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Antioxidant and Anti-Inflammatory Potency of Mesua Ferrea Linn

KEYWORDS: Mesua ferrea, Antioxidants, Anti-inflammatory

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ABSTRACT *The present study was conducted to evaluate antioxidant and anti-inflammatory potency of stem bark of Mesua ferrea L. an ethno medicinally important plant. Evaluation of in vitro antioxidant activity was carried out by Total antioxidant, DPPH, Ferric reducing, ABTS and nitric oxide assays. In vitro anti-inflammatory assays was also studied through inhibition of HRBCs membrane stabilization, heat induced hemolysis, Proteinase inhibitory activity and albumin denaturation assay. Results revealed that the methanolic extracts have significantly higher antioxidant activity scavenging for DPPH assay (89.70%), ABTS assay (77.64%), and Nitric oxide scavenging 89.28%. Total phenolics-content found to be 33,600 mg/100g plant material, total flavonoids 164 µg/ml and total tannins content 156 µg/ml were significantly higher in methanol extract. The methanol extract of the plant exhibited significant anti-inflammatory activity for HRBCs membrane stabilization (78.20%), heat induced hemolysis (47.40%), Albumin Denaturation (70.58%) and for Proteinase inhibitory activity (50.73%).*

INTRODUCTION:-

Mesua ferrea (Clusiaceae) is commonly known as Nagakesara (Kannada). It is an important medicinal plant which finds varied use in Ayurveda, Siddha and Unani. Bark and roots in decoction or infusion or tincture is a better tonic and are useful in gastritis and bronchitis (Sandeep et.al, 2009), in fever, itching, nausea, leprosy, skin disorders, erysipelas, bleeding piles, menorrhagea, excessive thirst, and sweating (Bhavna Panchal et.al, 2012). Leaves are used in the form of poultice which is applied to head in severe colds. Dried flowers are used in bleeding piles as well as dysentery with mucus, irritability of the stomach, excessive perspiration, cough with much expectoration, dyspepsia, etc. Leaves and flowers are astringent and stomachic and are also used for snake bite and scorpion stings. Seed oil is applied for sores, skin scabies, wounds and as an embrocation in rheumatism (Ali et.al, 2004).

Worldwide, traditional medicine is being re-evaluated by extensive research on different plant species and their therapeutic principles. The present study was conducted to evaluate antioxidant and anti-inflammatory potency of stem bark of Mesua ferrea L. petroleum ether and methanol extracts.

MATERIALS AND METHODS:

Collection, identification and extraction of plant material:
Plant material was collected from Shettihalli reserve forest, Western Ghats Shivamogga, Karnataka, identified and authenticated and a voucher specimen is deposited in Departmental herbaria. The plant material was shade dried and powdered mechanically, filtered (sieve no-45); 250 g of plant material was subjected to soxhlation using petroleum ether and methanol solvents for about 48 hours. Extract was filtered, concentrated to dryness in vacuum under reduced pressure using rotary flash evaporator (IKA-German). Crude extract was subjected to preliminary phytochemical screening following the methods described in (Kokate, 2001).

Estimation of total phenolics:
Total phenol content was evaluated by the method described by Singleton et.al, 1974. Gallic acid standards were prepared (0-500 µg), 0.5 ml of FC-Reagent was added and kept for incubation for 6 min at room temperature. Then 1 ml of Na_2CO_3 mixed thoroughly and incubated at room temperature for 15 mins and absorbance was measured at 765 nm against reagent blank. A standard curve was plotted using different concentrations of Gallic acid. From the standard curve the concentration of Phenolics present in the extracts was estimated and expressed as mg phenols/100 g of plant material. Preparation of plant extract: about 100 mg plant extracts were weighed and dissolved in 10 ml of methanol and used for further study.

Estimation of total flavonoids contents:
Total flavonoids content was evaluated by the method described by Manashi Bagchi et.al 1999. Catachin standards were prepared (0-100 µg) to this 0.3 ml of 5% $NaNO_2$ and 0.3 ml of 10% Al_2Cl_3 was added, incubated at RT for 6 min. After incubation, 2 ml of 1 M NaOH was added and volume was made upto 10 ml with distilled water. The absorbance of the mixture was measured at 510 nm spectrophotometrically.

