxidant activity (Phospho-Molybdenum antioxidant assay)

The total antioxidant activity of the *Mesua Ferrea* bark extracts was assessed by Phospho-Molybdenum assay method [10] which is based on the reduction of Mo VI to Mo V by the extracts and formation of green phosphate-Mo V complex in acidic condition.

Procedure

An aliquot of 0.1ml of extract (1mg/ml) / ascorbic acid equivalent to 500µg was combined with 1ml of regent solution (0.6ml sulphuric acid, 28mM sodium phosphate and 4mM ammonium Molybdate). The reaction mixture was incubated at 95^0C for 90 minutes. The absorbance of solution was measured at 695nm using UV visible spectrophotometer against blank (0.3ml of methanol is used as blank in the place of extract), after cooling to room temperature.

The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

2.5.2 DPPH free radical scavenging assay

DPPH (1, 1-diphenyl -2-picryl hydrazyl) free radical scavenging assay is an established assay and is widely used to assess the radical scavenging activity of antioxidant natural and chemical compounds. This assay is based on the reduction of DPPH in methanol in the presence of hydrogen donating antioxidant constituents of plant extracts, due to the formation of the non-radical form of DPPH . This transformation results in color change from purple to yellow which is measured spectrophotometrically. The disappearance of purple color is monitored at 517nm.

Procedure

An aliquot of 1ml, 0.3mM DPPH ethanolic solution was added to 2.5ml of plant extract and allowed to incubate at room temperature in dark conditions. After 30 minutes the absorbance values were measured at 517nm. Ethanol was used as a blank. DPPH solution (1ml, 0.3mM) plus ethanol (2.5ml) serves as negative control [11]. Ascorbic acid (100µg/ml) was used as standard.

The percentage of DPPH inhibition was calculated using the formula

$$Percentage\ of\ inhibition = \left(\frac{O.D.of\ control - O.D.of\ test}{O.D.of\ control}\right) \times 100$$

2.5.3 ABTS radical cation decolorization assay

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity was measured by the ABTS cation decolorization assay method and is used to determine the antioxidant activities of phyto compounds from medicinal plants [12].

Procedure

7mM of ABTS solution was reacted with freshly prepared 2.45mM ammonium persulphate solution and kept in dark for 12-16h, to produce a dark colored solution containing ABTS radical cations. The initial absorbance was measured at 734nm. This stock solution was diluted to give a final absorbance value of about 0.7 (±0.02). 0.1mL of different

concentrations (2-20µg/ml) of *Mesua Ferrea* stem bark extracts were allowed to react with 2mL of ABTS solution and the absorbance was measured at 734nm.

The percentage of inhibition was calculated using the formula

$$Percentage\ of\ inhibition = \left(\frac{O.D.of\ control - O.D.of\ test}{O.D.of\ control}\right) \times 100$$

2.5.4 NBT Riboflavin photo reduction method (Superoxide radical scavenging activity) (Dasgupta & De, 2004)

The superoxide radical scavenging activity of *Mesua Ferrea* stem bark extracts were determined by the method of Mc Cord and Fridvich (1969), modified by R. Kuttan et al., 1996 [13] which depends on the light induced superoxide generation by Riboflavin and the corresponding reduction of NBT to a purple formazan.

Procedure

The assay mixture contained different concentrations of the *Mesua Ferrea* stem bark extracts and EDTA (6mM containing 3µg sodium cyanide), nitro blue tetrazolium (50µm) riboflavin (2µM) and phosphate buffer (58µM, pH 7.8) in total volume of 300µl. The reaction was initiated by adding 0.4ml of 1mM hydroxylamine hydrochloride and incubated for 20 minutes. The absorbance was measured by 560nm using spectrophotometer.

The percentage of inhibition was calculated using the formula

$$Percentage\ of\ inhibition = \left(\frac{O.D.of\ control - O.D.of\ test}{O.D.of\ control}\right) \times 100$$

An IC50 was determined using line equation of the curve plotted concentration against percentage inhibition

2.5.5 Nitric oxide radical scavenging assay

Nitric oxide (NO) is a major secretory product of mammalian cells that initiates host defense, homeostatic and development functions by either direct effect or intercellular signaling. NO is formed from amino acid L-arginine by nitric oxide synthase [14]. As a direct effector, NO is thought to activate regulatory proteins, kinases and proteases which are directed by reactive oxygen intermediates [15]. As a messenger molecule, NO covalently interacts with target molecules based on redox potential [16]. Nitric oxide assay was measured by spectrophotometric method described by Govindarajan et al., 2003[17]

