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Cyclooxygenase, Lipoxygenase, Nitric Oxide Synthase, Myeloperoxidase and Protease Inhibiting Activities of the Leaves and Flowers of *Mikania micrantha* Kunth

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ABSTRACT
Aim: To study *in vitro* anti-inflammatory activity of the leaves and flowers of the perennial climber *Mikania micrantha*.
Methods: Leaves and flowers of the plant was collected in February 2015. Dried and powdered plant materials was extracted with ethyl acetate at room temperature in an orbital shaker. Cyclooxygenase, 5-lipoxygenase, myeloperoxidase and nitric oxide synthase inhibiting activities of the ethyl acetate extract were tested in human monocytic cell line THP1 after activation with lipopolysaccharide. Protease inhibiting activities were tested using trypsin as the enzyme. Percentage inhibitions of enzyme activities were calculated and IC_{50} values were determined. Diclofenac sodium was used as the reference drug.
Results: Ethyl acetate extract of the leaves as well as flowers exhibited very good myeloperoxidase, nitric oxide synthase, cyclooxygenase and lipoxygenase inhibiting activities and showed moderate effect towards protease inhibition. The IC_{50} of inducible nitric oxide synthase activity was 5 and 11µg respectively for leaf and flower extracts. IC_{50} of myeloperoxidase activity was 4µg for flower extract. The inhibition of myeloperoxidase activity by leaf extract was so high that IC_{50} could not be determined at the tested concentration. IC_{50} of cyclooxygenase activity was 25 and 89µg and lipoxygenase activity was 98 and 185µg respectively for leaf and flower extracts. The IC_{50} of leaf and flower extracts towards protease inhibition were 1180 and 1250 µg respectively.
Conclusion: This study revealed that *Mikania micrantha* contains principles which can inhibit diverse enzymes of inflammatory pathway and is a potential target for the development of new anti-inflammatory drug.

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INTRODUCTION

Inflammation refers to the response of the immune system of an organism to microbial infection, physical trauma or irritation by chemicals. It arises as a healing response of the body to different insults but the persistence of the process may lead to various illnesses. Inflammatory response can be divided into two – acute and chronic. Acute inflammation, which is of short duration and has rapid onset, is a defence mechanism of the body for killing microbes while still facilitating wound healing. There is increased permeability of blood capillaries leading to the infiltration of plasma and leukocytes into the damaged tissue for a short period. In normal conditions the immune system resolves the inflammatory response spontaneously and the normal tissue function is restored. Failure of the resolution of the acute inflammation will lead to chronic inflammation. Also autoimmunity, the condition in which the immune system mistakenly attacks the body of the host itself will similarly lead to chronic inflammation. Chronic inflammation if persistent, leads to degenerative diseases such as asthma, rheumatoid arthritis, Alzheimer's disease,

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multiple sclerosis, atherosclerosis, inflammatory bowel disease, cancer, aging and gout. In chronic inflammation there is increased production of pro-inflammatory cytokines, activation of NF kappa B, increased expression of cyclooxygenase, 5-lipoxygenase, phospolipase A2 and inducible nitric oxide synthase and production of large amounts of reactive oxygen species [1-3]. Currently used drugs for the treatment of various inflammatory disorders act by inhibiting or decreasing the production of these mediators.
Besides this, proteinases (or proteases) are emerging as new targets to control inflammation. Traditionally proteinases are considered as digestive enzymes which degrade protein. But now a day they are also recognised as signalling molecules involved in various physiological as well as pathophysiological processes. Among different proteases, serine proteases are now known to be involved in cell signalling via the activation of a family of G-protein coupled receptors known as proteinase activated receptors (PARs). Four PARs have been identified till date – PAR 1, PAR 2, PAR 3 and PAR 4. Thrombin activates PAR 1, PAR 3 and PAR 4 while PAR 2 is activated by trypsin and mast cell tryptase. PAR 2 controls inflammation and nociception in pulmonary system, cardiovascular system, gastrointestinal system and nervous system [4,5].
Mikania micrantha is a fast growing perennial creeping weed belonging to the family Asteraceae. The plant is native to central and South America but is now widely distributed across different continents. In Jamaica, poultice of the leaves is used for scorpion stings and snake bites. Decotion of the leaves is used to treat skin itches and athlete's foot, as wound dressings and to bath rashes [6,7]. The plant has been reported to possess antibacterial activity [7-8], anti-stress activity [9] and antidermatophytic activity [10].
The leaves and inflorescences of the plant have been reported to possess anti-inflammatory activity in mouse ear edema test [11]. In a preliminary study conducted the plant was found to possess protease inhibiting activity [10]. Present paper reports cyclooxygenase (COX), 5-lipoxygenase (LOX), myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and protease inhibiting activities of the leaves and flowers of *Mikania micrantha*.

