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Original Article

EVALUATION OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY OF STEM BARK EXTRACTS OF MESUA FERREA LINN.

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ABSTRACT

Objective: Phytochemical evaluation and *in vitro anti-inflammatory* activity of stem bark extracts of *Mesua ferrea* Linn.

Methods: Petroleum ether, chloroform, and 80% ethanol extracts were used for *in-vitro* bioassays: such as Human red blood cells membrane stabilization, protein inhibitor, inhibition of egg albumin and anti-lipoxygenase assay to evaluate their anti-inflammatory potential.

Results: Phytochemical analysis revealed the presence of phenols, flavonoids, alkaloids, steroids, saponins, tannins, glycosides, and terpenoids. The petroleum ether, chloroform, and 80% ethanol extracts showed significant reduction in inflammation at different 100, 200 and 500µg/ml compared to standard drug indomethacin (100µg/ml).

Conclusion: Based on the results, it can be concluded that the 80% ethanol extract of stem bark of *Mesua ferrea* Linn. (*M. ferrea*) exhibited significant anti-inflammatory activity compared to petroleum ether and chloroform extracts.

Keywords: *Mesua ferrea* Linn. Phytochemicals, Anti-inflammatory.

INTRODUCTION

Inflammation is a complex response that protects the host against tissue injury and microbial invasion. The redness, swelling, heat, pain, and loss of functions are considered as symptoms of inflammation. However, prolonged inflammation can be harmful contributing to the pathogenesis of many diseases such as asthma, arthritis, multiple sclerosis and even cancer [1, 2].

Inflammation can be classified as acute and chronic status depending on onset time. Acute inflammation is the primary response of the body to injurious stimuli and is involved in vascular changes i.e. vasodilatation, increased capillary permeability and migration of leukocytes. Chronic inflammation is prolonged inflammation characterized by progressive destruction and retrieving of injured tissue from the inflammatory process [3, 4].

Generally, Non-steroidal anti-inflammatory drugs (NSAIDs) are used to reduce the pain, inflammation and fever since many years and their prolonged use often lead to gastric intolerance, bone marrow depression, water and salt retention. Therefore, naturally originated agents with fewer adverse effects are desirable to substitute chemical therapeutics [5, 6].

Mesua ferrea Linn. (Clusiaceae) is a medicinal plant commonly known as Nagasampige. It is distributed in tropical countries like India, Burma, Thailand, Indochina and New Guinea. Traditionally, various parts of *Mesua ferrea* used for the treatment of inflammation related diseases such as skin diseases, wounds, ulcers, hemorrhoids, piles and snake bite [7, 8]. Bark and roots are used for the treatment of gastritis and bronchitis, buds useful in dysentery, flowers powder mixed with ghee applied externally in bleeding piles, leaves as a poultice applied on the forehead in severe colds, bark astringent, decoction with ginger used as sudorific [7, 9]. The pharmacological investigation on *Mesua* species have shown that they exhibit many biological properties such as antioxidant [9, 10], antiulcer [10], antimicrobial [11-14], analgesic [15, 16], antibacterial [17], anti-inflammatory [18, 19], wound healing [20], anti-arthritic [21], hepatoprotective [22], antifungal [23], immuno-modulatory [24], antispasmodic[25], antineoplastic[26], anti-convulsant [27] and antivenom activity [28]. Hence, the present study was carried out to evaluate the phytochemical analysis and in vitro anti-inflammatory activity of *Mesua ferrea* Linn.

MATERIALS AND METHODS

Drugs and chemicals

Petroleum ether, chloroform, ethanol and Folin-ciocalteu reagents were purchased from Merck (Germany). Gallic acid, quercetin, sodium hydroxide, sodium nitrate, aluminum chloride, trypsin, perchloric acid and indomethacin were procured from Himedia (India). Lipoxidase enzyme and linoleic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used were of analytical grade.

Collection of plant material

The stem bark of *Mesua ferrea* Linn. was collected from the Agumbe reserve forest area of Western Ghats, Shimoga, Karnataka state, India with the permission of forest department (Megaravalli, Karnataka). Stem bark material was washed under running tap water to remove adhered dirt, followed by rinsing with distilled water, shade dried and pulverized in a mechanical grinder to obtain coarse powder and stored in an airtight container until use.

Extraction of the plant material

About 250 gm of dried powder stem bark of *M. ferrea* was subjected to soxhlation. It was first defatted with petroleum ether then sequential extraction with chloroform and 80% ethanol solvent in a soxhlet apparatus for 48 h. The temperature was maintained at 50-60 °C. Extracts were concentrated to dryness by using rotary flash evaporator and kept in a desiccator for further use.