Procedure

Sodium nitroprusside (5mM) in phosphate buffer saline was mixed with different concentrations of *Mesua Ferrea* stem bark extracts dissolved in methanol and incubated at 250C for 30 minutes. After 30 minutes 1.5ml of the incubation solution was removed and diluted with 1.5ml of Griess reagent (1% sulphanilic acid, 2% phosphoric acid and 0.1% N-1-napthylethylene diamine dihydrochloride). The absorbance was measured at 546nm. The ascorbic acid was taken as standard

The percentage of nitric oxide radical scavenging activity was calculated using the formula

$$Percentage\ of\ inhibition = \left(\frac{O.D.of\ control - O.D.of\ test}{O.D.of\ control}\right) \times 100$$

2.6 Phytochemical analysis

2.6.1 Qualitative Phytochemical analysis

The phytochemical studies of successive extracts of *Mesua ferrea* bark were tested for the presence of bioactive compounds by using following standard phytochemical methods [18,19].

2.6.1.1 Test for alkaloids

Hager's test

Few drops of Hagers reagent were added to each filtrate and observed for the formation of yellow precipitate which indicates the presence of alkaloids [20].

Wagner's test

Few drops of Wagner reagent (solution of potassium iodide) were added to each filtrate and observed for the formation of reddish brown precipitate which indicates the presence of alkaloids [21].

2.6.1.2 Test for tannins

Ferric chloride test

Add ferric chloride ($FeCl_3$) solution drop by drop to the extract. Bluish black precipitate indicates presence of tannins [22].

2.6.1.3 Test for saponins

Foam test

5ml of extract was shaken vigorously with water and warmed. Formation of stable foam indicates saponins [23].

2.6.1.4 Test for phlobatannins

Precipitate test

2ml of extract was added to 1ml of 1% HCl and the mixture was boiled. Deposition of red precipitate indicates phlobatannins [24].

2.6.1.5 Test for flavonoids

Alkaline Agent test

Add 2ml of 2% Solution of NaOH to the extract leads to formation of intense yellow color and colorless after addition of dilute acid indicates flavonoids [25].

2.6.1.6 Test for terpenoids

Salkowski test

5ml of 1mg extract was mixed with 2ml of chloroform and 3ml of Concentrated H_2SO_4 was carefully added to form a layer. Reddish brown interface was formed implying the presence of terpenoids [26].

2.6.1.7 Test for glycosides

Liebermann's test

The extract was added with equal amount of chloroform and acetic acid. The mixture was cooled then H_2SO_4 was carefully added, the color change from violet blue to green indicates glycosides [27].

2.6.1.8 Test for cardiac glycosides

Keller Kilani test

To the extract add 2ml glacial acetic acid containing few drops of ferric chloride solution. The mixture was transferred to another test tube containing 1ml of concentrated sulphuric acid. The formation of brown ring at the interface indicates cardiac glycosides [28].

2.6.1.9 Test for steroids

Salkowskis test

To the extract chloroform and concentrated H_2SO_4 were added and shaken well. Reddish brown color appearance in the lower layer indicates sterols [26].

Liberman-Burchard test

To the extract add few drops of acetic anhydride. Boiled and cooled, then add concentrated sulphuric acid slowly to the sides of test tube. Brown ring at the junction of the two layers indicates sterols [27].

2.6.1.10 Test for quinines

a) Extract was treated with concentrated HCl. Formation of yellow precipitate indicates quinones.
b) Extract was treated with concentrated H_2SO_4. Formation of red color indicates quinines [28].

2.6.1.11 Test for coumarins

To the extract add 10% NaOH and then add Chloroform. Formation of yellow color indicates coumarins [28].

2.6.2 Quantitative Phytochemical studies

2.6.2.1 Determination of total phenolic content

The total phenolic content in the various organic plant extracts of *Mesua ferrea* were measured by using Folin-Ciocalteu reagent based on the procedure described by Singleton et al., 1999[29], with some modifications. Folin-Ciocalteu reagent was used as oxidising agent and galic acid was used as a standard in determination of total phenolic content in the plant extracts (Skerget M., Kotnik P., Food Chem, 2005, Chye FY-int J. Pharamacol, 2009).