MATERIALS AND METHODS

Chemicals

THP1 cell line was obtained from NCCS, Pune. RPMI 1640 was purchased from HIMEDIA Laboratories, Mumbai. Trypsin was purchased from Sisco Research Laboratories, Mumbai. Diclofenac sodium, Glutathione, Arachidonic acid, Sodium linoleate, Guaiacol, Thiobarbituric acid and Hydrogen peroxide were purchased from Sigma – Aldrich, USA. Haemoglobin standard was from Erba Mannheim, Germany. All other chemicals used were of analytical reagent grade.

Plant materials

The leaves and flowers (with seeds) of *Mikania micrantha* were collected from Kottayam district, Kerala in February 2015. The plant materials were washed with water, shade dried, powdered in a kitchen blender and stored in air tight bottles until use. The plant was authenticated at St.Thomas College, Pala, Kerala, India and a voucher specimen (SBSBRL 19) has been maintained at the author's institute.

Table 1: IC_{50} values for various enzyme activities

IC_{50} in µg	ELMM	EFMM	Diclofenac sodium
COX activity	25	89	4
LOX activity	98	185	5
MPO activity	ND[a]	4	ND[a]
iNOS activity	5	11	6
Trypsin activity	1180	1250	15

[a] Not determined because inhibition was so high than 50 % at the lowest tested concentration

Solvent extraction

In the preliminary study conducted ethyl acetate extract was found to possess protease inhibiting activity [10]. Hence in this study, ethyl acetate was used as the solvent for extraction. The powdered plant materials (25g) were extracted with ethyl acetate at room temperature in an orbital shaker for 7 days. The extracts were then filtered using Whatman No 1 filter paper, evaporated to dryness and stored at 4^0C until use. Ethyl acetate extract of the leaves and flowers of *Mikania micrantha* were named as ELMM and EFMM respectively.

Study of anti-inflammatory activity on cell line

COX, LOX, MPO and iNOS inhibiting activities were studied using human monocytic cell line THP1. The study was conducted in 2015. RPMI 1640 supplemented with 10% heat inactivated foetal bovine serum, 1.5% sodium bicarbonate and antibiotics a penicillin (100 Units/mL) and streptomycin (100µg/mL) was used as the media for culturing as well as testing the cell line. The cells were grown till 60% confluency and then activated with 1µL of lipopolysaccharide (1µg/mL). Stimulated cells were then exposed with different concentrations of extracts - 6.25 µg, 12.5µg, 25 µg, 50 µg, 100µg and 200µg and incubated for 24 hours. The cells were collected by spinning at 6000 rpm for 10 minutes. Then 200µL of cell lysis buffer (1MTris HCl, 0.25M EDTA, 2M NaCl, 0.5% Triton X-100) was added and incubated at 4^0C for 30

minutes. Different inhibitory assays were conducted using this lysed cell suspension. DMSO (0.1%) was used as negative control and diclofenac sodium was used as the reference drug (positive control). The percentage inhibition of enzyme activities was calculated as,

$$\frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

Control and tests were done in triplicate and the results are expressed as mean ± SD, where n=3 (Calculated using Microsoft Office Excel 2007). IC_{50} values were calculated by plotting percentage of inhibition against concentration of test materials.

Study of cyclooxygenase inhibiting activity

Activity of cyclooxygenase was measured by TBARS assay. The assay system consisted of cell lysate (enzyme source), Tris-HCl buffer (100 mM, pH 8), haemoglobin (5µM) and glutathione (5mM). To this 200 µM arachidonic acid was added and incubated for 20 minutes at 37^0C. Reaction was stopped by adding 0.2mL of 10%Trichloro acetic acid in 1N HCl. Then 0.2mL of 1% thiobarbituric acid was added and heated in a boiling water bath for 20 minutes. After cooling and centrifugation the absorbance of supernatant was measured at 532 nm [12,13]. Percentage inhibition of cyclooxygenase activity was calculated and IC_{50} was determined.

