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RESEARCH ARTICLE

Dual inhibition of arachidonic acid pathway by mulberry leaf extract

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Abstract The present work investigates the anti-inflammatory, analgesic and antipyretic activity of methanolic extract of mulberry leaves of variety S-1, S-13 and S-146. The S-146 extract was further evaluated for its efficacy against adjuvant arthritis in albino rats followed by inhibitory potential for COX 1, COX 2 and 5 LOX. The HPLC analysis enumerated the presence of morin, reversterol, scopoletin and 7-hydroxy coumarin as the major constituents. The anti-inflammatory, antipyretic and analgesic activity observed in the present experiment could be accredited to the dual inhibition in the AA pathway. The inhibition of COX and LOX enzymes could be imparted to the presence of resveraterol, morin, scopoletin and 7-hydroxy coumarin.

Keywords Adjuvant arthritis · Cyclooxygenase · Inflammation · Lipoxygenase · *Morus alba*

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Introduction

Morus alba L (Mulberry) (Family: Moraceae) is one of the food plants of silkworm that subsist of over 150 species (Srivastava et al. 2006). In many countries like Turkey and Greece, *M. alba* and other mulberries are grown for fruit production that have variable pertinence in traditional medical system (Ercisli 2004). The fruits of *Morus alba* are used traditionally for the treatment of diabetes, bald head, hangover, hypertension and inflammation. Recent studies have proclaimed the anti-inflammatory activities of *Morus alba* and active compound oxyresveratrol via inhibition of leukocyte migration (Chen et al. 2013). In spite of divulged anti-inflammatory activity, no literature enumerates the mechanism of mulberry for the underlying anti-inflammatory potential. Henceforth, the present work was endeavored to enumerate possible mechanism underlying the anti-inflammatory effect for three mulberry varieties viz S-1, S-146, and S-13 developed by the central sericulture research and training institute, Mysore, India. As most of the anti-inflammatory agents are analgesic and antipyretic in nature, the work also extends its horizons to delve into the same using suitable animal models.

Materials and methods

Plant material

About 1,000 g of 4th, 5th and 6th positioned matured mulberry leaves of different varieties, namely S-13, S-146 and S-1 below apical part were collected after 90 days. The mulberry leaves were shade dried and grinded into powder. The sieved powder was stored in airtight container and kept at room temperature for further study.

Preparation of extracts

The dried mulberry leaves powder samples (100 g) were extracted with methanol for about 48 h and acidified with 0.1 % HCl. The residues were again dissolved in methanol (twice) and filtered. All filtrates were pooled together and evaporated in water bath. The yield of the S-1, S-13 and S-146 extracts was 2.24, 1.53 and 2.97 g, respectively, per 100 g of the crude material.

Animals

The wistar strain of albino rats (100–150 g) and swiss albino mice (20–40 g) was obtained from the central animal house, Sidharth Institute of Pharmacy, Dehradun, Uttrakhand. Animals were housed under standard conditions of temperature (25 ± 1 °C) with 12 h light/dark cycle and had free access to commercial pellet diet and water ad libitum. The study was approved from the institutional animal ethics committee of Siddhartha Institute of Pharmacy, Dehradun (1435/PO/a/11/CPCSEA).

Drugs and chemicals

Complete freund's adjuvant (CFA), prostaglandin E_2 (PGE_2) and leukotriene (LTB_4 methyl ester) were supplied by Sigma Aldrich, USA. Carrageenan, histamine and AA were procured from SD fine chemicals, Baroda, India. Kaempferol, scopoletin, morin, reverasterol and 7-hydroxycoumarin were procured from Sigma-Aldrich, USA. All other chemicals were of analytical grade.