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Estimation of Tannins content:
Total tannin contents were estimated by the method described by Oyaizu, 1986. Tannic acid standards were prepared (0-100 µg), to this 5 ml of Folin-Denis reagent and 1 ml of Na_2CO_3 solution was added and made up to 10 ml with distilled water. Reagent blank was prepared with distilled water. Optical density was measured at 700 nm spectrophotometrically.

Total antioxidant activity assay (TAC):
Total antioxidant activity of crude extracts were evaluated as described by Lissi et.al, 1995. 0.3 ml of sample was mixed with 3.0 ml reaction mixture (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM $(NH_4)_2MoO_4$). Then incubated at 95° C for 90 min on a water bath. After incubation 300 µl of the content was transferred into a microtitre plate and absorbance was measured at 695 nm. TAC was expressed as the number of equivalents of ascorbic acid in mg.

DPPH radical scavenging assay:
DPPH radical scavenging activity of crude extracts were evaluated as described by Blois, 1958. Ascorbic acid standards (0-100 µg) and test samples were prepared in methanol, 2 ml of 10 mM DPPH prepared in methanol was added, and then incubated at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm, using UV-visible spectrophotometer.

ABTS radical scavenging assay:
ABTS radical scavenging activity was evaluated as described by Re et.al, 1999. To generate ABTS radical cation, 50 ml of 2 mM ABTS and 0.3 ml of 17 mM $K_2S_2O_8$ was mixed together and incubated in the dark for 12-16 hrs to develop Prussian blue colored $ABTS^{\cdot+}$ solution which has an absorption maxima at 734 nm. To evaluate scavenging activity of pet ether and methanol plant extracts, different concentrations of extracts were added to 1.6 ml of ABTS solution. The absorbance was measured at 734 nm. Ascorbic acid (20-100 µg) was used as standard.

Scavenging by ferric reducing power assay (FRAP):
Reducing power of crude extracts was evaluated by the method described by Benzie et.al 1996. Tannic acid standards were prepared (0-100 µg), in 2.5 ml with phosphate buffer (200 mM pH 6.6) to this 2.5 ml of 1% $K_3[Fe(CN)_6]$ was added. The mixture was placed in water bath for 20 min at 50^{0}C, cooled and added 2.5 ml of 10% TCA. Then centrifuged at 3000 rpm for 10 min. 5 ml of supernatant was mixed with 5 ml of distilled water and 0.1% of ferric chloride solution, the amount of Iron (II)-ferricyanide complex was determined by measuring the absorbance 700 nm after 10 min. The higher absorbance of the reaction mixture indicated increased reducing power.

Nitric oxide radical scavenging Assay:
Nitric oxide radical scavenging was evaluated by the method described by Marococci et.al, 1994. 10 mM Sodium nitroprusside was prepared in 0.2 M PBS (pH 7.4) and was mixed with different concentrations of extracts and incubated at 25^0 C for 150 min. Then added 2 ml of Griess reagent. The absorbance formed was measured at 546 nm spectrophotometrically.

ANTI-INFLAMMATORY ACTIVITY:
The HRBC membrane stabilization method:
HRBC membrane Stabilization assay was performed by the method described by Sakat et.al, 2010. Assay system consisted of the test drug (at various concentrations) 1 ml phosphate buffer (0.15 M, pH 7.4), 2 ml of hyposaline (0.36%) and 0.5 ml of HRBC suspension. Standard was prepared by using Diclofenac sodium (50mg/ml) & control consisted of distilled water instead of hyposaline. The assay systems were incubated at 37^0 C for 30 min and centrifuged at 3000 rpm for 20 min. The Hb content of the supernatant solution was estimated spectrophotometrically at 560 nm. The percentage stabilization of HRBC membrane was calculated by using the formula,

$$\% \text{ Protection} = 100 \frac{\text{Optical density of test}}{\text{Optical density of control}}$$

Heat induced hemolysis:
Heat induced hemolysis was carried out according to the method described by Shinde et.al, 1999. The reaction mixture (2 ml) consisted of 1 ml of test drug solution and 1 ml of HRBCs suspension. Control was prepared by adding saline instead of drug. Aspirin was used as standard drug. All the tubes containing reaction mixture was incubated in a water bath at 56^0 C for 30 min, at the end of the incubation, the tubes were cooled and centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was measured at 560 nm.

