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ANTI-INFLAMMATORY ACTIVITY OF COUMARIN AND STEROIDAL FRACTIONS FROM LEAVES OF *MORINGA OLEIFERA*

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Abstract
The extracts from leaves of *Moringa oleifera* Lam. were evaluated for Anti-inflammatory activity in rats with formalin induced paw edema. A maximum inhibition (50.85%) of formalin induced paw edema was shown by methanolic leaf extract. Steroidal fraction SF (18.64%) and coumarin fraction CF (44.06%) from leaves also showed response showing to possess antiproliferative and antiarthritic activities similar to indomethacin, a cyclooxygenase inhibitor. Against formalin induced increase in lysosomal enzymes, the methanolic leaf extract ($P<0.001$), coumarin fraction CF M 1-4 and C 1-3 ($P<0.001$) and steroidal fraction SF (C 1-3) ($P<0.01$ to 0.001) from methanolic leaf extract showed significant response. Thus the anti-inflammatory activities of extract and some of the fractions may be because of the restabilization of lysosomal membrane. The coumarin fraction from the leaves may thus be further explored for characterization and quantitative pharmacological correlation.
Keywords: *Moringa oleifera*, Lysosomal Enzymes, Indomethacin.

Introduction

Moringa oleifera Lam. (family Moringaceae) is a small genus of quick-growing trees distributed in India, Arabia, Asia Minor and Africa. This tree is indigenous to northwest India. It is widely cultivated and naturalized in tropical Africa, tropical America, Sri Lanka, Mexico, Malabar, Malaysia and the Philippine Islands. Two species are recorded from India of which one *Moringa oleifera* is widely cultivated in the tropics for its edible fruits. It is also known by different name e.g *Moringa pterygosperma* Gaertn, Drum stick tree, Horse Radish tree, Shobhanjana (Sans.), Mungna, Sainjna, Shajna (Hindi), Sainjana, Soanjana (Punjabi), sigru, Moringa (Malayalam) (1).

All parts of the tree are considered medicinal and used in treatment of ascites, rheumatism, and venomous bites and as cardiac and circulatory stimulants. The root of young tree and also root bark are rubefacient and vesicant. The leaves are rich in Vit. A and C and are considered useful in scurvy and catarrhal affections; they are also used as emetic. A paste of leaves is used as an external application for wounds. Flowers are used as tonic, diuretic and cholagogue (1, 2). Seeds are considered as antipyretic acrid and bitter. Seed oil is used as anti-inflammatory (3) in rheumatism and gout. The flowers, leaves, and roots are used in folk remedies for tumors and the seed for abdominal tumours. The root decoction is used in Nicaragua for dropsy. Root juice is applied externally as rubefacient or counter-irritant. Leaves are applied as poultice to sores, rubbed on the temples for headaches, and said to have purgative properties (1). Bark, leaves and roots are acrid and pungent, and are taken to promote digestion. Oil is somewhat dangerous if taken internally, but is applied externally for skin diseases. Bark is regarded as antiscorbutic, and exudes a reddish gum with properties of tragacanth and is sometimes used for diarrhoea. Roots are bitter, act

as a tonic to the body and lungs, and are emmenagogue and expectorant.

The fatty oil from seeds of Moringa *pterigosperma* were found to contain the glycerides of palmitic acid (9.3%), stearic acid (7.4%), behenic acid (8.6%) and oleic acid (65.7%) (4). α and γ-tocopherol have been found in leaves, flowers and fresh beans from *Moringa oleifera* by High-performance liquid chromatography method (5).

The role of the aqueous extract of *Moringa oleifera* leaf (300 mg/kg body weight) havebeen studied on mean ulcer index, enterochromaffin cell (EC) count and serotonin content of experimental ulcer model using aspirin (500 mg/kg, po), cerebellar nodular lesion and applying cold stress. The results suggested that the protective effect of MO on ulceration is mediated by increased EC cell count and 5-HT levels which may act via 5-HT3 receptors on gastric tissue (6).

All though *Moringa oleifera* has been mentioned as anti-inflammatory, the detailed anti-inflammatory activity for various constituents and fractions has not been carried out. The same hasbeen taken up for investigation.

Materials and methodology

Moringa oleifera leaves and flowers were collected from local fields of Lucknow, Uttar Pradesh, India. A voucher specimen was deposited at taxonomy lab, ethnopharmacognosy division, National Botanical Research Institute (NBRI), Lucknow, India for future reference (No: NBRI/CIF/Re./08/2008/32). After qualitative and quantitative morphological, microscopical and physicochemical evaluation extraction was done with solvents of different polarity by percolation or continuous extraction and phytochemical and preliminary pharmacological profiling was done.