In-vivo anti-inflammatory activity

Inflammatory mediator-induced paw oedema

The anti-inflammatory activity of S-13, S-146 and S-1 methanolic extracts (MME) was evaluated against carrageenan- and AA-induced inflammation in albino rats. The results demonstrated significant anti-inflammatory activity for MME (S-146) against carrageenan- and AA-induced paw oedema. Considering the same, the MME (S-146) was further evaluated against a variety of phlogistic agents/oedemogens. The respective strength of oedemogens, the volume injected and the time for determination of oedema are shown in the parenthesis; 1 % carrageenan (1 % in normal saline, 0.1 ml, 3 h); PGE_2 (10^{-8} gm/ml, 0.1 ml, 30 min); AA [0.5 % in 0.2 M carbonate buffer (pH 8.43–8.56), 0.1 ml, 30 min]; LTB_4 (1×10^{-6} gm/ml, 0.1 ml, 30 min); histamine (1×10^{-3} gm/ml, 0.1 ml, 60 min) (Kaithwas et al. 2011). The oedemogens were injected in the hind paw of rat after 60 min of administration of MME (S-146) (3 and 6 mg/kg) or standard drugs or control (distilled water, 3 ml/kg) orally to groups of fasted rats (Winter et al. 1962). The standard drugs used in different oedema models are enumerated in Table 1.

Analgesic activity (acetic acid-induced writhing)

Swiss albino mice of either sex were divided into five groups of six animals each. Group I served as control and received distilled water (3 ml/kg, po); Groups II to III received MME (S-146) (3 and 6 mg/kg, po), respectively. Groups IV-V received aspirin (100 mg/kg, po) and nalbuphine (25 mg/kg, po). After 60 min, 0.6 % v/v acetic acid solution in normal saline (10 ml/kg, ip) was administered. Immediately after the acetic acid administration, numbers of writhings or stretches (a syndrome characterized by the wave of contraction of the abdominal muscle followed by the extension of the hind limb) were counted for 15 min. Reduction in writhings as compared to control was considered as the evidence for the presence of the analgesia (Koster et al. 1959).

Antipyretic activity

The albino wistar rats were divided into four groups of six animals each. Experimental animals were acclimatized for 1 h at 28–32 °C before the experiment. Group I served as a control and received only typhoid paratyphoid A/B vaccine, at dose of 1 ml/kg (sc). Groups II to III received MME (S-146) (3 and 6 mg/kg, po), in addition to the vaccine, while group IV received a standard drug, paracetamol (100 mg/kg, po) along with the vaccine. Rectal temperature was measured at the time of administration of vaccine, followed by hourly measurement up to 4 h (Kaithwas et al. 2011).

CFA-induced arthritis

Different groups of animals ($n = 6$) received toxic control vehicle (normal saline, 3 ml/kg, po), MME (S-146) (3, 6 mg/kg, po) and standard drug (diclofenac sodium, 30 mg/kg, po), 1 day before the CFA injection, and daily treatment continued for 21 days. The left foot pad of each rat was injected subcutaneously with 0.05 ml (0.5 % w/v) of CFA (Kaithwas et al. 2012; Newbould 1963). The oedema of the left and right hind paws was evaluated at 7, 14 and 21 days post-CFA injection using micrometre screw gauge (Winter et al. 1962). The blood samples were collected on the 21st day and subjected to estimations of IL-1β (Catalog no. K0331212P), IL-6 (Catalog no. K033122P) and TNF-α (Catalog no. K0331196P) using radioimmunoassay kits (Koma Biotech, Seoul, Korea).

Table 1 Effect of MME on inflammatory mediators induced paw oedema, and COX and LOX enzymes