Proteinase inhibitor assay:
The test was performed according to the modified method of Oyedepo et.al, 1995. The reaction mixture (2 ml) was prepared by 0.06 mg/ml trypsin, 1 ml 20 mM Tris-HCl buffer (pH 7.4) and 1 ml test sample of different concentrations. The reaction mixture was incubated at 37^0 C for 5 min and then added 1 ml of 0.8% (w/v) Casein. Again incubated for 20 min. then added 2 ml of 70% percholoric acid to terminate the reaction. The resultant cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The percentage inhibition of Proteinase inhibitory activity was calculated by using the formula

Inhibition of albumin denaturation assay:
The study was carried by following the method of Muzushima and Kabayashi with slight modification. The Standard drug and test compound was dissolved in Dimethyl formamide and diluted with phosphate buffer (0.2 M pH, 7.4). Final concentration of DMF in all solutions was less than 2.5%. Test solution (1 ml) was mixed with 1 ml of 1 mM albumin solution prepared in phosphate buffer and incubated at 27^0 C for 10 min. Denaturation was induced by keeping the reaction mixture at 60° C in a water bath for 10 min. After cooling, the turbidity was measured at 660 nm spectrophotometrically. A control was prepared in which no drug and was used for the calculation of percentage of inhibition of denaturation (Sakat et.al, 2010).

DISCUSSION:
Based on the preliminary phytochemical analysis quantitative estimation of phytoconstituents was carried out. Total phenolic content of M. ferrea petroleum ether and methanol extract was found to be 9000 mg and 33,600 mg/100g of plant extract respectively, reflecting the potency of the plant in scavenging free radicals. Phenols are very important plant constituents. There is a highly positive relationship between total phenols and antioxidant activity of many plant species, because of the scavenging ability of their hydroxyl groups (Vinson et.al, 1998). Literature reveals that antioxidant activity of plant extract is mainly due to presence of phenolic compounds, which may exerts antioxidant effects as free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators (Okawa et.al. 2001).

Flavonoids are regarded as one of most spread groups of natural constituents found in plants. The flavonoids content of M. ferrea petroleum ether and methanol extract was found to be 160 and 328 µg respectively at concentration of 1mg/ml of plant extract. It has been revealed that flavonoids actively participate in antioxidant scavenging and the mechanism of action of flavonoids is through scavenging or chelation process which terminates free radical. These are very important phytoconstituents because of their hydroxyl groups confer scavenging ability (Arora et.al, 1998).

The total tannins content of M. ferrea extract was found

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to be 90 µg and 312 µg respectively at concentration of 1 mg/ml of plant extract. Tannins and tannins like substances are widespread in nature and are probably present in all plant materials. Tannins could affect the inflammatory response via their radical scavenging activities (Lin et.al, 1997).

The total antioxidant capacity of pet ether and methanol extracts of M.ferrea was found to be 200 and 960 µg/g of ascorbic acid. Total antioxidant capacity by Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The Phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid (Prieto et.al, 1999).

In the present study, the DPPH scavenging activity of pet ether and methanol extracts of M. ferrea was found to be 37.8% and 89.7% respectively at concentration of 500 µg/ml of plant extract. The DPPH scavenging ability of the extracts were found to be dose dependent. Among the two extracts tested, methanolic extract showed the highest activity than the petroleum ether extract. The study showed that the extracts have the proton-donating ability and could serve as free radical scavenger, acting possibly as primary antioxidant.

The $ABTS^{\cdot}$ scavenging activity of pet ether and methanol extracts was found to be 52.94% and 77.64% respectively, at concentrations of 250 µg and 15 µg respectively (Graph-IIA and IIB). Among the two extracts tested methanol extract is more efficient towards the scavenging of $ABTS^{\cdot}$.

In the reducing power assay, the presence of antioxidants (reductants) in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity. This would have the effect of converting free radicals to more stable products and thus terminating free radical initiated chain reaction (Benzie et.al, 1996). In our study, reducing power of crude extracts was found to increase with the dose. The reducing capacity of extracts may serve as significant indicators of its potential antioxidant activity. The reducing power assay correlated with the results obtained by the DPPH assay i.e., the methanol extract showed highest activities than petroleum ether extract. These activities are due to the presence of Phenolics equivalents. It is in accordance with the findings of Sakagami and Satoh 1997 who reported that Phenolics increases reducing power with the increase in its concentration.

