Melastoma malabathricum Linn attenuates complete freund’s adjuvant-induced chronic inflammation in Wistar rats via inflammation response

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Abstract

Background: Natural products use for arthritis treatment is gaining importance in the medical worldl. Various studies reports medical importance of Melastoma malabathricum Linn. (MM) (Melastomataceae), also known as “putki,” has a broad range of health benefits, for its free radical scavenging constituents. The current investigation scrutinizes the antioxidant and anti-inflammatory effect of MM against adjuvant-induced arthritis in experimental rats.

Methods: High-performance thin layer chromatography (HPTLC) was used for estimation of phytochemical-constituents present in the MM extract. Protective effect of MM extract in Wistar rats was estimate du s i n g C F A - i n d u c e d model. The rats were divided into different groups with six rats in each group. All animals received oral administration of MM and indomethacin for 28 days. The body weight and arthritic score were scrutinized at regular intervals. At the end of experimental protocol, the rats were sacrificed, and blood samples were used for antioxidant, hematological parameters, pro-inflammatory and inflammatory mediator, respectively. Histopathological observation was used to evaluate the protective effect of MM Extract.

Result & discussion: Current study confirmed the preventive effect of MM against adjuvant-induced paw edema, paw redness and arthritic progression. MM significantly (P < 0.001) modulated the oxidative stress parameters as well as hematological parameter induced by CFA. The result also altered the distorted level of proinflammatory mediators and inflammatory mediator, which further reinforce the implication of MM in CFA induced arthritis. Histological analyses of rats of rats showed a reduction in the synovial hyperplasia and mononuclear infiltration in the MM treated group which provides evidence for the antiarthritic effect of MM.

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Background

Rheumatoid arthritis (RA) is a chronic immune-mediated inflammatory disease, which finally results in damage of ligaments, peripheral joints and tendons. RA affects around 1% people in developed countries [1–5]. RA is further defined as the inflammatory condition of joints, which produces the pain, immobility and inflamed condition with excess deposition of synovial fluid (accumulation of polymorphonuclear leukocytes) [6, 7]. NSAIDs (Nonsteroidal anti-inflammatory drugs) are well established class of drugs used by many physicians on their patients as a first line antiarthritic drug, but they have side effects such as gastric erosion, ulceration, liver toxicity, inhibition of platelet activation and gastrointestinal hemorrhage, which consequently result in increased risk of hemorrhage [8, 9]. A lot of drugs have been developed for the treatment of RA over the past two decades, but researchers are still in search for more specific drugs with minimum side effects. Researchers were able to explain the one of the herbal formulation, piaseclidine, which is the mixture of avocado and soybean oils for relief of inflammatory arthritis symptoms. Several other small anti-inflammatory compounds isolated from natural sources have been scrutinized for the development of new lead molecule in the treatments for RA, but scientific data of their antiarthritic efficacy is still deficient and need more thorough study.

Melastoma malabathricum Linn. (Melastomataceae) (MM), commonly known as Phutki in India, is a branched shrub available in subcontinent, especially in northeastern region. Traditionally, its leaves are chewed and applied to cuts and wounds for their coagulating properties. The leaves are used to treat hemorrhoid, dysentery, diarrhea and to prevent scarring from the pox. The shoots are used as a toothbrush for toothache. Leaves and roots powder can be applied to pox scars and wounds to expedite the healing process or to alleviate the uneasiness of hemorrhoids. Flowers are used to treat stomach-ache [10, 11, 12]. MM plant has been utilized in the treatment of inflammation and rheumatism by the local people in Dibrugarh district, Assam. The plant is rich source of different chemical constituents such as α-amyrin, patriscabatine and auranamide [13], kaempferol-3-O-(2′,6′-di-O-p-trans-coumaroyl)glucoside, quercitrin, Mefloquine, (+) 3,4-Dehydroproline amide, 2-(3,5-Diphenyl-pyrazol-1-yl) benzothiazole, Kaempferol, Kaempferol-3-O-(2′, 6′-di-O-p-trans-coumaroyl)-β-D-glucopyranoside [14, 52].

Researchers have proved the antinociceptive, anti-inflammatory, antipyretic [15, 16], antimarial [17], antimicrobial [18], antiproliferative and antioxidant [19], antulcer activity [20], acute toxicity evaluation, bactericidal, antioxidant and immunomodulatory effects and hepatoprotective activity [21, 22] of the plant. However, this plant is not scientifically explored for its antiarthritic activity. Hence, efforts were made by the authors to screen the antiarthritic potential of MM.

Methods

Drugs and chemicals

CFA was procured from Sigma Chemical Company, USA and Indomethacin from Micro Lab Pvt. Ltd., India. Rest of the chemicals used in the experimental protocol was purchased as an analytical grade from the approved vendor.

Plant material

MM leaves were collected from the Herbal Garden, Dibrugarh University, Dibrugarh, Assam and authenticated by the Botanical Survey of India, Shillong and specimen voucher of plant sample was deposited for future reference.