Procedure

2mg of extract was taken and dissolved in the distilled water to get a sample concentration 2mg/ml. Plant extracts were solubilized with 2% DMSO (Di methyl sulfoxide). Briefly 0.5ml of plant extracts (2mg/ml) was mixed with 1.5ml (1:10 V/V diluted with distilled water) of Folin-Ciocalteu's reagent and allowed to stand for 5 minutes at room temperature, then 2ml of sodium carbonate (Na_2CO_3, 7.5% W/V) was added and the mixture was kept steady for another 1 1/2 hr., and kept in dark with intermittent shaking. Then the absorbance of blue colour developed was measured at 760nm using UV spectrophotometer. The experiment was carried out in triplicates. Galic acid was used for constructing the standard curve (50-500µg/ml) and the total phenolic concentrations in the plant extracts were expressed as milligrams of galic acid equivalent per gram of dry weight (mg of GAE/g) of plant.

2.7 Statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM).

3. Results and Discussion

3.1 Dose-dependent effect of MFBHE, MFBEE and MFBME on 5-LOX activity

The percentage of 5- Lipoxygenase enzyme inhibitory activity of *Mesua Ferrea* stem bark extracts at concentrations of 25, 50 and 100µg/ml were shown in **table 3.1**. The 5-LOX inhibitory activities of MFBHE, MFBEE and MFBME at 100µg/ml were found to be 43.24%, 76.58% and 61.46% respectively and zileuton was taken as a positive control for comparing 5-LOX inhibitory activity of plant extracts and its percentage of inhibition at 10µg/ml was found to be 61.78%. The IC_{50} values of MFBHE, MFBEE, MFBME and zileuton were found to be 174.35µg/ml, 46.65µg/ml,69.67µg/ml and 6.85µg/ml respectively. It was observed that MFBEE showed significant inhibitory activity than MFBHE and MFBME.

The 5-LOX inhibitory activity of *Mesua ferrea* bark extracts exhibits the following order

MFBHE < MFBME < MFBEE

It has been shown that the secondary metabolite flavonols such as quercetin, morin and kaempferol were found to have 5-LOX inhibitory activities. The results obtained from 5-LOX inhibitory activities of *Mesua ferrea* bark extracts exhibits dose-dependent activities on 5-LOX enzyme. The most promising anti-inflammatory activity was exhibited by MFBEE among the solvent extracts; this may be due to the presence of secondary metabolites such as flavonoids, terpenoids, glycosides, cardiac glycosides, steroids/ phytosterols, quinones and coumarins.

Table 3.1: Inhibitory effect of *Mesua ferrea* bark extracts on 5-LOX activity

Solvent extracts/Standard	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
MFBHE	25	18.97±0.939	174.35
	50	22.61±0.75	
	100	43.24±0.854	
MFBEE	25	36.35±1.599	46.65
	50	42.95±0.994	
	100	76.58±0.683	
MFBME	25	23.15±0.584	69.67
	50	38.59±0.936	
	100	61.46±2.483	
Zileuton	2.5	26.19±1.408	6.85
	5	43.32±0.988	
	10	61.78±1.653	

3.2 Protective effect of *Mesua ferrea* bark extracts on HRBC stabilization:

HRBC stabilization activity of *Mesua ferrea* bark extracts (MFBHE, MFBEE, MFBME) has been evaluated by HRBC

stabilization method developed by Ranjan et al., 2013[30]. HRBC stabilization activity of *Mesua ferrea* bark extracts was performed with different doses viz., 50, 100, 250 and 500µg/ml along with the standard diclofenac sodium as shown in **table 3.2.** A dose-dependent HRBC stabilization activity of *Mesua ferrea* bark extracts was observed and evaluated by % hemolysis and % stabilization of HRBC. The MFBEE has shown significant HRBC stabilization activity than MFBHE and MFBME. The MFBEE has even shown more protective effect at the concentration of 500µg/ml with 77.13% stabilization, whereas MFBHE and MFBME have shown protective effect at the concentration of 500µg/ml with 51.77% and 65.45% stabilization respectively, whereas, diclofenac sodium has shown protective effect at the concentration of 500µg/ml with 87.38% stabilization. Similarly the % hemolysis was found to be significantly reduced in a dose-dependent manner, when HRBC is treated with MFBEE compared to MFBHE and MFBME.

The *Mesua ferrea* extracts exhibited membrane stabilization by inhibiting hypotonicty-induced hemolysis. The ethrocyte membrane is similar to lysosomal membrane and its stabilization implies that the extracts of *Mesua ferrea* will even stabilize the lysosomal membranes [31]. Stabilization of lysosomal membrane helpful in preventing the release of inflammatory mediators from activated neutrophils, which can cause tissue inflammation upon extracellular release. The anti-inflammatory activities of MFBEE are due to the ability of its interference with the early phase of inflammation by preventing the release of histamine and serotonin vasodilators [32].