Phlogistic agent	Paw oedema assay (values represented as mean change in paw diameter)								COX and LOX inhibitions assay (inhibition percentage)			
	Group											
	Control (Dist. water, 3 ml/kg, po)	S-13 (3 mg/kg)	S-1 (3 mg/kg)	S-146 (3 mg/kg)	S-146 (6 mg/kg)	Ibuprofen (20 mg/kg, po)	Cyclizine (25 mg/kg, po)	Ketoconazole (14 mg/kg, po)	Compound	COX-1	COX-2	5-LOX
Carrageenan	0.77 ± 0.11	0.59 ± 0.09^{X}	0.71 ± 0.06	0.27 ± 0.02^{Z}	0.19 ± 0.02^{Z}	0.34 ± 0.02^{Z}	–	–	MME (S-146) (30 µg/ml)	78.24 ± 5.12	85.24 ± 3.45	71.32 ± 3.14
AA	0.81 ± 0.12	0.78 ± 0.04	0.71 ± 0.01	0.31 ± 0.02^{Z}	0.29 ± 0.01^{Z}	0.42 ± 0.02^{Z}	–	0.62 ± 0.06^{X}	Morin (30 µg/ml)	45.12 ± 3.13^{c}	67.12 ± 2.78	09.78 ± 0.97^{c}
Histamine	0.59 ± 0.06	0.52 ± 0.03	0.63 ± 0.02	0.53 ± 0.04	0.51 ± 0.01	–	0.32 ± 0.03^{Y}	–	Reverasterol (30 µg/ml)	19.18 ± 1.32^{c}	11.09 ± 1.11^{c}	45.23 ± 1.72^{b}
PGE_2	0.68 ± 0.12	–	–	0.41 ± 0.01^{Z}	0.31 ± 0.04^{Z}	0.29 ± 0.02^{Z}	–	–	Scopoletin (30 µg/ml)	71.12 ± 4.68^{c}	65.31 ± 6.51^{c}	11.15 ± 1.23^{c}
LTB4	0.73 ± 0.06	–	–	0.29 ± 0.01^{Z}	0.26 ± 0.02^{Z}	–	–	0.37 ± 0.03^{Z}	7-hydroxy coumarin (30 µg/ml)	67.12 ± 7.13	56.98 ± 4.19^{a}	67.52 ± 3.67
–	–	–	–	–	–	–	–	–	Aspirin (30 µg/ml)	87.98 ± 5.98^{c}	91.23 ± 3.21^{c}	23.12 ± 2.17^{c}
–	–	–	–	–	–	–	–	–	Ketoconazole (30 µg/ml)	1.54 ± 2.57^{c}	0.98 ± 0.01^{c}	76.12 ± 3.67

(Values are mean ± SEM), each group contains six animals

Statistical significance compared to control using Student-Newman-Keul's Test ($^{X}P < 0.05$, $^{Y}P < 0.01$, $^{Z}P < 0.001$)

Statistical significance compared to MME (S-146) using Student-Newman-Keul's Test ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$)

Phytochemical analysis

The MME (S-146) was subjected to phytochemicals screening through high-performance liquid chromatography (HPLC) (Hertog et al. 1992, Hakinen et al. 1998 and Kalt et al. 1999). The HPLC analysis comprises Analytical Waters HPLC system equipped with Waters 2958 Photodiode Array Detector, C18 column (250 × 4.6 mm inner diameter, 5 µm, Varian, USA), Waters 717 plus Autosampler and Waters 515 HPLC Pumps. The data were collected and analyzed on IBM computing system equipped with empower programming software. For the analysis, mobile phase A was a water solution of 5 mm ammonium acetate and mobile phase B was 100 % HPLC grade acetonitrile. The elution conditions were as follows: 90 % A, 0–1 min; 40 % A, 1–15 min; 20 % A, 15–30 min; 90 % A, 30–40 min with a flow rate 1.0 ml/min. Column and oven temperature was set at 25 °C. The 10 µl of the standards and samples were injected into the HPLC system. PDA spectra were recorded in the wavelength range from 200 to 450 nm (detection wavelength was 254 and 320 nm). The HPLC analysis revealed the presence of morin, reverasterol and scopoletin as major phytochemical followed by 7-hydroxyl coumarin (Fig. 1).

In vitro COX and LOX inhibition test

To further elucidate the possible anti-inflammatory mechanism, MME (S-146) was tested for COX 1, COX 2 and 5 LOX inhibitory activity, using a COX-(ovine) and 5 LOX inhibitor screening kit (catalog no. 760111, 760700, Cayman Chemicals, USA) according to the manufacturer's instructions. The stock solution of the MME (S-146), reverasterol, morin, scopoletin and 7-hydroxy coumarin was prepared in dimethy sulphoxide and further dilutions were made up to concentration 30 µg/ml. Percent inhibition was calculated by comparing the absorbance intensities, measured spectrophotometrically with a 96-well plate reader (BioTek Microplate Reader, ELX 800) at 590 and 490 nm for COX and LOX, respectively. Each assay was performed in triplicate. Aspirin and celecoxib were used as reference standards (Gaffney 1996; Kulmacz and Wang 1995; Jang et al. 1997).