Preparation of extracts

MM leaves were thoroughly cleaned with tap water to eliminate extemporaneous matter and dried in the shade. The dried MM (1 kg) leaves samples were exhaustively extracted with methanol in Soxhlet extractor for 72 h [23, 24]. The extract was filtered using the Whatman filter paper and the filtrate was concentrated under reduced pressure at low temperature. The extract was stored at 4 °C until further use. Before experimentation, MM extract was freshly prepared by dissolved in 1% carboxyl methyl cellulose (CMC) for experimental study.

Chromatography experiment

Pre-coated silica gel plate 60 F254 with a width (6 mm band) used for the HPTLC. Camag microliter syringe with a Linomat 5 applicator was used for injecting the sample over the plate under a flow of Nitrogen gas. The mobile phase (Hexane/ethyl acetate/formic acid (5:4:1, v/v/v)) was used in linear ascending development. The saturation time for mobile phase was about 20 min in

Conclusion: From above parameters our study states that the MM is capable of restraining the alteration produced via adjuvant-induced arthritis in aminals. The repressing effect of MM could be attributed, at least in part, to antioxidant, hematological and anti-inflammatory effect.

Keywords: Melastoma malabathricum Linn, Complete Freund’s Adjuvant, Pro-inflammatory mediator, Inflammatory mediator
saturation chamber to obtain the best results of tested and reference samples at room temperature (27 ± 20 °C) with 50% ± 2 relative humidity. Finally, the TLC (Thin layer chromatography) plate was dried at room temperature and the TLC plate image was developed at the distance of up to 80 mm length.

HPTLC (High-Performance Thin Layer Chromatography) photo-densitometry
HPTLC study of MM extract was carried out according to reported method of Kumar et al. with minor modification [25]. Quercetin (10 mg) was dissolved in 10 mL of methanol for preparing the standard solution and MM (2.0 g) extract solution was dissolved in 50 mL of methanol and was subjected to filtration using the Whatman filter paper. The filtrate was completely evaporated under reduced pressure and the obtained remains were re-dissolved in 1 mL of methanol. The prepared solution of quercetin and MM extract was carefully spotted on the precoated TLC plates using Camag microliter syringe with constant application 150 nL/s.

Molecular docking studies
The molecular docking study was performed on cyclooxygenase 2 (3D structure) complex enzyme using Maestro 9.0 program (Schrodinger Inc. USA) by 64 bits operating systems under Windows 7 with an HCl computer [Intel (R) Core (TM) i5-2400 CPU @ 3.10 GHz, 8GB memory]. The molecular docking study was performed on enzymes cyclooxygenase, which was taken from the protein data bank (PDB ID: 1cx2). The protein is having the 96% similarity with the human cell enzyme with all the active site residues. Before performing the molecular docking, the enzyme structure was scrutinized for missing bonds, atoms and contacts. All residues and water molecules were manually removed from the structure. The grid box was used for the estimation of the active site of the protein. The minimum energy was selected and used for energy minimization.

Animals
Swiss albino adult Wistar rats (weighing 150–220 g; both sexes, kept per cage) were obtained from the departmental animal house and were used according to departmental ethical committee and care of animals (National Institutes of Health). The animals were housed in the animal house under standard conditions (relative humidity (55 ± 10%), temperature (23 ± 1 °C), 12 h/12 h light/dark cycles) they received the standard pellet diet and water ad libitum.

Acute toxicity study of MM
Oral acute toxicity study of MM extract was performed according to the reported method of Kumar et al. with minor modifications [25]. Swiss albino Wistar rats of both sexes (weighing 100 to 220 g, body weight) were used for the experimental purpose. The rats were rav- enous overnight and divided into the four groups (each group of rats contained three male and three female rats): Group I: (normal control) treated with vehicle only (1 mL water) and Group II (tested) received MM (0.5 g/kg); Group III received MM (1.0 g/kg, body weight (b.w.)) and Group IV received MM (2.0 g/kg b.w.), respectively. All group animals were examined for 24 h, for the confirmation of signs and symptoms of autonomic, neurological and behavioral changes [26].

Sub acute toxicity study of MM
Swiss albino Wistar rats (weighing 100 to 220 g; both male and female) were randomly selected randomly for the study. The animals were divided into four groups and each group contains animals (five male and five female): Group I- normal controls received vehicle (1 mL water); Group II- treated MM (2000 mg/kg b.w.); Group III- treated MM (1000 mg/kg b.w.); Group IV- treated MM (2000 mg/kg b.w.). All animals treated with predetermined oral treatment and MM extract which was suspended in distilled water.