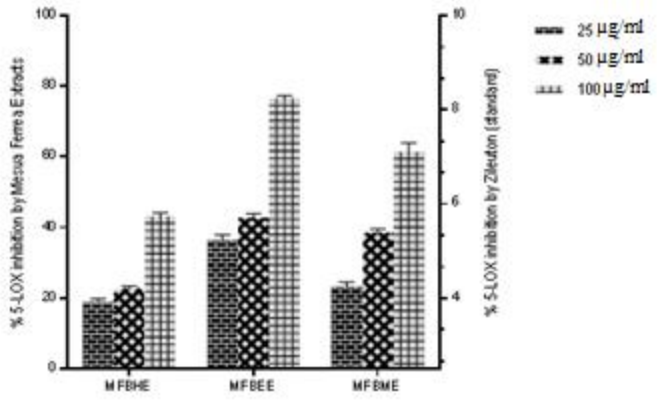

Figure 3.1: 5-lipoxygenase enzyme inhibition by organic solvent extracts of *Mesua ferrea* at different concentrations. Values are mean of three replicates ± SEM

Table 3.2: *In vitro* anti-inflammatory activity of *Mesua ferrea* stem bark extracts by HRBC membrane stabilization method

Organic solvent plant Extract	Extract concentration (µg/ml)	HRBC		IC_{50} (µg/ml)
		% Hemolysis	% Stabilization	
MFBHE	50	78.96	21.04±0.24	388.76
	100	66.36	33.64±1.29	
	250	54.36	45.64±0.47	
	500	48.23	51.77±1.42	
MFBEE	50	66.84	33.16±2.38	112.01
	100	50.15	49.85±1.24	
	250	32.93	67.07±0.44	
	500	22.87	77.13±0.98	
MFBME	50	69.7	30.30±1.03	162.69
	100	54.08	45.92±1.84	
	250	43.36	56.64±1.65	
	500	34.55	65.45±1.89	
Diclofenac Sodium	50	39.79	60.21±1.57	19.25
	100	29.68	70.32±2.37	
	250	20.22	79.78±1.39	
	500	12.62	87.38±1.52	

3.3 Preliminary qualitative and quantitative phytochemical analysis of *Mesua ferrea* bark extracts

3.3.1 Qualitative phytochemical analysis [28]

The preliminary qualitative phytochemical screening of *Mesua ferrea* stem bark extracts was done to assess the presence of the bioactive compounds responsible for anti-inflammatory and antioxidant activities. There is a need to

choose different solvents with varying polarity to prepare the extracts so as to dissolve the different phytoconstituents present in the plants. Based on the above reason three solvents of varying polarities viz., hexane, ethyl acetate, methanol were used for preparation of extracts. The phytochemical screening of these solvent extracts shown the presence and absence of phytoconstituents according to polarity of solvent used for extraction. The results were shown in table 1. Alkaloids, tannins, saponins and phlobatannins were found to be absent in all the three extracts of *Mesua ferrea*. While all the three extracts of *Mesua ferrea* showed presence of glycosides, cardiac glycosides, sterols and quinones. The ethyl acetate and methanolic extracts accounted for the presence of all the secondary metabolites such as flavonoids and terpenoids. The potential anti-inflammatory and antioxidant activities of *Mesua ferrea* were found to be due to the presence of compounds such as flavonoids, terpenoids, steroids (glycosides, cardiac glycosides) and quinones.

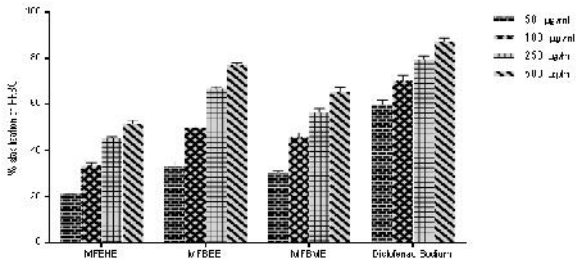

Figure 3.2: Protective effect of various concentrations of *Mesua ferrea* bark extracts and diclofenac sodium (standard) on HRBC membrane stabilization. Values are mean of three replicates ± SEM

Table 3.3: Preliminary phytochemical screening of *Mesua ferrea* stem bark extracts

S. No.	Phytochemicals	Hexane Extract	Ethyl Acetate Extract	Methanol Extract
1.	Alkaloids	-ve	-ve	-ve
2.	Tannins	-ve	-ve	-ve
3.	Saponins	-ve	-ve	-ve
4.	Phlobatannins	-ve	-ve	-ve
5.	Flavonoids	-ve	+ve	-ve
6.	Terpenoids	-ve	+ve	+ve
7.	Glycosides	+ve	+ve	+ve
8.	Cardiac Glycosides	+ve	+ve	+ve
9.	Steroids/Phytosterols	+ve	+ve	+ve
10.	Quinones	+ve	+ve	+ve
11.	Coumarins	-ve	+ve	-ve