Phytochemical studies

The preliminary phytochemical screening was performed by using standard methods [29, 30]. The presence of phytoconstituents such as phenols, flavonoids, alkaloids, steroids, saponins, tannins, glycosides and terpenoids were confirmed in stem bark petroleum ether, chloroform and 80% ethanol extract..

Quantitative analysis of stem bark extracts

Determination of total phenol content

Total phenol content in petroleum ether, chloroform, and 80% ethanol extracts was measured by the Folin–Ciocalteu method of

Chang *et al.*[31]. Briefly, 1 ml of each extract of (100µg) was mixed with Folin–Ciocalteu reagent (2 ml) (diluted 1:10, v/v) followed by the addition of 2 ml of sodium carbonate (7.5% w/v) and mixed, allowed to stand for 90 min. at room temperature and absorbance was measured against the blank at 750 nm using a spectrophotometer. Total phenol content of the extract was expressed in terms of equivalent to Gallic acid.

Determination of total flavonoids content

Total flavonoid content of petroleum ether, chloroform, and 80% ethanol extracts was determined according to the modified method of Zhishen *et al.*, [32]. Briefly, 2.5 ml of extract (100 µg) was mixed with 300 µl of 5% sodium nitrite and 300 µl of 10% aluminum chloride followed by the addition of 2 ml of 1 M sodium hydroxide after the incubation of reaction mixture at room temperature for 6 min. The volume in each test tube was made up to 10 ml by adding 2.4 ml of Millipore water. Absorbance was measured at 510 nm against the blank. Total flavonoid content of the extract was expressed in terms of equivalent to quercetin.

Evaluation of *in vitro anti-inflammatory* activity

Human red blood cells (HRBCs) membrane stabilization method

The blood was collected from a healthy human volunteer who had not taken any NSAIDS for two 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) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline,, and a 10% suspension was made. Various concentrations of extracts were prepared (100, 200 and 500 µg/ml) using distilled water and to each concentration, 1 ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of HRBC suspension were added. It was incubated at 37 °C for 30 min. and centrifuged at 3,000 rpm for 20 min. and the hemoglobin content of the supernatant solution was estimated using UV spectrophotometer at 560 nm. Indomethacin (100µg/ml) was used as the reference standard, and control was prepared by omitting the extracts [33]. The experiment was performed in triplicates. Percentage inhibition of HRBCs membrane stabilization was calculated by using the following formula:

Percent inhibition = $[(A_{control} - A_{test})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance of the control reaction with extract. IC_{50} value was calculated using the formula: $IC_{50} = [(\Sigma C/\Sigma I) \times 50]$, where ΣC is the sum of extract concentrations used for testing and ΣI is the sum of the percentage of inhibition at different concentrations.

Protein Inhibitory activity

The test was performed according to the modified method of Oyedepo *et al.,*[34] and Sakat *et al.,* [35]. The reaction mixture of 2 ml containing 0.06 mg/ml trypsin, 1 ml 20 mM Tris-HCl buffer (pH 7.4) and 1 ml of test sample of different concentrations (100, 200 and 500 µg/ml) were incubated at 37 °C for 5 min. and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against parallel blank. The experiment was performed in triplicates. Percentage inhibition of protein denaturation was calculated by using the following formula:

Percent inhibition = $[(A_{control} - A_{test})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance of the control reaction with extract. IC_{50} value was calculated using the formula: $IC_{50} = [(\Sigma C/\Sigma I) \times 50]$, where ΣC is the sum of extract concentrations used for testing and ΣI is the sum of the percentage of inhibition at different concentrations.

Inhibition of albumin denaturation

The anti-inflammatory activity of *M. ferrea* Linn was studied inhibition of albumin denaturation technique according to the method of Mizushima *et al.*, [36] with minor modifications. The reaction mixture consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using a small amount of 1N HCl. The sample extracts were incubated at 37 °C for 20 min. and then heated to 51 °C for 20 min. after cooling the samples, the turbidity was measured at 660 nm. The experiment was performed in triplicates. Percentage of inhibition was calculated using the following formula:

Percent inhibition = $[(A_{control} - A_{test})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance of the control reaction with extract. IC_{50} value was calculated using the formula: $IC_{50} = [(\Sigma C/\Sigma I) \times 50]$, where ΣC is the sum of extract concentrations used for testing and ΣI is the sum of the percentage of inhibition at different concentrations.