All group animals were observed for adverse reactions, side effects, mortality and any other changes at end of the sub acute toxicity study. The food consumption, water intake and body weight of all group animals were scrutinized at regular intervals. At the end of protocol animals were sacrificed by cervical dislocation using the anesthesia condition, all the vital organs of animals were scrutinized visibly for evidence of any morphological deformity and blood samples of all group animals were collected and were used for the estimation of serum biochemistry like RBC (red blood cells), WBC (white blood cells), Hb (hemoglobin) and platelets levels and the biochemical parameters like AST (Aspartate aminotransferase), ALP (Alkaline phosphatase), ALT (Alanine transaminase), total cholesterol, LDL (Low-density lipoprotein), VLDL (Very low-density lipoprotein), HDL (High-density lipoprotein), triglyceride, bilirubin, albumin, creatinine, total protein and serum urea were also estimated [53]. The vital organs such as kidney, heart and liver were excised, removed and weighed. Samples of extraneous tissues were removed, fixed in 10% formalin and dehydrated for histopathological examinations [27].

Oral administration of drugs
The flexible metal rodent feeding tube was used for oral administration of tested and reference drugs. Mis-feeding of tested and reference drugs into trachea was perceived by signs of an irregular breathing pattern, irritation and choking. When the misfeeding transpired (less than 5% of all feedings), the rats were carefully observed for their revival [28].

Complete Freund’s Adjuvant-induced arthritis in rats
The rats were divided into six groups (each group 6 rats). Group I was treated with vehicle (1% (w/v) CMC);
II received CFA; III received CFA + MM (100 mg/kg); IV received CFA + MM (250 mg/kg); V received CFA + MM (500 mg/kg) and Group VI received CFA + indomethacin (10 mg/kg). CFA (0.5% w/v) was inducted into the supplanter region of left hind paws to induced the arthritis. All the animals received the predetermined treatment till 28 days [29, 30] with paw edema of rats subjected to scrutinized at a regular interval using the screw gauge micrometer [31].

**Evaluation of arthritis swelling**

The joint swelling (arthritis index) was evaluated by the reported method of Kumar et al. [25] with minor modifications. The arthritis index was assessed on scale 0–4, 0: no change, 1: mild swelling and erythema of limb, 2: erythema of limb and moderate swelling, 3: erythema of limb and coarse swelling, 4: the inability of limb and gross deformity. The score of the four limbs was counted. Score more than one, confirmed arthritis and limited score of arthritis was set to 16. The arthritis score day of onset and frequency of arthritis was also recorded [32].

**Blood and tissue sampling**

Rats of all groups were sacrificed on 29th day, and blood and tissue samples were collected in test tubes with anticoagulation agent. The collected blood samples were centrifuged at 15,000 rpm, and the serums were obtained for the estimation of biochemical parameters.

**Estimation of proinflammatory and inflammatory mediators**

The proinflammatory mediators such as interlukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) inflammatory mediators such as cyclooxygenase 2 (COX-2) were estimated using the manufacturer instructions of Enzyme Linked Immunosorbent Assay kits.

**Histopathology examination**

The left paw of all groups rats were cut above and below the 0.5 cm of joints, the muscle and skin of joints were trimmed away to leave the intact synovial membrane. All tissues were processed with the 3% hydrochloric acid (HCl) solution for 5–7 days, with periodic change of HCl every 24 h for complete decalcification of joints. After that, the joints were fixed in the neutral buffered formalin (10%) for 2 days. Decalcified joints of rats were again dehydrated in different series of alcohol and joints were cleared and embedded in liquid paraffin. Decalcified joints were sliced g into 5 μm pieces with hematoxylin and eosin used for staining the tissue and preparing the slide. The slides were prepared for microscopic evaluation under light microscope (original magnification 40×, DXIT 1200, Nikon, Japan). H&E stained joint slides were examined for bone and cartilage destruction by synovial proliferation, cellular infiltration, and cartilage erosion by the following scoring system.

a. **Synovial proliferation**

For the estimation of synovial proliferation, the following score system was used. No change: Score 0; mild proliferation with 2–4 layers of synoviocytes = Score 1; moderate proliferation with four or more layers of synoviocytes, absent synovial cell invasion of adjacent connective tissue and bone with enhanced mitotic activity = Score 2; provide proliferation distinguished by adjacent cartilage and enchancement of joint space, connective tissues, and bones = Score 3.

b. **Cellular infiltration**

Cellular infiltration, no changes: Grade 0; the small number of focal infiltrates: Score 1; extensive focal infiltration: Score 2; widespread infiltrates assaulting the capsules with combined formation: Score 3.

c. **Cartilage erosion**

No changes: Score 0; localized cartilage ruin in more than one region: Score 1; cartilage ruin: Score 2; thick cartilage ruin different locations.

**Statistical analysis**

All the results were presented as mean ± SEM (standard error mean) using one-way ANOVA (Graph Pad Prism 5.0, Graph Pad Software, San Diego, CA, USA). The values of the result were considered to be significant, when the P value was p < 0.05, p < 0.01 and p < 0.001.

**Results**

**HPTLC analysis**

Various composition and amalgamations of the mobile phase were used for the required resolution of phytoconstituent (quercetin). Mobile phase solvent combination (Hexane, Ethyl acetate and formic acid (5:4:1, v/v/v)) gave a compact and dense spot. The presence of quercetin in the MM was confirmed by comparison of Rf value of standard quercetin. The sample bands at Rf 0.62 corresponded to quercetin (Figs. 1 and 2).