(+ve) presence of constituents; (-ve) absence of constituents

3.3.2 Quantitative phytochemical analysis

3.3.2.1 Total Phenolic content

The total phenolic content in *Mesua ferrea* bark extracts was quantified with standard curve prepared using gallic acid. The phenolic content was higher in MFBEE than MFBHE and MFBME. The phenolic content of *Mesua ferrea* bark extracts was expressed in terms of mg of gallic acid equivalents (GAE) per gram of *Mesua ferrea* bark extracts. As shown in the **table 3.4** the MFBEE contain higher total phenolic content (37.34mg/gm) than MFBHE (19.80mg/gm) and MFBME (23.68mg/gm). The anti-inflammatory and antioxidant activities of MFBEE are due to its high phenolic content.

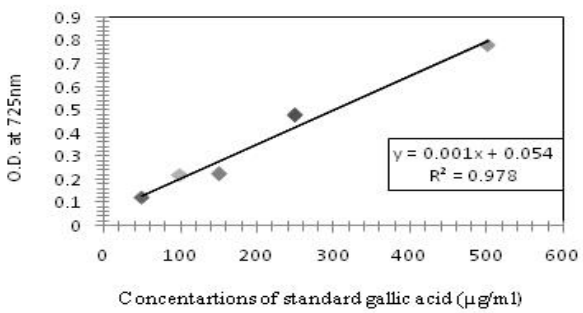

Figure 3.3: Calibration curve for total phenolic content using gallic acid as standard

Table 3.4: Quantitative phytochemical screening of *Mesua ferrea* bark extracts

Plant extract	TPC in GAE mg/gm extract			
	1	2	3	Mean±SEM
MFBHE	18.92	21.58	18.9	19.80±0.89
MFBEE	36.04	38.67	37.31	37.34±0.75
MFBME	21.43	24.29	25.32	23.68±1.16

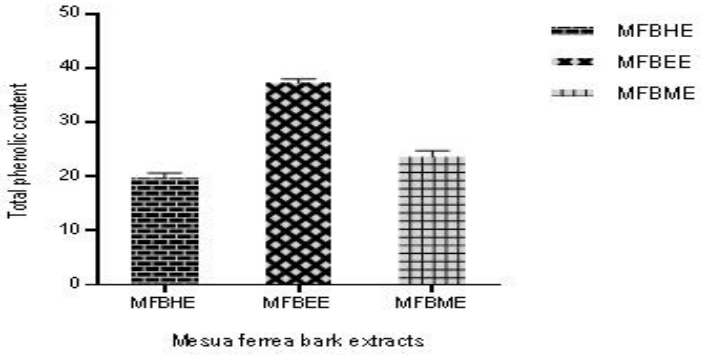

Figure 3.4: Total phenolic content of *Mesua ferrea* bark extracts (MFBHE, MFBEE and MFBME) using gallic acid as a standard

3.4 Total antioxidant activity of *Mesua ferrea* bark extracts

Secondary metabolites of plant sources such as phenolic compounds, flavonoids, terpenoids and alkaloids showed a wide range of *in vitro* and *in vivo* free radical scavenging activity [33]. These plant originated antioxidants avert the body from oxidative stress caused by reactive oxygen and nitrogen species. Mesua ferrea is a good source of phenolic compounds, flavonoids, and terpenoids. From the previous reports Mesua ferrea leaf ethanolic extract contain high amount of phenolic and alkaloid content so that it exhibit dose-dependent inhibition of free radicals. The total antioxidant activity of *Mesua ferrea* bark extracts of different concentrations is based on the reduction of Molybdenum-VI to Molybdenum-V, a green phosphate Molybdenum-V is formed. This green phosphate color intensity was measured maximum absorption at 695nm. The total antioxidant capacity was increased by increasing the concentration of *Mesua ferrea* bark extracts. The total antioxidant capacity of Mesua ferrea bark extracts was expressed as a number of equivalents of ascorbic acid. As shown in the **table 3.5** the MFBEE contain higher total antioxidant capacity (32.25mg/gm) than MFBHE (9.21mg/gm) and MFBME (11.37mg/gm).