Statistical analysis

All the data are presented as mean ± SD and analyzed by one-way ANOVA followed by Dunnet Test for the possible significance identification between the various groups. $P < 0.05$ was considered statistically significant. Statistical analysis was carried out using Graph Pad Prism 3.0 (Graph pad software, San Diego, CA, USA).

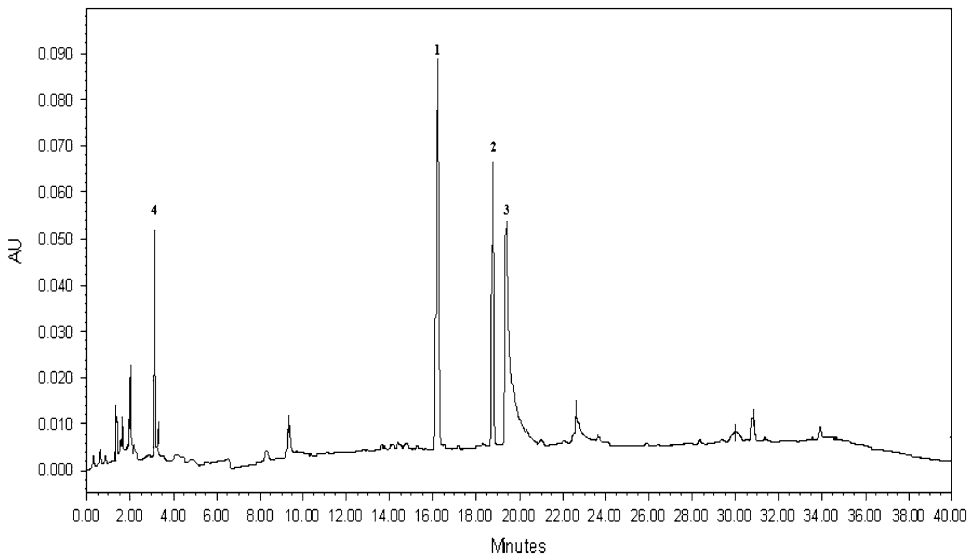

Fig. 1 HPLC chromatogram for the MME (S-146). *1* Morin; *2* Reverasterol; *3* Scopoletin; *4* 7- hydroxyl coumarin

Results and discussion

The present study establishes the anti-inflammatory, analgesic and antipyretic activity of the mulberry leaf extract credited to COX and LOX inhibition. The MME (S-1, S-13, S-146) was firstly appraised against carrageenan- and AA-induced paw oedema. Carrageenan mediates a non-specific inflammation characterized by three distinct phases of mediator release, including histamine and serotonin in the first phase, kinins in the second phase and prostaglandin in third phase, whereas AA is a precursor for PGs and LTs, generated through COX and LOX pathway (Kaithwas and Majumdar 2013; Di rosa 1971). Out of the three varieties tested, only S-146 manifested a marked 64.94 and 61.73 % inhibition of carrageenan- and AA-induced paw oedema, respectively (Table 1). The result implied the anti-inflammatory potential of S-146, which could be arbitrated through the inhibition of histamine, serotonin, kinins and/or PG's, LT's. To explore further, the MME (S-146) was further figured out against histamine, PGE_2 and LTB_4 induced paw oedema. It is noteworthy that MME (S-146) did not manifest significant competency against the histamine-induced paw oedema (13.56 %). However, 54.41 and 64.38 % inhibition of PGE_2- and LTB_4-induced paw oedema was contemplated (Table 1). The inhibition of AA-, PGE_2- and LTB_4-induced oedema by MME (S-146) suggests that the anti-inflammatory activity could be mediated through the inhibition of COX or LOX pathway or both.