**Molecular docking study**

We have performed the molecular modeling docking studies via automated docking method into the ATP-binding site of COX-2 with s58 at 3.0 Å resolution complex (PDB code: 1cx2). The binding pose found by Maestro 9.4 for quercetin against COX-2, presented in Fig. 3. It can be observed from the molecular docking studies, that these molecules are located in such a way that the quercetin hydroxyl group creates hydrogen bond with the backbone of OH reside (Ser 530) and NH of His 90 in the hinge section. For further refinement of results, we superimposed the quercetin with s58 analyses and found the orientation of quercetin same as s58 in the COX-2 catalytic domain.
Fig. 1 High performance thin layer liquid chromatography (HPTLC) profiles of *Melastoma malabathricum* Linn. methanolic extract

Fig. 2 High performance thin layer liquid chromatography (HPTLC) profiles of Quercetin
For better understanding, the binding mode of quercetin at the molecular level, the molecular docking simulations of quercetin at the COX-2 catalytic ligand-binding site was carried. The Schrodinger software suite was used for the estimation of simulation. The ligand was prepared in the 3D format using ligprep. The protein was obtained from the protein data bank (PDB ID: 1cx2), and made ready by removing the solvent, adding hydrogen and further minimization the bound ligand (s58) (Fig. 4). The re-docking study was carried for validation of co-crystal ligand parameters (s58) at the protein catalytic site, and re-docked pose was found to be 0.249 Å (Fig. 5).

**Oral toxicity study**

Oral administration of MM (500 to 2500 mg/kg b.w.) did not produce any change in the clinical or behavioral signs and symptoms, mortality or any other types of
adverse reactions at the end of study. The experimental animals did not show any change in food consumption and body weight during the study period. Similar results were obtained during a subacute toxicity study, when compared to normal rats, confirm the safety of MM even upon continuous oral administration for 28 days, irrespective of the sex of rats. The results of subacute study on relative body weight of kidney (0.78 ± 0.02 g), liver (3.73 ± 0.46 g) and heart (1.98 ± 0.07 g) in male Wistar rats and the relative body weight of kidney (0.54 ± 0.02 g), liver (2.45 ± 0.31 g) and heart (1.34 ± 0.17 g) in female Wistar rats. The relative organ weight of all MM (2500, 1250 and 625 mg/kg, b.w.) treated groups was almost similar to normal rats. These results indicate the body weight of normal male rats was enhanced from 140.3 ± 3.43 to 189.5 ± 8.34 with growth gain rate of 1.76 ± 0.23 and the body weight of normal female Wistar rats increased from 123.4 ± 2.83 to 156.2 ± 6.43 with 1.17 ± 0.19 growth gain rate. The body weight of MM (2500 mg/kg b.w.) treated rats confirmed the proper range of hepatic and renal parameters as compared to normal rats. There was no significant alteration in lipid profile such as triglycerides, cholesterol, LDL, HDL and VLDL as compared to normal control by MM (Table 1).

The histology of normal and MM treated rats did not show any change in the appearance of the rat organs (Fig. 6).

**Effect of MM on adjuvant-induced arthritis**

The rats developed the swelling and redness over 24 h after the adjuvant treatment and reached extreme intensity on day 4 (first stage of swelling developed). Consequently, redness and swelling slowly augmented until the 8th day and subsequently, rats re-developed the redness and swelling. Arthritis developed during the secondary swelling, with more predominant swelling and redness, as a comparison to first stage. The secondary stage swelling was maximum by 25th day of study. Pre-treatment of MM significantly \((P < 0.001)\) censored the swelling and repressed the paw edema/redness in dose dependent manner (Fig. 7a). MM (500 mg/kg) showed the better recovery of the rats as compared to indomethacin treated group. Clinical assessment of rat ankles signified that AC rats confirmed the maximum arthritis score which was considerably higher than the normal rats. AC rats treated with MM significantly \((P < 0.001)\) produced a dose dependently inhibition of arthritic score as compared to AC rats (Fig. 7b). Considering the arthritic score, MM (500 mg/kg) and indomethacin significantly \((P < 0.001)\) showed the better protection of rats during the arthritis, but the
treatment by MM (500 mg/kg) was superior in comparison to indomethacin.

The cartilage erosion, synovial proliferation, and cell infiltration assessment between the different groups resulted in AC rats confirmed the higher score as compared to normal rats. Pre-treatment with MM, considerably restrained the histologic score as compared to AC group rats. Prominently, MM (500 mg/kg b.w.) inhibited the histologic score compared to Indomethacin group. Both MM (500 mg/kg) and indomethacin showed the protection/superiority over the arthritis disease but MM (500 mg/kg) demonstrated more superiority as compared to indomethacin group, which was confirmed by the histological observation. MM illustrated the average score 0.67 and indomethacin showed the average score 0.84 (Table 2).