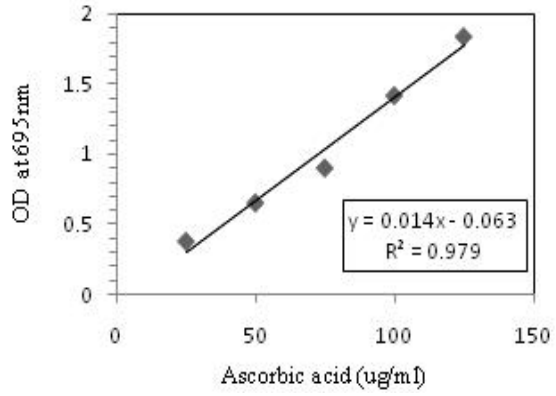

Figure 3.5: Calibration curve for total antioxidant capacity using ascorbic acid as standard

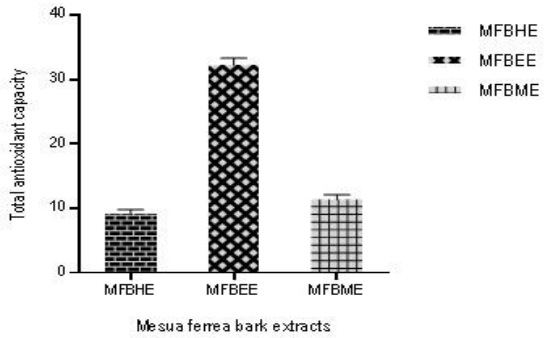

Figure 3.6: Total antioxidant content of Mesua ferrea bark extracts (MFBHE, MFBEE and MFBME) using ascorbic acid as a standard

Table 3.5: Total antioxidant capacity of *Mesua ferrea* bark extracts

Plant extract	Ascorbic acid equivalent mg/gm of extract			
	1	2	3	Mean±SEM
MFBHE	8.44	10.45	8.74	9.21±0.62
MFBEE	34.29	31.91	30.55	32.25±1.09
MFBME	10.09	12.67	11.35	11.37±0.74

3.5 DPPH scavenging effect of *Mesua ferrea* bark extracts

DPPH free radical scavenging activity of *Mesua ferrea* bark extracts was determined by the method of Lamaison JL et al., 1991[34]. Antioxidant activity of *Mesua ferrea* bark extracts (MFBHE, MFBEE and MFBME) was carried out with different doses viz., 25, 50 and 100µg/ml. As shown in **table 3.6** a dose-dependent free radical scavenging activity was obtained. The DPPH free radical scavenging activity of MFBHE, MFBEE and MFBME at 100µg/ml were 38.39%, 58.54% and 48.31% respectively, whereas the standard, ascorbic acid exhibited 73.49% at 100µg/ml. The IC_{50} values of MFBHE, MFBEE, MFBME and ascorbic acid were 237.40, 67.35, 103.66 and 4.95µg/ml respectively.

Table 3.6: Determination of scavenging activity of *Mesua ferrea* bark extracts by DPPH assay

Extracts / Standard	Concentration (µg/ml)	% Inhibition of DPPH radical	IC_{50} (µg/ml)
MFBHE	25	17.87±1.00	237.40±16.00
	50	25.03±1.08	
	100	38.39±0.77	
MFBEE	25	29.46±0.68	67.35±1.86
	50	43.33±0.45	
	100	58.54±0.67	
MFBME	25	24.63±0.95	103.66±2.43
	50	39.72±0.58	
	100	48.31±0.44	
Ascorbic acid	2.5	21.58±0.90	4.95±0.08
	5	55.84±0.95	
	10	73.49±1.91	

Table 3.7: Determination of scavenging activity of *Mesua ferrea* bark extracts by ABTS assay

Extracts / Standard	Concentration (µg/ml)	% Inhibition of ABTS radical	IC_{50} (µg/ml)
MFBHE	25	18.57±0.63	214.76±9.38
	50	27.13±0.69	
	100	39.25±0.59	
MFBEE	25	28.37±0.22	73.69±3.38
	50	41.42±1.12	
	100	56.75±1.22	
MFBME	25	23.84±0.78	108.13±7.81
	50	37.53±0.54	
	100	48.45±1.28	
Ascorbic acid	2.5	22.64±0.39	4.81±0.07
	5	57.15±1.07	
	10	74.29±0.45	

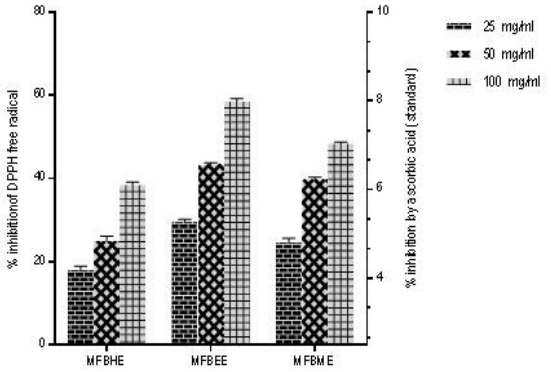

Figure 3.7: Scavenging activity of Mesua ferrea bark extracts at three different concentrations by DPPH assay