The nitric oxide scavenging activity of M. ferrea pet ether and methanol extract was found to be 45.71% and 89.28% respectively at concentration of 500 µg/ml (Graph-II and Table-II). Nitric oxide is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Nishaa et.al. 2012). In this assay NO is a free radical produced from the sodium nitroprusside in aqueous solution at physiological pH. It reacts with oxygen to form oxides of nitrogen in the reaction medium. The scavenging activity of the plant extracts against nitric oxide formation was significant & it was dose dependent. Even in this study also methanol extract has showed the better activity than the petroleum ether extract.

The HRBC membrane stabilization effect (by inhibiting hypotonicity induced lyses of erythrocyte membrane) of methanol extract at a concentration of 500 µg/ml was 34.20±0.2% and 78.20±0.2% and standard drug Diclofenac was showed 73.00% protection.

Stabilization of lysosomal membrane is important in limiting the anti-inflammatory response by preventing the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release (Murugasan 1981). The erythrocyte membrane is analogous to lysosomal membrane (Chou 1997) and its stabilization implies that the extract may as well stabilize lysosomal membranes. Some of the NSAIDs are known to possess membrane stabilization properties which may contribute to the potency of their anti-inflammatory effect. The HRBCs membrane stabilization method was used to evaluate the potency of the extract for its anti-inflammatory activity (Gandhisan et.al, 1991).

Denaturation of proteins by heat induced hemolysis is a well documented cause of inflammation. The inflammatory drugs/salicylic acid, henylbutazone etc, have shown dose dependent ability to thermally induced protein denaturation. Similar results were observed from many reports from plant extract (Sakat et.al, 2010). The heat induce denaturation of protein was effectively inhibited by pet ether and methanol extracts respectively 23.52±2% and 47.40±2% respectively (Table-II). Protein denaturation has been employed as an in vitro screening method for antiphlogistic agents. Mizushima and Kobayashi 1968, who reported that anti-inflammatory drug, inhibit protein denaturation. Drug binding to plasma albumin may inhibit thermal denaturation of albumin which perhaps Ø-NH_2 Groups in case of Histidine decarboxylase or may displace urate from albumin (Skidmore et.al, 1965).

Proteinase inhibitor activity of pet ether and methanol extracts was found to be 40.66±0.2% and 50.73±0.2% respectively at the concentration of 500 µg/ml of plant extract. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Das et.al, 1995).

The present findings exhibited a concentration dependent inhibition of protein (albumin) denaturation by the pet ether and methanol extract (23.5±0.2% and 70.58±0.2% respectively) at the concentration of 500 µg/ml of plant extract. Denaturation of proteins is a well documented cause of inflammation. Phenylbutazone, salicylic acid, flufenamic acid etc, show dose dependent ability to thermally induced protein denaturation (Mizushima et.al, 1968).

CONCLUSION:

Based on the results of this study, it was revealed that both the petroleum ether and methanol extracts have potential in vitro antioxidant activity against various antioxidant systems due to the presence of various Phytoconstituents. And also showed potent anti-inflammatory activity. All the results revealed that both the extracts showed dose dependent activity. The present study provides a scientific base for the ethanomedicinal claims of the plant. Further study on identification of the lead molecules of molecular mechanism studies of Mesua ferrea is under process.

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TABLES AND GRAPHS:

Table-I: Quantitative Estimation of Phenolics, Flavonoids and Tannins of Mesua ferrea Petroleum ether and Metha-

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nol Extract.