Body weight declined in AC rats at the end of experimental study (Fig. 8). AC rats treated with MM and Indo significantly (P < 0.001) improved the body weight in dose dependent manner. (Fig. 8).

Effect of MM on hematological parameters
CFA made a compelling upsurge in fructification of WBC (8 ± 0.32 cells/cu.mm). The MM treatment conferred important protection from the same, just about consistently (Fig. 6). Similarly, outstanding enhancement of ESR was noted in AC control (14 ± 0.46 million/cu.mm (gm%)) in counterpart to normal control (9.8 ± 0.29 million/cu.mm (gm%)). Concomitant administration of MM significantly (P < 0.001) altered the WBC and ESR. Compelling subsidence in the hematological parameters of RBC as perceived in AC (4.4 ± 0.25 million/cu.mm). Similarly, the outstanding decline in Hb was noted in AC (9.6 ± 1.37 cells/cu.mm), it is notable that treatment with MM helped to restore the hematological parameters of RBC and WBC synchronously (Fig. 9).

Effect of MM on antioxidant enzymes
We also examined the antioxidant parameters (Fig. 10). As expected, CFA treatment considerably increased the

### Table 1 Effect of administration of *Melastoma malabathricum* Linn. (MM) (28 days) on biochemical and hematological parameters in Wistar rats (Male and Female)

| S. No | Parameters             | Normal control | MM (500 mg/kg, b.w.) | MM (1000 mg/kg, b.w.) | MM (2000 mg/kg, b.w.) |
|-------|------------------------|----------------|----------------------|-----------------------|-----------------------|
| 1     | Body Weight (gm)       | 189.5 ± 8.34   | 176.3 ± 6.34         | 198.3 ± 9.76          | 201.2 ± 11.34         |
| 2     | Water Intake (mL)      | 102            | 83                   | 94                    | 104                   |
| 3     | Food Intake (gm)       | 52             | 32                   | 44                    | 54                    |
|       | RBC (10^6/cu mm)       | 6.43 ± 1.21    | 5.98 ± 1.04          | 6.87 ± 1.65           | 7.75 ± 1.83           |
| 4     | WBC (10^3/cu mm^3)     | 8.99 ± 1.43    | 11.2 ± 1.65          | 10.33 ± 1.64          | 9.01 ± 1.59           |
| 5     | Hb (g dL^-1)           | 13.02 ± 2.34   | 12.54 ± 1.19         | 13.06 ± 2.53          | 14.03 ± 2.83          |
| 6     | Platelet (10^5/cu mm)  | 4.01 ± 0.73    | 6.2 ± 2.01           | 5.21 ± 1.03           | 4.50 ± 0.94           |
| 7     | Eosinophils (mm^3)     | 796 ± 293      | 498 ± 165            | 454 ± 214             | 731 ± 421             |
| 8     | Lymphocyte (mm^3)      | 3990 ± 850     | 2983 ± 594           | 3214 ± 843            | 4013 ± 985            |
| 9     | Neutrophils (mm^3)     | 1596 ± 748     | 1374 ± 398           | 1602 ± 67             | 1545 ± 938            |
| 10    | ALP (UL^−1)            | 221 ± 21.83    | 298 ± 13.78          | 276.2 ± 23.73         | 245.5 ± 21.6          |
| 11    | ALT (UL^−1)            | 61.3 ± 2.34    | 90.3 ± 6.65          | 93.1 ± 8.76           | 75.9 ± 6.87           |
| 12    | AST (UL^−1)            | 103.2 ± 7.65   | 71.3 ± 8.76          | 74.5 ± 10.4           | 79.5 ± 7.54           |
| 13    | Bilirubin (mg dL^-1)   | 0.52 ± 0.09    | 0.68 ± 0.08          | 0.79 ± 0.14           | 0.72 ± 0.21           |
| 14    | Creatinine (mg dL^-1)  | 0.71 ± 0.27    | 0.86 ± 0.22          | 0.79 ± 0.14           | 0.72 ± 0.21           |
| 15    | Urea (mg dL^-1)        | 36 ± 3.21      | 41.3 ± 3.43          | 39.3 ± 3.54           | 36.2 ± 3.43           |
| 16    | Cholesterol (mg dL^-1) | 71.3 ± 1.76    | 82.5 ± 1.93          | 79.6 ± 2.45           | 75.3 ± 4.32           |
| 17    | Triglyceride (mg dL^-1)| 74.5 ± 1.87    | 86.7 ± 1.65          | 92.2 ± 2.43           | 78.4 ± 3.45           |
| 18    | HDL (mg dL^-1)         | 26.4 ± 1.41    | 26.8 ± 2.73          | 22.4 ± 1.54           | 25.3 ± 2.43           |
| 19    | LDL (mg dL^-1)         | 37.1 ± 1.23    | 51.79 ± 2.13         | 53.69 ± 1.86          | 58.01 ± 1.98          |
| 20    | VLDL (mg dL^-1)        | 14.9 ± 1.34    | 17.7 ± 1.67          | 17.66 ± 1.98          | 18.06 ± 1.34          |

**Table 1** Effect of administration of *Melastoma malabathricum* Linn. (MM) (28 days) on biochemical and hematological parameters in Wistar rats (Male and Female)
MDA enzyme activity and decreased the activities of SOD, GSH and GPx. AC rats treated with MM significantly ($P < 0.001$) altered the antioxidant enzymes to near the normal rats in dose dependent manner.