Anti-Lipoxygenase assay

Anti-lipoxygenase assay was studied using linoleic acid as substrate and lipoxidase as an enzyme. The test solution was dissolved in 0.25 ml of 2M borate buffer pH 9.0 and added 0.25 ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 min. at 25 °C. After which, 1 ml of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234 nm. Indomethacin was used as the reference standard [37]. Percentage inhibition of Lipoxygenase assay was calculated by using the following formula: Percent inhibition = $[(A_{control} - A_{test})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance of the control reaction with extract. IC_{50} value was calculated using the formula: $IC_{50} = [(\Sigma C/\Sigma I) \times 50]$, where ΣC is the sum of extract concentrations used for testing and ΣI is the sum of the percentage of inhibition at different concentrations.

Data analysis

Results are expressed as mean±SEM. The statistical analysis was carried out using one-way ANOVA followed by Tukey's t-test. The differences in values at $p<0.05$ or $p<0.01$ were considered as statistically significant. Statistical analysis was performed by ezANOVA 0.98 version.

RESULTS AND DISCUSSION

The qualitative screening confirms the presence of phenols, flavonoids, alkaloids, steroids, saponins, tannins, glycosides and terpenoids (table 1) and the quantitative analysis of stem bark extracts has shown, the highest total phenol (58.9 µg/ml) and flavonoids content (6.0 µg/ml) in 80% ethanol extract when compared to chloroform and petroleum ether extracts is shown in the table 2. The above extracts were screened for anti-inflammatory activity using various *in vitro* methods. The prevention of erythrocyte lysis is known to be a very good index of anti-inflammatory activity of extracts. HRBC membrane is similar to lysosomal membrane components. Lysosomal enzymes are being released during inflammation produce a variety of disorders which leads to the tissue damage and inflammation. The inflammatory responses get limited by preventing the release of lysosomal constituents of activated neutrophils thereby the damage to the tissue is reduced. This extract may inhibit the release of the lysosomal content of neutrophils at the site of inflammation [38, 39]. Previous studies reported that *M. ferrea* exhibited significant anti-inflammatory activity for HRBCs membrane stabilization, albumin denaturation and proteinase inhibitory activity [9].

As a result (table 3), HRBC membrane stabilization was effectively inhibited by petroleum ether, chloroform and 80% ethanol extracts (55.2%, 59.2% and 70.6% respectively) at the concentration of 500µg/ml and standard drug indomethacin (38.7%) which showed significant inhibition at the concentration of 100 µg/ml. IC_{50} value of petroleum ether, chloroform, and 80% ethanol extracts of *M. ferrea* Linn. were found to be 335.2, 309.8 and 247.3 µg/ml respectively.

Denaturation of proteins is responsible for the cause of inflammation. Neutrophils are known to be a rich source of proteinases which carry in their lysosomal granules, and it was previously reported that leukocytes proteinase plays an important role in the development of tissue damage during the inflammatory response. Prevention of protein denaturation may also help in preventing inflammatory conditions [35, 40]. The inhibitory effect of different concentrations (100, 200 and 500 µg/ml) of all the extracts of *M. ferrea* on protein denaturation is shown in table 4. Petroleum

ether, chloroform and 80% ethanol extracts (46.4%, 49.1% and 80.6% respectively)at the concentration of 500 µg/ml and standard drug Indomethacin (34%) at the concentration of 100 µg/ml showed significant inhibition of protein denaturation when compared with control. IC_{50} value of petroleum ether, chloroform and 80% ethanol extracts of *M. ferrea* were found to be 464.6, 428.7 and 237.8 µg/ml respectively. Whereas in albumin denaturation assay (table 5), the maximum inhibition ($p<0.01$) was observed in 80% ethanol extract (72.5%) followed by petroleum ether (39.3%) and chloroform (38.6%) at the concentration of 500 µg/ml. while the standard drug Indomethacin showed 28.8% inhibition at the concentration of 100 µg/ml. Petroleum ether, chloroform and 80% ethanol extracts of *M. ferrea* Linn. showed IC_{50} value of 491.5, 488.8 and 217.2 µg/ml respectively.

Anti-lipoxygenase assay was carried out using soybean lipoxidase as an enzyme and linoleic acid as a substrate. Lipoxygenase enzyme (LOXs) is sensitive to antioxidants and involved in pathophysiology of several allergic and inflammatory diseases [41]. Previous studies confirm the inhibition of lipoxygenase enzyme can inhibit the synthesis of leukotrienes. Leukotrienes are one of the inflammatory mediators released from mast cells. They have direct toxic effects which phagocytosize the damaged tissue and in the process lead to further release of inflammatory mediators [37]. Therefore, inhibition of lipoxygenase enzyme by *M. ferrea* leads to inhibition of synthesis of leukotrienes, one of the mechanisms of anti-inflammatory activity [42].