Serial No.	Name of the Assay	Petroleum ether extract	Methanol Extract
1.	Total Phenolics	9,000 mg/100g plant extract	33,600 mg/100g plant extract.
2.	Flavonoids	160 µg/ml	312 µg/ml
3.	Tannins	90 µg/ml	332 µg/ml

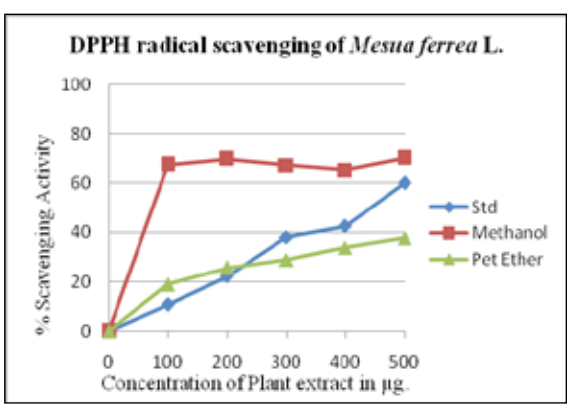
Table-II: Antioxidant assays of Mesua ferrea Petroleum ether and Methanol Extract:

Serial No.	Name of the Assay	Petroleum ether extract	Methanol Extract
1.	TAC assay	200 µg/g of Ascorbic acid	960 µg/g of Ascorbic acid
2.	DPPH assay	37.80±2%	89.70±2%
3.	ABTS assay	52.94±2%	77.64±2%
4.	Nitric oxide assay	45.71±2%	89.28±2%

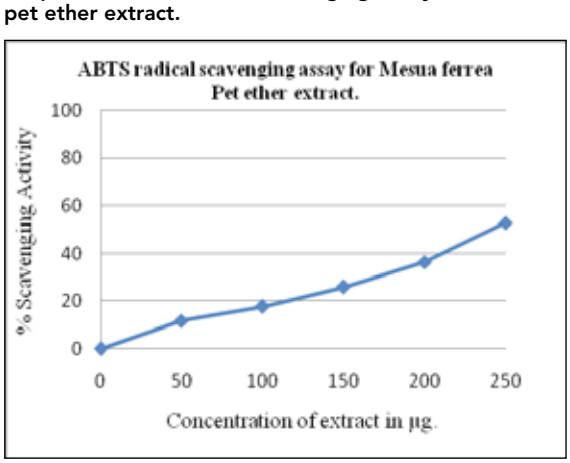
Table-III: Antiinflammatory assay of Mesua ferrea Petroleum ether and Methanol Extract

Serial No.	Name of the assay	Petroleum ether extract	Methanol extract
1.	Heat Induced Hemolysis	23.38±2%	47.40±0.2%
2.	Membrane Stabilization	34.20±0.2%	78.20±0.2%
3.	Albumin Denaturation	23.5 ±0.2%	70.58±0.2%
4.	Proteinase inhibitor assay	40.66±0.2%	50.73±0.2%

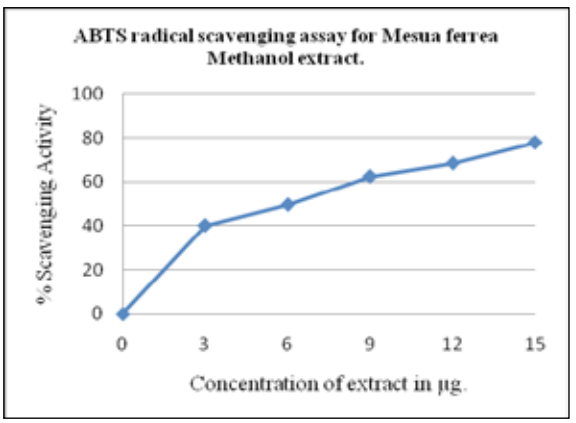
Graph-I: DPPH scavenging assay of Mesua ferrea Petroleum ether and Methanol Extract.



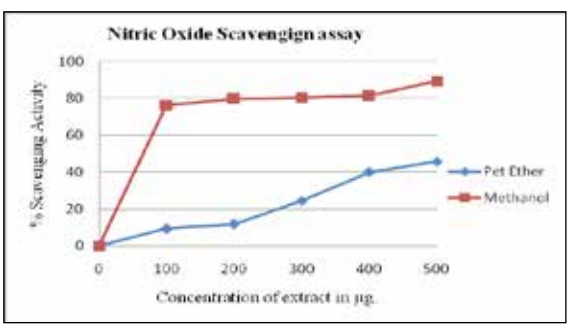
Graph-IIA: ABTS radical Scavenging assay Mesua ferrea pet ether extract.



Graph-IIB: ABTS radical Scavenging assay Mesua ferrea methanol extract.



Graph-III: Nitric Oxide Scavenging assay for Mesua ferrea.



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