**Effect of MM on inflammatory mediators**

The levels of COX-2, IL-6, TNF-α and IL-1β were augmented in the serum after CFA treatment. AC rats received oral administration of MM apparent supplementary increase of the same (Fig. 11).

**Effect of MM on histopathology of CFA-induced rats**

The histopathology observation of normal control and CFA-induced rats are showed in Fig. 12. The normal rat histopathology explained clean articular surface among 1–4 layers of synovial cells and normal articular smooth surface with a loose connective tissue. This also contained the fatty tissue below the synovial membrane. AC control rat histopathology showed the hyperplasia in the articular region with the loose connective tissue of the same. The synovial defect showed the distortion of pannus formation of bone and cartilage. The deformity of the joint was widespread resulted due to increased sizes and connectivity of synovial membrane. AC showed the loose arrangement of connectivity of tissue due to hyperemia, edema and infiltration with provocative cells (Fig. 12b). AC rats treated with MM showed the remarkable restoration of all histology characteristics in dose dependent manner. MM dose (500 mg/kg b.w.) displayed enhanced histopathology as compared to MM dose (100 and 250 mg/kg b.w.) (Fig. 12 c-e).

**Discussion**

Arthritis is a chronic painful inflammatory disease, affecting more than 1% population in developed countries. On the basis of biochemical markers, CFA-induced...
Table 2 Effect of administration of *Melastoma malabathricum* Linn. (MM) on the scores of histopathological changes in CFA induced arthritic group rats

| S. No | Histological changes | Groups | AC | AC + MM (100 mg/kg) | AC + MM (250 mg/kg) | AC + MM (500 mg/kg) | AC + Indo (10 mg/kg) |
|-------|----------------------|--------|----|----------------------|---------------------|---------------------|----------------------|
| 1     | Synovial proliferation |        |    |                      |                     |                     |                      |
|       | Grade 0              | -      | -  | -                    | 2                   | 1                   |                      |
|       | Grade 1              | -      | -  | -                    | -                   | 1                   |                      |
|       | Grade 2              | -      | 2  | -                    | 2                   | 2                   | 2                    |
|       | Grade 3              | 5      |    | 1                    | -                   | -                   | -                    |
|       | Total                | 15     | 10 | 7                    | 4                   | 5                   |                      |
|       | mean                 | 2.5    | 2.3| 2.3                  | 1                   | 1.25                |                      |
| 2     | Cartilage erosion    |        |    |                      |                     |                     |                      |
|       | Grade 0              | -      | -  | 2                    | 4                   | 3                   |                      |
|       | Grade 1              | -      | 2  | 2                    | 1                   | 1                   |                      |
|       | Grade 2              | 2      | 1  | -                    | -                   | -                   | -                    |
|       | Grade 3              | 3      |    | -                    | -                   | -                   | -                    |
|       | Total                | 13     | 4  | 2                    | 1                   | 1                   |                      |
|       | mean                 | 2.6    | 1.33| 0.50                 | 0.20                | 0.25                |                      |
| 3     | Cellular infiltration|        |    |                      |                     |                     |                      |
|       | Grade 0              | -      | -  | -                    | 3                   | 3                   |                      |
|       | Grade 1              | -      | 1  | 2                    | 2                   | 1                   |                      |
|       | Grade 2              | -      | 2  | 2                    | 1                   | 2                   |                      |
|       | Grade 3              | 5      | 1  | -                    | -                   | -                   | -                    |
|       | Total                | 15     | 10 | 6                    | 4                   | 5                   |                      |
|       | mean                 | 3      | 2.5| 1.5                  | 0.67                | 0.84                |                      |
Arthritis is clinically classified into four phases, initial phase developed during the first week after injecting the CFA with acute local inflammation and having some effects on liver; in the second week afterward, extend the reduction of acute inflammation and start the periarteritis; third-fourth week develops the arthritis with chronic inflammation, osteogenic and periarteritis activity, therefore, the fifth week onwards (forever), with permanent articular deformity and minimal inflammation [33, 34].

Our study showed the credible protecting impact of this plant against the chronic inflammation induced by CFA. The results confirmed the use of MM extract as an antiarthritic agent in Indian folk medicine on the scientific basis. The conclusion of this study clearly explains that the MM extract is a potent antiarthritic remedy against chronic phases of inflammation. Topical and systemic application of MM is commonly observed for the treatment of inflammation, predominantly, arthritis (ama vata) which has been pursued for many years in the practice of Indian system of medicine (Ayurveda).