Study of lipoxygenase inhibiting activity

Lipoxygenase activity was measured according to Axelrod et al, 1981 using linoleic acid as the substrate [14]. Seventy mg of sodium linoleate and 70 mg of tween 20 were dissolved in 4mL of oxygen free water and mixed. Then 0.5N NaOH was added to this until the solution became clear and made up to 25mL with oxygen free water (This was divided into 0.5mL portions and flushed with nitrogen gas before closing and kept frozen for further use). 0.2mL of this solution was added to a quartz cuvette followed by 2.75mL Tris- HCl buffer and 50µL of cell lysate. Increase in absorbance at 234 nm due to the formation of conjugate double bonds in the product linoleic acid hydroperoxide was measured. Percentage inhibition of lipoxygenase activity was calculated and IC_{50} was determined.

Study of myeloperoxidase inhibiting activity

The assay was conducted according to the method of Renlund *et al*., 1980 [15]. Cell suspension was mixed with 0.5% solution of Hexadecyl trimethylammonium bromide (HTAB) in potassium phosphate buffer 50mM (pH- 6). After freeze thawing 3 times, the samples were centrifuged at 2000g for 30 minutes at 4^0C. To 0.1 mL of the supernatant 2.9 mL of potassium phosphate buffer containing 0.167mg/mL of guaiacol and 0.0005% H_2O_2 were added. Oxidation of guaiacol into tetraguaiacol was monitored by increase in absorbance at 460nm [13,16].

One unit of myeloperoxidase activity was defined as the amount of enzyme catalysing the formation of one micromole of tetraguaiacol per minute (can be calculated using the molar extinction coefficient of tetraguaiacol which is equal to 26600 mol/cm). From the activities of control and test samples, percentage reduction of myeloperoxidase activity was calculated and IC_{50} was determined.

Study of inducible nitric oxide synthase inhibiting activity

In mammalian cells nitric oxide synthase produces nitric oxide. During inflammation nitric oxide is produced by inducible nitric oxide synthase which is not typically expressed in resting cells [17]. In *in vivo* conditions, the final products nitric oxide are nitrite and nitrate. Hence cellular nitrite level is a measure of nitric oxide produced which in turn is a measure of inducible nitric oxide synthase activity. Level of cellular nitrite was estimated using the method of Lepoivre *et al*., 1989 as modified by Kakadiya and Shah, 2010 [18,19]. Griess reagent converts nitrite into a deep purple coloured azo compound whose absorbance can be measured at 540 nm.

0.5 mL of cell lysate was mixed with 0.1 mL of 3% sulphosalicylic acid (to precipitate proteins) and centrifuged at 5000 rpm for 15 minutes. To 200 µL of the supernatant 30 µL of 10% NaOH and 300 µL Tris-HCl buffer (100mM, pH 8) were added and mixed. To this 530 µL of Griess reagent was added, incubated for 15 minutes in dark and absorbance was read at 540 nm. The amount of nitrite present in the samples was determined from a standard curve (sodium nitrite solution 100 – 1000 µg) and percentage of inhibition and IC_{50} were determined.

Protease inhibition assay

The assay was conducted using trypsin as the protease and albumin as its substrate. To 50 µL of test samples (in DMSO) having different concentrations (10, 50, 100, 500, 1000 and 2000µg) 100 µL of Trypsin (1mg/ml in 1mM HCl) was added and made up to 1mL with Tris – HCl buffer (50 mM, pH 7.8 containing 1mM $CaCl_2$). After incubation at room temperature for 10 minutes 1 mL of BSA was added to all tubes and digestion was carried out at 37^0C in a water bath for 20 minutes. Reaction was stopped by the addition of 3mL of 5 % TCA, centrifuged at 2500 rpm and absorbance of supernatant was read at 280 nm. Appropriate controls and blanks were also run [20]. Diclofenac sodium was used as the reference drug and the percentage of inhibition and IC_{50} values were determined.