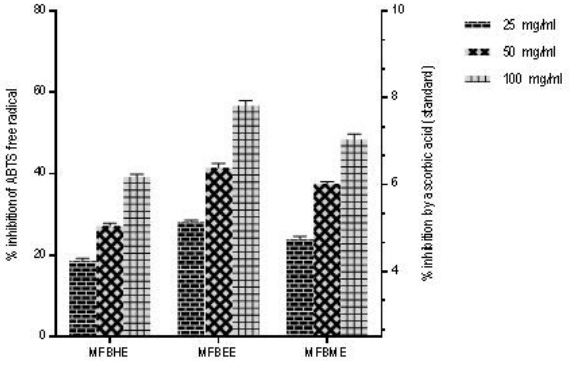

Figure 3.8: Scavenging activity of Mesua ferrea bark extracts at three different concentrations by ABTS assay

3.6 ABTS scavenging effect of Mesua ferrea bark extracts

ABTS radical scavenging activity Mesua ferrea bark extracts was determined by the method of Pellegrini et al., 1999{35]. Antioxidant activity of Mesua ferrea bark extracts (MFBHE, MFBEE and MFBME) was carried out with different doses viz., 25, 50 and 100µg/ml. As shown in the **table 3.7** a dose-dependent free radical scavenging activity was obtained. The ABTS free radical scavenging activity of MFBHE, MFBEE and MFBME at 100µg/ml were 39.25%, 56.75% and 48.45% respectively, whereas the standard, ascorbic acid exhibited 74.29% at 100µg/ml. The IC50 values of MFBHE, MFBEE, MFBME and ascorbic acid were 214.76, 73.69, 108.13 and 4.81µg/ml respectively.

3.7 NBT Riboflavin scavenging effect of *Mesua ferrea* bark extracts

Superoxide scavenging ability of *Mesua ferrea* bark extracts was determined by the method of Mc Cord & Fridvich et al., 1969 [36] and modified by R. Kuttan et al., 1996[37]. This activity depends on light induced super oxide generation by riboflavin and corresponding reduction of NBT. Antioxidant activity of *Mesua ferrea* bark extracts (MFBHE, MFBEE and MFBME) was carried out with different doses viz., 25, 50 and 100µg/ml. As shown in **table 3.8** a dose-dependent free radical scavenging activity was obtained. The superoxide free radical scavenging activity of MFBHE, MFBEE and MFBME at 100µg/ml were 37.12%, 53.15% and 45.60% respectively, whereas the standard, quercetin exhibited 72.18% at 100µg/ml. The IC_{50} values of MFBHE, MFBEE, MFBME and quercetin were 275.15, 87.53, 127.71 and 5.02µg/ml respectively.

Table 3.8: Determination of scavenging activity of *Mesua ferrea* bark extracts by NBT Riboflavin assay

Extracts / Standard	Concentration (µg/ml)	% Inhibition of NBT radical	IC_{50} (µg/ml)
MFBHE	25	17.85±0.58	275.15±39.11
	50	25.24±0.41	
	100	37.12±1.08	
MFBEE	25	26.47±0.45	87.53±2.51
	50	38.29±0.39	
	100	53.15±0.48	
MFBME	25	21.49±0.48	127.71±2.77
	50	34.11±0.42	
	100	45.60±0.21	
Quercetin	2.5	21.93±0.95	5.02±0.04
	5	55.22±0.51	
	10	72.18±0.45	

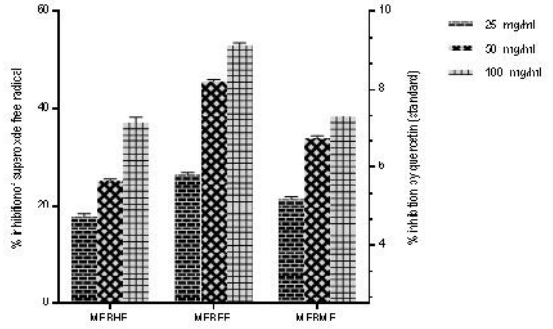

Figure 3.9: Scavenging activity of *Mesua ferrea* bark extracts at three different concentrations by NBT Riboflavin assay