MM extract is a rich source of the flavonoids (quercetin). Flavonoids play an important role to inhibit the inflammation via apoptotic mechanisms (calcium dependent) or cyclin-dependent kinase inhibitors; modify the cell cycle capture at the G1/S phase (Extracellular growth signals stimulate progression through the first gap phase G1, and into S, where genomic DNA is replicated); inhibit the cell-survival kinase and inflammatory transcription factors; down-regulate the antiapoptotic gene products [34, 35].

The CFA-induced arthritis model is the most extensively use model to investigate the clinical and pathogenic changes, which are similar to human arthritis [25]. Further this method helps in determining the correlation between efficacy of therapeutic agents and RA model [36, 37]. The adjuvant-induced inflammatory process involves fenestration of microvasculature, leakage of element into the interstitial space and passage of macrophages, lymphocytes into inflamed tissue; where adjuvant inoculation releases the number of inflammatory mediators like cytokines, histamine, 5 hydroxytryptamine, bradykinin,
various chemotactic factor, interferons and prostaglandin into inflamed area. These mediators are liable for demolition and destruction of bone, cartilage and associated with pain, which can lead to several disabilities. Other mechanisms involves leukocytes and phagocytes, preparing a complex with the mediators, releasing the lysosomal enzymes and causing the injury cartilage and other tissue. CFA affected the soft tissue and created the swelling near the ankle joints confirm the expansion of arthritis which was measured as edema of the particular tissues [38, 39]. In the current study, the efficacy of MM treated rats was evaluated in the proliferative phase of inflammation, showing the minimization of the chronic swelling induced by CFA. The first indication of the antiarthritic effect of MM was observed when the administered extract (100, 250 and 500 mg/kg) showed a significant dose-dependent reduction of paw edema with 59.17%, 74.16%, and 87.72%, as compared to AC rats.

Fig. 10 Effect of Melastoma malabathricum Linn. (MM) on CFA induced arthritic rats. Arthritic was assessed in terms of modulation of (a) MDA, (b) GPx, (c) SOD and (d) GSH as described in Materials and methods. ns = non-significant, CFA = Complete Freund Adjuvant, NC = Normal Control, AC = Arthritic Control, MDA = Malonaldehyde, GPx = Glutathione peroxidase, SOD = Superoxide dismutase, GSH = Glutathione. The comparisons were made by ANOVA followed by Dunnett’s test. *P < 0.05 is considered as significant, **P < 0.01 is considered as very significant, ***P < 0.001 is considered as extremely significant

Fig. 11 Effect of Melastoma malabathricum Linn. (MM) on CFA induced arthritic rats. Arthritic was assessed in terms of modulation of proinflammatory mediators such as (a) TNF-α, (b) IL-6, (c) IL-1β and (d) COX-2 as described in Materials and methods. ns = non-significant, CFA = Complete Freund Adjuvant, NC = Normal Control, AC = Arthritic Control, TNF-α = Tumor necrosis factor-α, IL-6 = Interlukin-6, IL-1β = Interlukin 1β. The comparisons were made by ANOVA followed by Dunnett’s test. *P < 0.05 is considered as significant, **P < 0.01 is considered as very significant, ***P < 0.001 is considered as extremely significant
respectively. However, MM received rats did not show enhancement of joint diameter, which suggests that mechanisms involved, is inhibition of inflammatory autacoids. The most likely mechanism for MM extract may be inhibition of proinflammatory mediators, which could have lead to an alteration in the immunological situation during the delayed phase of the response. The determination of body weight and foot thickness of CFA-induced arthritis animals has been used to evaluate the antiarthritic effect of drug treatment. We found increased body weight of rats with decreased thickness of rat paw as compared to AC rats.

AC rats confirmed the decline level of RBC, Hb and increased levels of WBC, ESR. WBC is an important component of an immune system which is associated with induction of inflammation and is related to other infectious diseases [40]. During the arthritic condition, 1 L-18 mediated increase in WBCs, cause the growth of colony stimulating factors and production of inflammatory macrophages and granulocyte [41]. MM treated rats significantly suppressed the migration of inflammatory macrophages and granulocytes in dose dependent manner. On the other hand, increased ESR levels start the deprivation of synovial fluid and induce the depolymerization of hyaluronic acid which turns the loss of viscosity in joints [47]. The human body has an efficient mechanism to avoid and deactivate the free radical generation/production damage caused by endogenous antioxidants such as SOD, GSH, GPx and CAT. SOD is metalloprotein that is the first line antioxidant marker involve in antioxidant defense. Decreased levels of RBCs in arthritic conditions increase the production of superoxide (O₂) and hydrogen peroxide radicals (H₂O₂) due to accumulation of inflammatory macrophages and granulocytes in the inflamed area [47, 48]. Lipid peroxidation (LPO) is a common feature of rheumatic arthritis. MDA (as an indicator of lipid peroxidation) is used to evaluate the LPO. MDA is a pro-oxidant factor which determines the oxidative stress present in the rats [48]. AC rats showed increased levels of MDA as compared to NC
rats, due to elevated d level, it also started the accumulation of neutrophils in the inflamed area. CFA-induced arthritic rats that received MM significantly (P < 0.001) declined the levels of MDA. GSH and SOD are the first line antioxidants against scavenging the free radicals. SOD converts the superoxide radical to H2O2 and is widely used to protect the cell damage during the toxic effects of O2- anion. First line antioxidants catalytically scavenge the hydrogen peroxide (H2O2) and superoxide (O2-) molecules. Endogenous antioxidant GPx reduced the H2O2 in the presence of GSH to form oxidized glutathione and water [49]. CFA-induced arthritic rats that received MM had significantly (P < 0.001) modulated the level of an antioxidant enzyme as compared to AC rats.