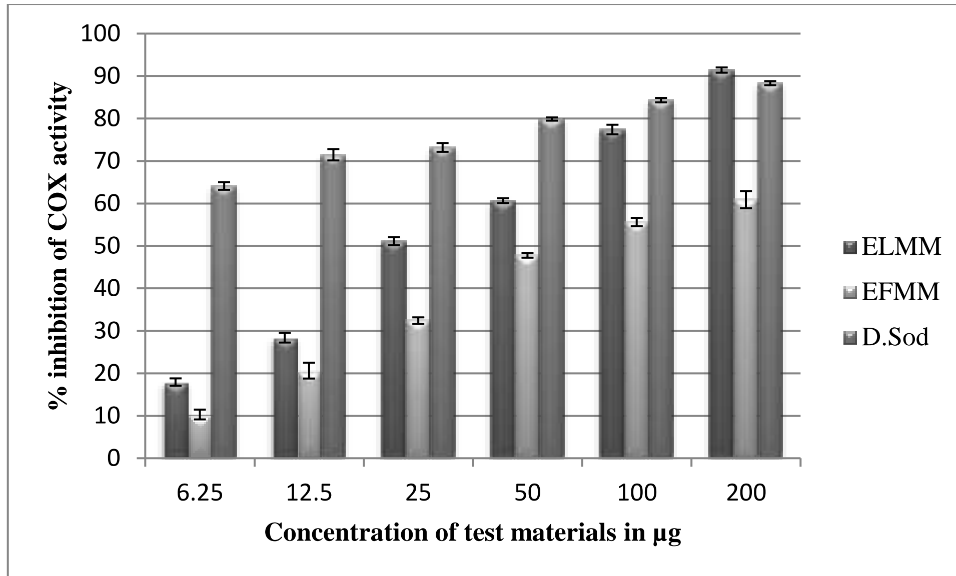

Figure 1: Percentage inhibition of cyclooxygenase activity by ethyl acetate extract of the leaves (ELMM) and flowers (EFMM) of *Mikania micrantha* and diclofenac sodium.
Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

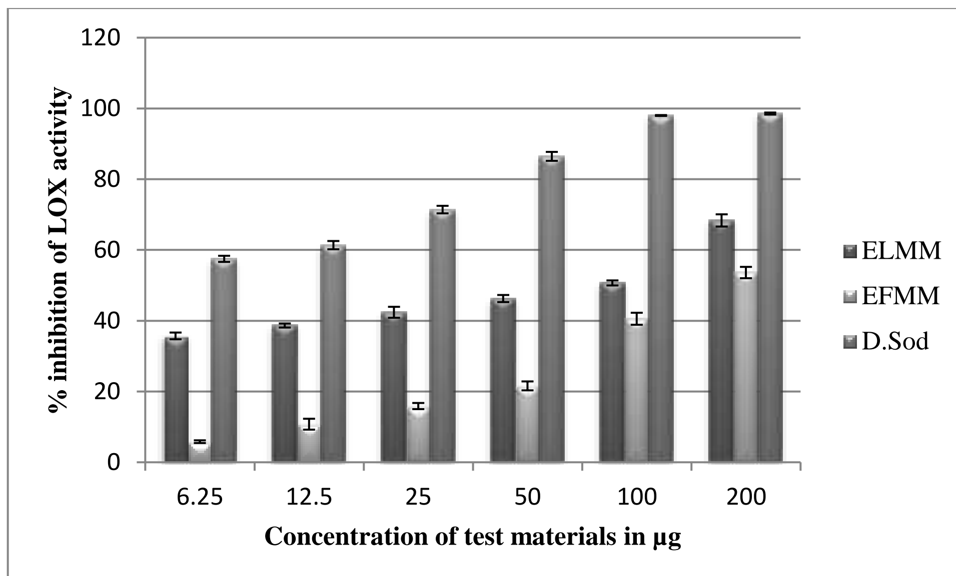

Figure 2: Percentage inhibition of lipoxygenase activity by ethyl acetate extract of the leaves (ELMM) and flowers (EFMM) of *Mikania micrantha* and diclofenac sodium.
Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