As most of the anti-inflammatory agents are analgesic and anti-pyretic, the MME (S-146) was further appraised against typhi-paratyphi A/B vaccine-induced pyrexia and acetic acid-induced writhing test. The results demonstrated dose-dependent inhibition of pyrexia and writhing count (Table 2). Painful sensation in acetic acid-induced writhing is elicited by localized peripheral inflammatory mediators released from the AA from tissue phospholipids (Ahmed et al. 2006; Duarte et al. 1988). The agent reducing the number of writhing will render analgesic effect, preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte et al. 1988; Ferdous et al. 2008). It is noteworthy that the nalbuphine, a centrally acting analgesic, was not efficacious in this test, pointing towards the peripheral analgesic activity of the MME (S-146). Following acetic acid-induced test, the MME (S-146) was evaluated for possible antipyretic activity. The extract demonstrated reduction in the temperature to an appreciable extent, in which inhibition of prostaglandin synthesis could be designated as one of the reasons (Feldberg and Saxena 1975) (Table 2)

Considering the NSAID's like activity and to evaluate the long-term anti-inflammatory effects, MME (S-146) was further evaluated against chronic inflammation, which is more akin to clinical situations, i.e., CFA-induced arthritis. Shortly after the administration of CFA into hind paw, pronounced swelling appears in the hind paw, which persists for weeks (primary reaction). After a few days, the contralateral paw as well as front paw also becomes swollen and arthritic nodules appear in ear and tail (delayed systemic response). The MME (S-146) exhibited antiarthritic activity which was maintained until the experiment was terminated on day 21. Mean change in paw diameter was 4.39 ± 0.11 mm in the control group and MME (S-146) significantly reduced the paw diameter on the 21st day in a dose-dependent manner with percentage protection of 71.01 and 74.48 % (Fig. 2). The MME (S-146) significantly decreased humoral immune response probably due to its ability to inhibit acute inflammation by reducing vascular permeability and inhibiting other mediators. The secondary inflammatory responses observed from the 10th day were also inhibited by MME (S-146) as effectively as diclofenac sodium. The secondary lesions have been shown to be

Table 2 Analgesic and antipyretic effect of MME (S-146) in animal models

S. no.	Groups (dose)	Typhi paratyphi A/B vaccine induced pyrexia (rectal temperature °C)					Writhing test
		1 h	2 h	3 h	4 h	5 h	Writhing's count
1	Control (NS, 3 ml/kg, po)	35.12 ± 0.13	37.42 ± 0.12	39.21 ± 0.27	41.11 ± 0.11	43.12 ± 0.16	35.17 ± 3.56
2	MME (3 mg/kg, po)	35.11 ± 0.16	37.11 ± 0.45	38.12 ± 0.81	40.18 ± 0.21	40.43 ± 0.11	15.87 ± 5.12***
3	MME (6 mg/kg, po)	35.16 ± 0.23	37.21 ± 0.32	37.11 ± 0.51	36.56 ± 0.41	36.11 ± 0.18**	13.11 ± 4.04***
4	Aspirin (100 mg/kg, po)	35.18 ± 0.54	38.34 ± 0.28	38.37 ± 0.26	36.78 ± 0.26	36.28 ± 0.21**	3.26 ± 2.09***
5.	Nalbuphine (2 mg/kg, sc)	35.01 ± 0.38	37.45 ± 0.38	39.42 ± 0.71	41.21 ± 0.25	42.39 ± 0.29	33.12 ± 3.19

Values are mean ± SEM, each group contains six animals

Statistical significance compared to control as per Student–Newman–Keul's Test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