Many researchers have shown that the proinflammatory mediators play a crucial role in the pathogenesis of arthritis [50]. TNF-α (pleiotropic inflammatory cytokines) plays a crucial role in pathogenesis of acute and chronic inflammation, associated with the neutrophils and lymphocytes in endothelial cells via inflamed tissue. TNF-α present in sera and synovial fluid of arthritic patients is a laboratory and clinical marker for estimation/confirmation of RA disease [51–53]. It also promotes the generation of macrophage chemotactic protein-1 (MCP-1), IL-6 and IL-1β, which are involved in increasing the inflammatory reaction. Macrophages release the proinflammatory IL-1β, which enhances the inflammatory reaction. Both inflammatory cytokines (IL-1β and TNF-α) activated through the osteoclasts and nuclear factor are responsible for resorption and obliteration of bones. The generation of proinflammatory cytokines such as IL-1β starts through the T-cells, fibroblasts and monocytes, which initiate the induction of osteoclast discrimination and stimulates the MCP-1. All these proinflammatory cytokines are also involved in enhancing the clinical signs of arthritis and expansion of arthritic symptoms as well as body weight loss and joint swelling [54, 55]. In the current study, we observed a considerable enhancement of serum TNF-α, IL-1β and IL-6 concentrations in AC group as compared to NC group. Treatment with MM decreased the serum levels of IL-1β, TNF-α and IL-6 without disturbing other inflammatory mediators.

Conclusion
In conclusion, Melastoma malabathricum Linn was effective in attenuating the CFA-induced arthritis rats in a dose-dependently manner, and therefore it could be investigated as a probable treatment for chronic arthritis conditions in humans. The results obtained from the current study confirm the use of MM extract in traditional Indian medicine for the management of painful inflammatory and arthritic conditions. Additional work is going on to isolate, characterize and identify the structure of phytoconstituents responsible for the antiarthritic activity.

Highlights
Melastoma malabathricum Linn reduced CFA induced activation of the proinflammatory cytokines.
Melastoma malabathricum Linn reduced the CFA induced activation of COX-2.
Melastoma malabathricum Linn significantly suppressed the paw edema.
Melastoma malabathricum Linn modulation of the CFA induced antioxidant parameters.

Abbreviations
AC: Arthritic control; ALP: Alkaline phosphatase; ASA: Acetylsalicylic acid; AST: Aspartate aminotransferase; b.w.: Body weight; CAT: Catalase; CFA: Complete Freund’s adjuvant; CMC: Carboxymethyl cellulose; COX-2: Cyclooxygenase 2; DNA: Deoxyribonucleic acid; ELISA: Enzyme Linked Immunosorbent Assay; ESR: Erthrocytes sedimentation rate; GPx: Glutathione peroxidase; H: Hour; Hb: Hemoglobin; HCO3: Hydrochloric acid; HDL: High-density lipoprotein; HPTLC: High-performance thin layer chromatography; IL-1β: Interleukin-1β; IL-6: Interleukin-6; Indomethacin; Kg: Kilogram; LDL: Low-density lipoprotein; LPO: Lipid peroxidation; MCP-1: Macrophage chemotactic protein-1; NSAIDs: Nonsteroidal anti-inflammatory drugs; O2: Superoxide; OA: Rheumatoid arthritis; RBC: Red blood cells; ROS: Reactive oxygen species; SEM: Standard error mean; SOD: Superoxide dismutase; TLC: Thin layer chromatography; TNF-α: Tumor necrosis factor-α; VLDL: Very low-density lipoprotein; WBC: White blood cells

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Availability of data and materials
The complete data are all contained within the paper and are also available from the corresponding author on reasonable request.

Authors’ contribution
VK and PCB premeditated and carried out the extraction of the Melastoma malabathricum Linn leaves. VK, NS, KS, AK, NKS, GK and FA carried out the biochemical estimations. VK, PCB, NS, GK, FA and AV analyses the statistical data and interpretation of the histological analysis. All the authors are involved in the critical evaluation of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not Applicable.

Ethics approval
The study was approved from the Siddhartha Institute of Pharmacy (1435/PO/a/11/CPSEA).

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