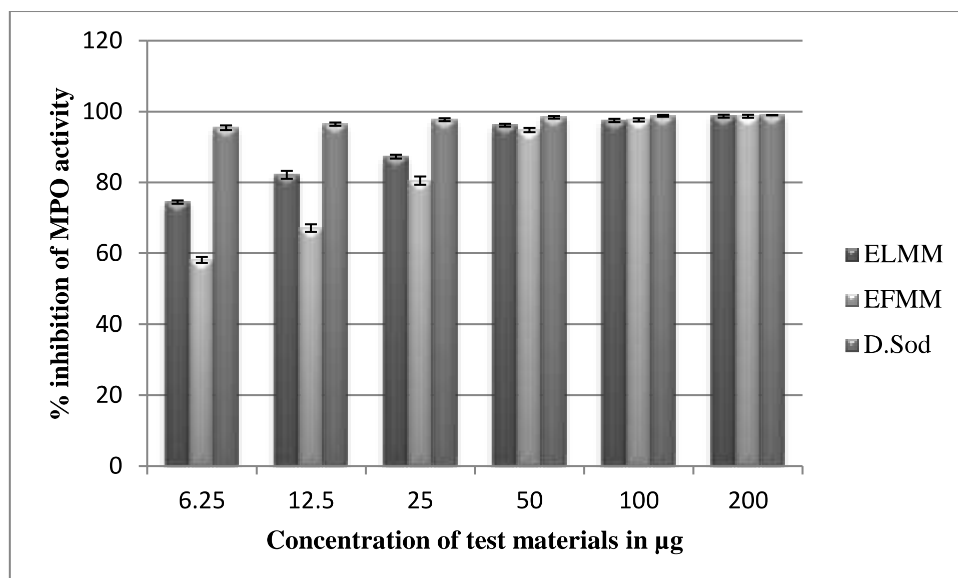

Figure 3: Percentage inhibition of myeloperoxidase activity by ethyl acetate extract of the leaves (ELMM) and flowers (EFMM) of *Mikania micrantha* and diclofenac sodium.
Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

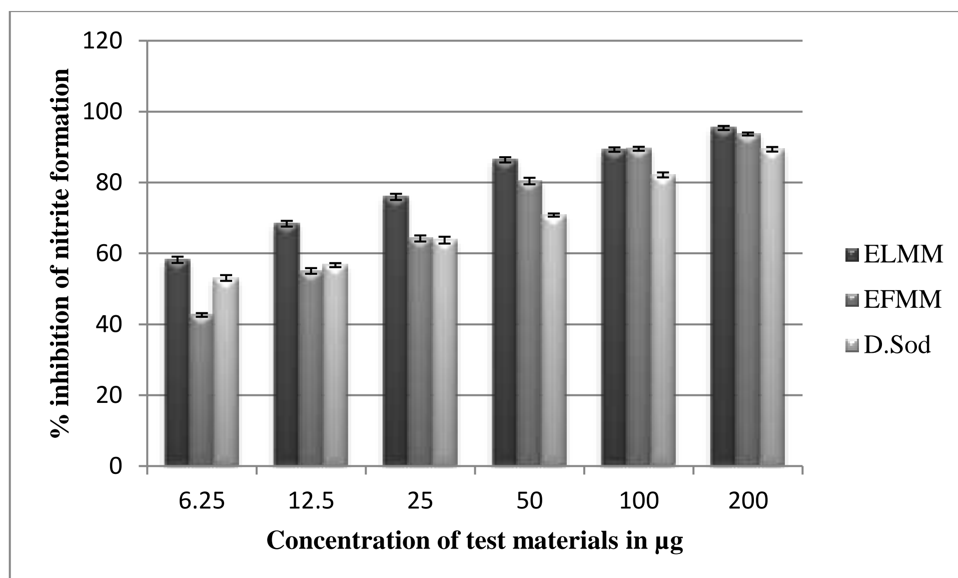

Figure 4: Percentage inhibition of nitrite formation by ethyl acetate extract of the leaves (ELMM) and flowers (EFMM) of *Mikania micrantha* and diclofenac sodium.
Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