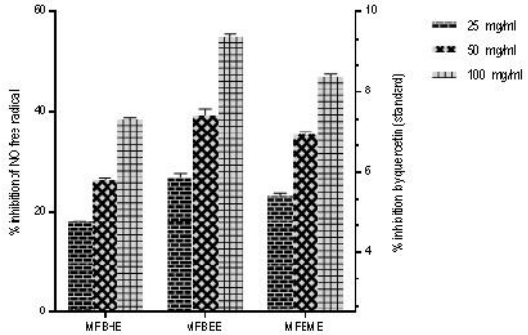

Figure 3.10: Scavenging activity of *Mesua ferrea* bark extracts at three different concentrations by NO assay

3.8 Nitric oxide scavenging activity by bark extracts of *Mesua ferrea*

Nitric oxide radical scavenging activity of bark extracts of *Mesua ferrea* was determined by the method of Griess illosvoy reaction developed by Garrat 1964. Antioxidant activity of bark extracts (MFBHE, MFBEE and MFBME) was carried out with different doses viz., 25, 50 and 100µg/ml. As shown in **table 3.9** a dose-dependent antioxidant activity was observed. The superoxide free radical scavenging activity of MFBHE, MFBEE and MFBME at 100µg/ml were 38.59%, 55.19% and 47.10% respectively, whereas the standard, quercetin exhibited 74.17% at 100µg/ml. The IC_{50} values of MFBHE, MFBEE, MFBME and quercetin were 227.33, 79.79, 117.87 and 4.82µg/ml respectively.

Table 3.9: Determination of scavenging activity of *Mesua ferrea* bark extracts by NO assay

Extracts / Standard	Concentration (µg/ml)	% Inhibition of NO radical	IC_{50} (µg/ml)
MFBHE	25	18.17±0.10	227.33±6.30
	50	26.42±0.35	
	100	38.59±0.32	
MFBEE	25	27.12±0.73	79.79±2.926
	50	39.45±1.13	
	100	55.19±0.54	
MFBME	25	23.22±0.56	117.87±2.45
	50	35.45±0.66	
	100	47.10±0.58	
Quercetin	2.5	23.41±0.50	4.82±0.02
	5	56.22±0.53	
	10	74.17±0.44	

Table 3.10: Percentage free radical scavenging activity at 25, 50 and 100µg/ml and IC_{50} values of Mesua ferrea bark extracts on DPPH, ABTS, NBT and NO

Antioxidant assay	MFBHE				MFBEE				MFBME			
	% Scavenging activity			IC_{50} (µg/ml)	% Scavenging activity			IC_{50} (µg/ml)	% Scavenging activity			IC50 (µg/ml)
	25µg/ml	50µg/ml	100µg/ml		25µg/ml	50µg/ml	100µg/ml		25µg/ml	50µg/ml	100µg/ml	
DPPH	17.87	25.03	38.39	237.40	29.46	43.33	58.54	67.35	24.63	39.72	48.31	103.66
ABTS	18.57	27.13	39.25	214.76	28.37	41.42	56.75	73.69	23.84	37.53	48.45	108.13
NBT	17.85	25.24	37.12	275.15	26.47	38.29	53.15	87.53	21.49	34.11	45.60	127.71
NO	18.17	26.42	38.59	227.33	27.12	39.45	55.19	79.79	23.22	35.45	47.10	117.87

4. Conclusion

Based on the results it was concluded that MFBEE shown significant *in vitro* anti-inflammatory and antioxidant activities. From *in vitro* anti-inflammatory studies on *Mesua ferrea* bark extracts, MFBEE exhibited dose-dependent 5-LOX inhibitory activity. The inhibitory activity of 5-LOX at 100µg/ml was found to be 76.58% with IC_{50} values of 46.65µg/ml. In the same manner, MFBEE has shown more protective effect (77.13%) in the HRBC stabilization at the concentration of 500µg/ml. From the results of *in vitro* antioxidant studies it was concluded that the *Mesua ferrea* bark ethyl acetate extract (MFBEE) has exhibited a dose-dependent DPPH, ABTS, NBT riboflavin and nitric oxide (NO) free radical scavenging activity with reference to standards. The phytochemical screening of *Mesua ferrea* bark ethyl acetate extract (MFBEE) accounted for the presence of all the secondary metabolites such as flavonoids and terpenoids, steroids (glycosides, cardiac glycosides) and quinones. Hence *Mesua ferrea* bark ethyl acetate extract (MFBEE) is a potential candidate for development of novel anti-inflammatory and antioxidant agents useful for the treatment of various inflammatory and oxidative stress related diseases. Further this study has been focused on isolation of anti-inflammatory compounds present in MFBEE by bioactivity-guided fractionation.

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