Methanolic extract from leaves showed significant preliminary anti-inflammatory activity by various methods and so was taken up for column chromatography and fractionation. A fixed quantity of extract was chromatographed on silica gel-G (120) column triturating with methanol as a stationery phase. After addition of the extract, proper time was allowed for partitioning of the constituents over the column. First elution was done with chloroform (50ml). The chloroform was added to the column and after allowing 20 min. for stabilization, 8 fractions (1-8) 10 ml each were collected in labeled test tubes at the rate of 15-20 drops /min. Second eluant was a mixture of chloroform and acetone (60: 40) of which 5 fractions (9-13) (10 ml each) were collected. The third eluant was mixture of chloroform and acetone (40: 60) of which 4 fractions (14-17) (10 ml each) were collected. The fourth eluant was Acetone. 4 fractions (18-21) (10 ml each) were collected. The fifth eluant was Methanol of which 5 fractions (22-26) were collected.

The fraction 1, 2, and 3 (chloroform fraction) of methanolic extract of the leaves of *Moringa oleifera*, showed single component TLC with Rf value 0.58 using mobile system Ethyl acetate : Formic acid : GAA : Water (100:11:11:27) or Toluene : Acetone : Methanol comparably with β-sitosterol. The fractions were processed and subjected to spectroscopic analysis. I.R spectroscopic peaks [peaks at 3446(s), 2947(s), 2358 (m), 1635, 1394, and 1035 cm-1] and mass spectroscopic data (m/z 93.9, 114.3, 29.0, 288.3) with 114.3 being largest fraction matched comparably with data for sterols e.g. cholesterol, showing the fraction to be steroidal in nature (confirmed also by chemical tests). The precipitated compound showed melting point 134-137˚C which matched with earlier reported (7). It was designated as steroidal fraction (SF).

Fraction M1, M2, M3 and M4 (methanolic fractions) of methanolic extract during column fractionation showed single component TLC with Rf value 0.46 using mobile phase (chloroform: GAA: Methanol: Water) and matched comparibily with scopoletin. The fractions were combined together and precipitated and subjected to spectroscopic analysis. The UV λmax values 227, 318.8 (main peak), 338.6 nm and IR peaks (peaks at 3440s, 2850m, 1647s, 1361, and 808 and 768 cm-1) and positive chemical tests for coumarins showed that the fraction may be coumarins (CF). Melting point of the precipitated compound was found to be 131-132˚C (7).

Animals:

Male albino rats weighing 100-150gm were used for study of anti-inflammatory activity. All the animals were kept under standard environmental condition. Animals were given standard diet of Hindustan Liver Limited and water ad libitum. All procedures were done as per approved CPCSEA guidelines and institutional animal ethics committee (approval no. BBDNITM/IAEC/clear/12/2008).

Formalin induced paw edema in rats:

Acute inflammation was induced by subaponeurotic injection of 0.1 ml of 2% formalin one hour after oral administration of extract, fraction, 10 mg/kg indomethacin, or vehicle (solution of 2.5% DMSO and 2.5% tween 20). The volume of paw was determined one, two, and four hours following the injection of formalin (8). The average volume (Vo) of the right hind paw of each rat was calculated from 3 readings that did not deviate more than 3%. After injection of the phlogistic agent, readings (Vt) were obtained for each rat at 30, 60, 120, 180, 240, 300 and 360 min, with the aid of a Ugo-Basil Plethysmometer. The edema was expressed as an increase in the volume

of paw. And the percentage of inhibition for each rat and each group was obtained as follows:

Percentage of inhibition = (Vt - Vo) control - (Vt - Vo) treated x 100

(Vt-Vo) control

Lysosomal enzymes (cathepsin) estimation:

The activity of lysosomal enzymes was investigated in blood serum. The blood withdrawn was centrifuged for 10 minutes at 2000 r.p.m. and separated plasma was taken for estimation of cathepsin which corresponds to lysosomal enzyme activity. For this, 0.9 ml of heamoglobin solution was added to 0.5 ml of plasma from various groups of animal and incubated at 37^0C for 2 hrs after then 1 ml of 10% Tri chloro-acetic acid was added and centrifuged at 2000 r.p.m for 10 minutes. Supernatant liquid was separated and taken 1.5 ml of it then 1 ml of 5% NaOH, 4.5 ml of 1% alkaline copper sulphate and 0.5 ml of Follin's reagent was added. A blank solution containing heamoglobin solution, alkaline copper sulphate, tri-chloro acetic acid and Follin's reagent was also prepared in another test tube and incubated same way. After 20 minutes the absorbance was measured at 620 nm for all (9).