As shown in table 6, 80% ethanol extract of *M. ferrea* significantly inhibited the enzyme in a concentration-dependent manner. The different concentrations of 80% ethanol extract (100, 200 and 500 µg/ml) exhibited 16.4%, 27.6% and 62.9% inhibition of Lipoxygenase enzyme activity respectively. From these results, the highest inhibition was observed at the concentration of 500 µg/ml when compared with standard drug Indomethacin (53.3% inhibition) at the concentration of 100µg/ml. IC_{50} value of 80% ethanol extract and indomethacin was found to be 374.2 and 93.8 µg/ml.

Table 1: Phytochemical analysis of stem bark extracts of *Mesua ferrea* Linn

Phytochemical Test	Petroleum ether extract	Chloroform extract	80% ethanol extract
Alkaloids	+	+	+
Flavonoids	+	+	+
Terpenoids	+	+	+
Steroids	-	-	+
Saponins	-	-	+
Tannins	-	+	+
Phenolics	+	+	+
Glycosides	+	+	+

+= present,-= absent.

Table 2: Quantitative estimation of stem bark extracts of *Mesua ferrea* Linn

	Total phenolic content (100 µg/ml)	Total flavonoids content (100 µg/ml)
Petroleum ether extract	18.1	1.0
Chloroform extract	43.9	2.3
80%ethanol extract	58.9	6.0

Table 3: Effect of *Mesua ferrea* Linn on HRBC membrane stabilization

	Concentration (µg/ml)	% inhibition	IC_{50} value
Control	-----	----	
Petroleum ether extract	100	18.6±12.8ns	335.2
	200	45.6±3.6ns	
	500	55.2±0.5**	
Chloroform extract	100	27.9±2.9ns	
	200	42.0±3.6ns	309.8
	500	59.2±2.0**	
80% ethanol extract	100	43.5±17.9ns	
	200	47.6±3.7*	247.3
	500	70.6±4.1 **	
Indomethacin	100	38.7±4.0	129.2

Each value represents the mean±SD. of triplicates. HRBC membrane stabilization by extracts was compared with standard drug **$p<0.01$, considered extremely significant; *$p<0.05$, considered significant; ns $p>0.05$, non-significant.

Table 4: Effect of *Mesua ferrea* Linn on inhibitory protein assay

	Concentration (µg/ml)	% inhibition	IC_{50}
Control	-----	------	
Petroleum ether extract	100	10.9±5.4**	464.6
	200	28.9±2.0ns	
	500	46.4±2.7**	
Chloroform extract	100	11.7±2.9**	
	200	32.4±0.8*	428.7
	500	49.1±0.3**	
80% ethanol extract	100	36.4±0.1**	
	200	51.2±4.7**	237.8
	500	80.6±2.3**	
Indomethacin	100	34.0±9.6	146.9

Each value represents the mean±SD. of triplicates. Inhibition of protein by extracts compared with standard drug ns $p>0.05$, non-significant; **$p<0.01$, considered extremely significant; *$p<0.05$, considered significant.

Table 5: Effect of *Mesua ferrea* Linn on albumin denaturation assay

	Concentration (µg/ml)	% inhibition	IC_{50}
Control	-----	------	
Petroleum ether extract	100	8.8±2.1**	491.5
	200	33.3±3.1ns	
	500	33.3±3.1ns	
Chloroform extract	100	20.2±3.3ns	
	200	29.9±2.7ns	488.8
	500	38.6±3.0ns	
80% ethanol extract	100	51.0±3.6*	
	200	60.6±7.1**	217.2
	500	72.5±1.7**	
Indomethacin	100	28.8±9.5	173.8

Each value represents the mean±SD. of triplicates. Inhibition of albumin denaturation by extracts was compared with standard drug ns p>0.05, non-significant; **p<0.01, considered extremely significant; *p<0.05, considered significant.

Table 6: Effect of *Mesua ferrea* Linn on anti-Lipoxygenase assay

	Concentration (µg/ml)	% inhibition	IC_{50} value
Control	-----	----	
	100	16.4±4.1**	
80% Ethanol extract	200	27.6±1.3**	374.2
	500	62.9±0.5*	
Indomethacin	100	53.3±0.7	93.8

Each value represents the mean±SD. of triplicates. Inhibition of Lipoxygenase by extract was compared with standard drug **p<0.01, considered extremely significant; *p<0.05, considered significant.

CONCLUSION

Based on the above results, it was concluded that *M. ferrea* Linn. possesses good anti-inflammatory activity which may be due to the strong presence of compounds such as phenols, alkaloids, flavonoids, tannins, glycosides and terpenoids. This research provides the scientific basis for the traditional usage of *M. ferrea* for various inflammatory diseases. However, further studies are required for isolation of bioactive compounds for analysis of the molecular mechanisms responsible for its anti-inflammatory potential and also *in vivo* anti-inflammatory activity of *M. ferrea* stem bark extract needs to be assessed.

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