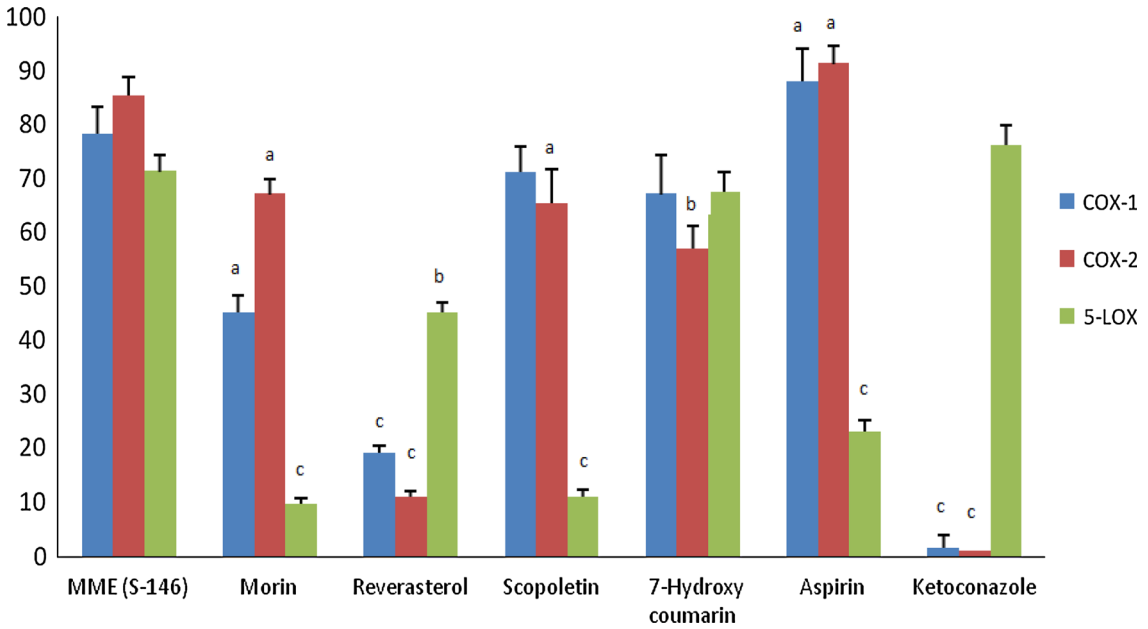

Fig. 2 In vitro cyclooxygenase and lipoxygenase inhibitory activity. Experiment was performed in triplicate. Statistical comparisons made with MME (S-146) on the basis of one way Anova followed by Student-Newman-Keul's Test ($^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$)

presumably due to a delayed hypersensitivity reaction (Singh and Majumdar 1996; Kaithwas and Majumdar, 2010), and MME (S-146) produced a marked effect on this. The increased expression of inflammatory cytokines, including TNF-α, IL-2 and IL-1β, was observed in the knee joint or serum samples from human osteoarthritis or RA patients (Kaneko et al. 2001). The TNF-α and IL-1β enhance the proliferation of fibroblasts, stimulate the production of PGE_2 (Arend and Dayer 1995), and increase in the expression of other cytokines and synthesis of collagen by synovial cells, contributing to cartilage and bone destruction (Dayer and Fenner 1992). The MME (S-146) demonstrated 80.08, 48.39 and 67.37 % inhibition of IL-1β, IL-2 and TNF-α expression in the sera of the CFA rats. Henceforth, it becomes evident that the MME (S-146) shifts the balance of the proinflammatory cytokine towards normal and, thereby, confirms its utility in chronic inflammatory conditions like RA. From the above discussion, it became evident that MME (S-146) exhibits anti-inflammatory, analgesic and antipyretic activity. The results also give the first line of evidences that the above-mentioned activities are mediated via inhibition of AA pathway, which we propose is mediated through inhibition of either COX/LOX pathways or both.

The HPLC analysis of the extract depicted the presence of resveratrol, morin, scopoletin and 7-hydroxy coumarin as the major constituents present in the MME (S-146). To confirm the exact mechanism underlying the above observed effects, the MME (S-146) and its major principles were subjected for COX and LOX inhibition assay in vitro. The in vitro evaluation of MME (S-146) against COX 1, COX 2 and 5 LOX demonstrated 78.24, 85.24 and 71.32 % inhibition, respectively (Table 1). The morin, 7-hydroxy coumarin and scopoletin also demonstrated COX inhibition, whereas reverasterol and 7-hydroxy coumarin exhibited 5 LOX inhibitory activity (Table 1).

To conclude, the anti-inflammatory, antipyretic and analgesic activity of MME (S-146) observed in the present experiment is due to dual inhibition of the AA pathway through inhibition of COX and LOX enzymes. The inhibition of COX and LOX enzymes could be imparted to the presence of resveraterol, morin, scopoletin and 7-hydroxy coumarin.

Conflict of interest Authors declare that we do not have conflicts of interest.

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