In the reaction the lysosomal enzymes, particularly cathepsin present in the blood of various animal groups, acts on heamoglobin and results into free tyrosine whose absorbance is measured at 620 nm after developing with Follin's reagent. For animal groups in which tyrosine absorbance readings are higher indicate higher level of cathepsin indicating higher level of lysosomal enzyme activity.

Animals treated with reference or therapeutically active fractions should show less tyrosine readings and lysosomal enzyme activity.

Lysosomal inhibiting activity was expressed as percentage inhibition and estimated by following formula reported in the Reference.

% Inhibition of lysosomal inzymes = A (disease control) - A620x 100 / A (disease control)

Statistical analysis:

All data were analyzed statistically and subjected to one way ANOVA and Newmans Keuls test to determine statistical significance and $p<0.05$ was considered as significant. All Groups were compared with control group.

Results and discussion:

Formalin induced paw edema in rats:

The methanolic extract of *Moringa oleifera* leaf showed maximum inhibition (36.84%) at the 1st hour followed coumarin fraction CF (28.94%) while other extract and fraction showed very less or no inhibition at 1st hour. At the 6th hour methanolic leaf extract showed 43.87% followed by 48.97% inhibition was shown by Coumarin fraction CF while no significant inhibition was shown by other extract and fractions. At the 24th hour a maximum inhibition (50.85%) was shown by methanolic leaf extract followed by steroidal fraction SF (18.64%) and coumarin fraction CF (44.06%) (Table: 1a & 1b) (Fig: 1).

Acute inflammation induced by Formalin results from cell damage, which provokes the production of endogenous mediators, such as, histamine, serotonin, prostaglandins, and bradykinin (10).

It is well known that inhibition of edema induced by Formalin in rats is one of the most suitable test procedures to screen antiarthritic and antiinflammatory agents, as it closely resembles human arthritis (11). Arthritis induced by Formalin is a model used for the evaluation of an agent with probable antiproliferative activity (12). As some of the extract and fractions significantly inhibited this model of inflammation, they can be thought to possess antiproliferative and antiarthritic activities similar to indomethacin, a cyclooxygenase inhibitor.

Lysosomal enzymes inhibition:

The methanolic extract of leaves of *Moringa oleifera* showed the significant activities ($P<0.001$). The fractions CF and SF from methanolic extract of leaves showed activities against lysosomal enzyme level by ($P<0.001$) and ($P<0.05$) respectively. Thus the anti-inflammatory activities of extract and some of the fractions may be because of the restabilization of lysosomal membrane. Lysosomal enzymes if allowed to circulate within plasma causes degradation in the cell wall and inner structure. Substances which inhibit release of lysosomal enzymes and stabilize lysosomal membranes, prevent wide spread damage to cellular and tissue structures which normally accompanies acute or chronic inflammatory conditions which show presence of large number of WBCs and activity of lysosomal enzymes (Table: 2) (Fig: 2)

Table: 1a. Effect of extract and fractions on Formalin induced paw edema in rats

S no.	Test group	Dose (mg/kg body wt)	Inflammation (Δ in ml)		
			1hr	6hr	24hr
1	Group I	0	0.38±0.05	0.98±0.1	0.59±0.02
2	Group II	10	0.21±0.02	0.48±0.03	0.26±0.01***
3	Group III	250	0.24±0.01	0.55±0.03	0.29±0.03***
4	Group IV	150	0.41±0.01	0.91±0.09	0.48±0.04*
5	Group V	150	0.27±0.02	0.50±0.04	0.33±0.01***

Group I= Disease control, Group II= diseased animals treated with reference (Indomethacin), , Group III= diseased animals treated with methanol (leaf extract), Group IV= diseased animals treated with steroidal fraction SF from leaves, Group V= diseased animals treated with coumarin fraction CF from leaves.
N= 6 Animals in each group
Values are expressed as Mean±SEM
*P<0.05; **P<0.01; ***P<0.001 when compared with disease control

Table: 1b. Percentage inhibition in inflammation induced by Formalin

S no.	Test group	Dose (mg/kg body wt)	Inflammation (Δ in ml)		
			1hr	6hr	24hr
1	Group I	0	-	-	-
2	Group II	10	44.74	51.02	55.93
3	Group III	250	36.84	43.87	50.85
4	Group IV	150	-7.8	7.14	18.64
5	Group V	150	28.94	48.97	44.06

Group I= Disease control, Group II= diseased animals treated with reference (Indomethacin), Group III= diseased animals treated with methanol (leaf extract), Group IV= diseased animals treated with steroidal fraction SF from leaves, Group V= diseased animals treated with coumarin fraction CF from leaves.
N= 6 Animals in each group
Values are expressed as Mean±SEM
*P<0.05; **P<0.01; ***P<0.001 when compared with disease control