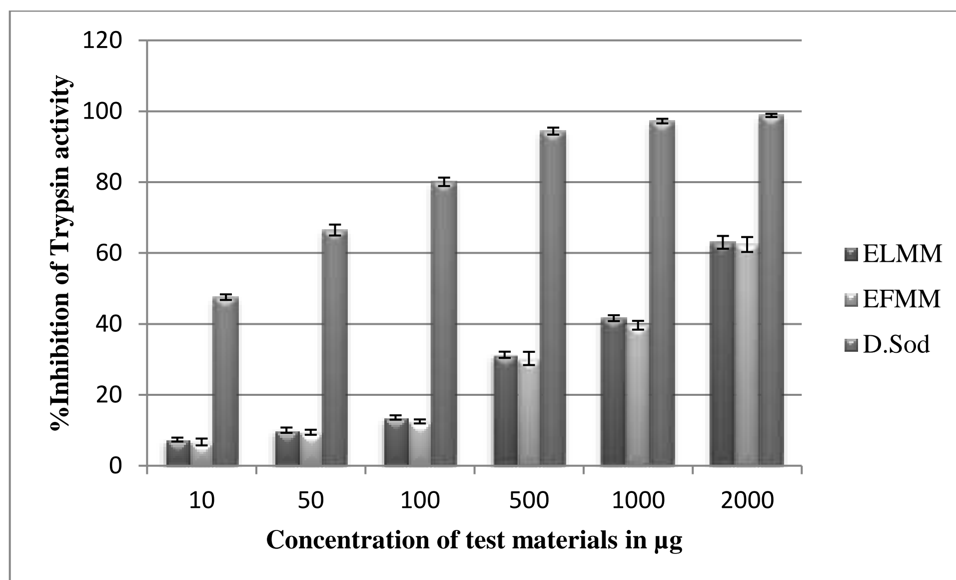

Figure 5: Percentage inhibition of trypsin activity by ethyl acetate extract of the leaves (ELMM) and flowers (EFMM) of *Mikania micrantha* and diclofenac sodium.
Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

RESULTS

Cyclooxygenase inhibition

ELMM and EFMM exhibited dose dependent inhibition of COX activity but ELMM exhibited greater inhibition than EFMM. The percentage inhibition of COX activity at different concentrations of ELMM and EFMM is shown in Figure 1 and the IC_{50} values are given in Table 1.

Lipoxygenase inhibition

ELMM exhibited greater inhibition of lipoxygenase activity than EFMM especially at lower concentrations. Percentage inhibition of LOX activity exhibited by the extracts is presented in Figure 2 and the IC_{50} values are given in Table 1.

Myeloperoxidase inhibition

Both the extracts exhibited excellent inhibition of myeloperoxidase activity. At lower concentrations (6.25 – 25 µg) activity of ELMM was slightly higher than that of EFMM but at higher concentrations both extracts exhibited very good inhibition (Figure 3). IC_{50} values are shown in Table 1.

Inducible nitric oxide synthase inhibition

Cellular nitrite level was found to be decreased in presence of the extracts ELMM and EFMM (Figure 4). This indicated the ability of the extracts to inhibit inducible nitric oxide synthase. IC_{50} values are shown in Table 1.

Protease inhibition

ELMM and EFMM exhibited similar inhibition of trypsin activity in dose dependent manner. The percentage inhibition of trypsin activity of ELMM and EFMM at different concentrations is depicted in Figure 5 and the IC_{50} values are given in Table 1.

DISCUSSION

Inflammation is a complex phenomenon executed in diverse ways via different mediators. Agents or mediators which can block any of these pathways can act as anti-inflammatory agents and can be used to treat chronic inflammation and associated illness. This study revealed cyclooxygenase, lipoxygenase, nitric oxide synthase, myeloperoxidase and protease inhibiting activities of leaves and flowers of *Mikania micrantha*.

ELMM and EFMM were found to possess cyclooxygenase and 5-lipoxygenase inhibiting activity. Nonsteroidal anti-inflammatory drugs (NSAIDS) execute their effect by inhibiting the enzyme cyclooxygenase which produce prostaglandins from arachidonic acid. There are two isoforms of cyclooxygenase – COX 1 and COX 2. It has been postulated that COX 1 is involved in homeostatic processes and COX 2 is involved in inflammation and pain. NSAIDS inhibit COX 1 as well as COX 2 and have sparing effect on renal function and gastric mucosa. Hence COX 2 inhibitors were developed which were intended to possess anti-inflammatory effect of NSAIDS but have no negative effect on kidney and gastric mucosa. But recently there are accumulating evidences that COX 1 and COX 2 are having overlapping actions and both are involved in homeostasis and inflammation. Also while COX synthesise prostaglandins from arachidonic acid, 5-LOX synthesise leukotrienes from it. Leukotrienes are potent mediators of inflammation including asthma. Inhibition of COX may lead to shunting of

arachidonic acid metabolism into 5-LOX pathway which results in increased production of leukotrienes and associated inflammation [21,22]. Inhibiting the production of prostaglandins as well as leukotrienes might have synergistic effect and will achieve optimum anti-inflammatory activity. ELMM and EFMM exhibited both COX and 5-LOX activity even though the COX activity was slightly higher than the 5-LOX activity. Lipoxygenase and cyclooxygenase inhibitors can also be used as cancer chemopreventive agents [21, 23]. Also dermatophytes produce prostaglandins and leukotrienes during infection [24]. Non steroidal anti-inflammatory drugs which inhibit COX have been found to inhibit the growth of dermatophytes [25,26]. Hence COX and 5-LOX inhibiting activity can intensify the reported antidermatophytic activity of the plant.

ELMM and EFMM exhibited very good myeloperoxidase inhibiting activity. Myeloperoxidase is a pro-inflammatory enzyme produced by neutrophils, monocytes and macrophages in response to leukocyte activation. Myeloperoxidase produces hypochlorus acid from hydrogen peroxide and chloride anion. The enzyme also oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as oxidizing agent. Hydrogen peroxide and tyrosyl radical are cytotoxic and are used by phagocytes to kill pathogens. But if released to outside of these cells the enzyme causes damage to adjacent tissues. It is capable of producing destructive oxidants which will oxidatively modify cellular structures leading to inflammation. It oxidizes low density lipoprotein (LDL) making it atherogenic and high density lipoprotein (HDL) rendering it dysfunctional. This leads to the deposition of cholesterol in the wall of the artery and MPO is regarded as a biomarker of inflammation in ischemic heart disease and acute coronary syndromes [27]. Multiple sclerosis is associated with elevated level of MPO where hypochlorus acid produced by the enzyme causes damage to axons [28]. MPO has also been found to be associated with transplant rejection [29], myocardial infarction [30] and atrial fibrillation [31]. Also it has been reported that, in addition to the inhibition of cyclooxygenase activity, anti-inflammatory effect of nonsteroidal drugs might also be due to free radical scavenger/antioxidant activity against MPO system [32]. ELMM and EFMM possessed both cyclooxygenase and myeloperoxidase activities which makes them an ideal target for efficient anti-inflammatory drug.

Nitric oxide (NO) is a small molecule with cytoprotective as well as cytotoxic properties. It is a double-edged sword being protective and beneficial at low concentration, and toxic and degenerative at high concentration. In the body it is produced by the enzyme nitric oxide synthase which has different isoforms. Under physiological conditions low level of NO is produced by constitutive nitric oxide synthase (cNOS). In pathological conditions high levels of NO is produced by inducible nitric oxide synthase (iNOS) which is pro-inflammatory and cytotoxic [33,34]. In this study ELMM and EFMM exhibited very good inhibition of nitric oxide synthase induced by lipopolysaccharide.

Diseases such as autoimmune disorders, asthma, arthritis, neurodegeneration, dementia, atopic dermatitis, Alzhemer's disease, ulcer and cancer are associated with various inflammatory disorders and require treatment for chronic inflammation. Currently various steroidal and non steroidal drugs are available to treat inflammation, but most of them are associated with side effects - renal failure, gastrointestinal harm and cardiovascular harm. Hence it is important to develop drugs which will promote resolution of inflammation in an efficient and homeostatic way but well tolerated by the host [3]. Also introduction of new therapeutic options is always appreciable in the treatment for any disease especially for conditions like inflammation which is multifactorial. A medicinal plant with scientifically proved anti-inflammatory activity is benefactory to traditional medicine as well as a novel source of therapeutic leads for modern pharmaceutical industry. In earlier days Willow trees (Salix species) which contain salicylic acid were used for treating pain and inflammation and this has lead to the discovery of the commonly used anti-inflammatory drug aspirin [3].

In this study, crude ethyl acetate extract of leaves and flowers of *Mikania micrantha* were found to inhibit multiple pathways of inflammation. This revealed the presence of anti-inflammatory principles in the leaves and flowers of the plant. Bioactivity guided fractionation and isolation of active principles is necessary to confirm whether the ability to inhibit diverse enzymes of inflammatory pathways is confined on a single molecule or attributed to different components. However leaves and flowers of *Mikania micrantha* are potential targets for developing anti-inflammatory formulations in traditional as well as modern medicine.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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