Table: 2 Lysosomal enzymes inhibition

S. No.	Test material	Absorbance (λmax 620nm)	% inhibition
1	Group I	0.096	-
2	Group II	0.778	-
3	Group III	0.170	78.14***
4	Group IV	0.577	25.83
5	Group V	0.193	75.19***

Group I= Disease control, Group II= diseased animals treated with reference (Indomethacin), Group III= diseased animals treated with methanol (leaf extract), Group IV= diseased animals treated with steroidal fraction SF from leaves, Group V= diseased animals treated with coumarin fraction CF from leaves.
N= 6 Animals in each group
Values are expressed as Mean±SEM
*P<0.05; **P<0.01; ***P<0.001 when compared with disease control

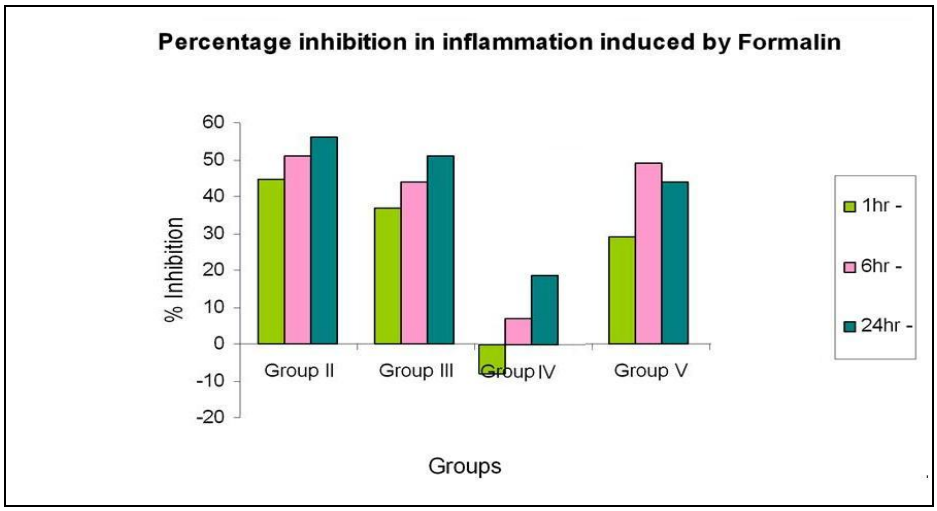

Fig: 1. Percentage inhibition in inflammation induced by Formalin

Group I= Disease control, Group II= diseased animals treated with reference (Indomethacin), Group III= diseased animals treated with methanol (leaf extract), Group IV= diseased animals treated with steroidal fraction SF from leaves, Group V= diseased animals treated with coumarin fraction CF from leaves.
N= 6 Animals in each group
Values are expressed as Mean±SEM
*P<0.05; **P<0.01; ***P<0.001 when compared with disease control

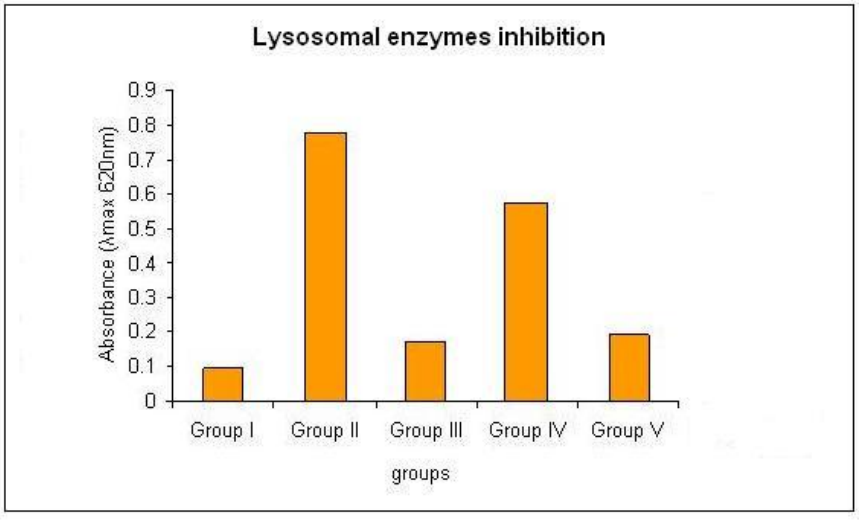

Fig: 2 Lysosomal enzyme inhibition by extracts/fractions
Group I= Disease control, Group II= diseased animals treated with reference (Indomethacin), Group III= diseased animals treated with methanol (leaf extract), Group IV= diseased animals treated with steroidal fraction SF from leaves, Group V= diseased animals treated with coumarin fraction CF from leaves.
N= 6 Animals in each group
Values are expressed as Mean±SEM
*P<0.05; **P<0.01; ***P<0.001 when compared with disease control

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