

Anti-Inflammatory and Analgesic Activity of Stem Bark of *Moringa Oleifera*.

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Summary

The present study evaluates the Anti-inflammatory and analgesic activities of various extracts of stem bark of *Moringa oleifera* using various experimental models.

The analgesic activity of stem bark of *Moringa oleifera* carried out using acetic acid-induced writhing in mice and tail flick test in rats. The anti-inflammatory activity was evaluated using carrageenan-induced rat paw edema and cotton pellet-granuloma formation in rats. The effects of the administration of reference standard (diclofenac) were also evaluated. Two different extracts (Petroleum ether and Methanolic) of *Moringa oleifera* at the dose level of 100,200 and 400 mg/kg, p.o. were tested. Treatment with Methanol extract (100, 200, and 400 mg/kg, p.o.) showed significant ($p < 0.01$) inhibition of carrageenan induced rat paw edema. Maximum inhibition was observed at 400 mg/kg dose as compared to the control, cotton pellet granuloma formation and acetic acid-induced writhing; however, pet ether and methanolic extracts (400 mg/kg, p.o.) were found to be more effective in increasing latency period in tail flick method. The results obtained indicate that *Moringa oleifera* has analgesic and antiinflammatory activities that supports the folk medicinal use of the plant.

Keywords: Acetic acid, carrageenan, granuloma formation, *Moringa oleifera*, tail flick

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Introduction

The use of herbal extracts and nutritional supplements either as alternative or complimentary medicine to the conventional chemotherapy for treatment of inflammatory diseases is well documented in Ayurveda, which is an alternative medicinal system that has been practiced primarily in the Indian subcontinent for 5000 years [1]. Inflammation is a normal response to any noxious stimulus that threatens the host and may vary from a localized response to a generalized one. The inflammatory process protects our body from diseases by releasing cells and mediators that combat foreign substances and prevent infection.[2]. In 1971 [3] the role of prostaglandins (PGs) in the inflammatory process was observed. PGs are synthesized from arachidonic acid which is released by the action of phospholipase A2 on damaged tissues. Arachidonic acid is converted by cyclooxygenase (COX) enzymes to cyclic PGG2 and PGH2 which cause vasoconstriction and pain. They, in turn, are converted to PGE2 and PGF2 α which cause vasodilatation and pain.[4]. Two isoenzymes of cyclooxygenase were postulated, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX- 2). COX-1 is a constitutive enzyme and is responsible for the production of the basic level of PGs. Inhibition of this enzyme by all older, non-selective non-steroidal antiinflammatory drugs is primarily responsible for a number of their side effects .(owever, the existence of COX-2 enzyme was confirmed.[5] It is an inducible enzyme which is induced in response to the release of several proinflammatory mediators, leading to the inflammatory non-selective NSAIDs response and pain.[6] *Moringa oleifera* (Moringaceae) is a bush of African savannah, commonly known as Drum stick used in folk Medicine for the treatment of rheumatic and articular pain. Its seeds shown analgesic activity[7], Antipyretic activity [8]. Its Leaves shown Wound healing activity [8], Analgesic activity[9], hepatoprotective [10,11] Antiulcer activity[12], Hypotensive [13]Diuretic activity [14].Roots have shown Antifertility activity [15]. Various phytoconstituents have been isolated from seeds as Alkaloid (Moringines) [16] from flowers Quercetin , kaempferol [10]from leaves Thiocarbamate [17]. Most clinically important medicines belong to steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation-related diseases. Though these have potent activity, long-term administration is required for treatment of chronic disease. Furthermore, these drugs have various and severe adverse effects. Therefore, naturally originated agents with very little side-effects are required to substitute chemical therapeutics.

Materials and Methods

Plant Material

Stem bark of *Moringa oleifera* Fam Moringaceae was collected from local region of Nashik, India in October 2008. The plant material was identified and authenticated by Dr. P. G. Diwakar Botanical survey of India, Pune (Ref no. BSI/WC/Tech/2009 /370).

Preparation of Extract

The plant material were cleaned, dried under shade and pulverized by using grinder. 500g of the powder of plant was successively extracted with Petroleum ether, chloroform, and methanol in order of their increasing polarity using Soxhlet apparatus. The yield of extracts obtained as Petroleum ether as 0.89 %, Chloroform as 3.6 % , Methanol as 16.63 %. From the

Preliminary Phytochemical study revealed that presence of sterols, glycosides, Alkaloids, Triterpenoids, Flavonoids and tannins in the extracts.

EXPERIMENTAL ANIMALS :

Albino rats of Wistar strain (150-200 g) and Swiss albino mice (25-30 g) of either sex were used in the entire study and were procured from Haffkine Institute, Mumbai. They were housed in standard polypropylene cages and kept under controlled room temperature (24 ± 2 °C; relative humidity 60%-70%) in a 12 h light-dark cycle. The animals were fed with standard laboratory diet of Pranav agro pvt .Ltd. and water *ad libitum*. Food was withdrawn 12 h before and during the experimental hours. The experimental protocol was approved by Institutional Animal Ethical Committee.

ANTI-INFLAMMATORY ACTIVITY:

Carrageenan induced rat paw edema

The anti-inflammatory activity using carrageenan induced hind paw edema was carried out as described by Winter *et al.*[18] Anti-inflammatory activity was evaluated using the Carrageenan induced rat paw edema according to the technique of Winter *et al.* . After 16h of fasting, the rats of 150-200 gm were divided into eight groups of six each. Group I served as control group and received distilled water (DW), orally. Group II received Diclofenac as standard at a dose of 5 mg/Kg. Group III, IV and V animals received Pet Ether extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg ; Group VI, VII, VIII received methanol extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg; After 1 h, 0.1 ml of 1% w/v Carrageenan suspension was injected subcutaneously in to the plantar surface of the right hind paw. The paw volume was measured using a Digital plethysmometer PLM-01 (Orchid Scientifics, India) immediately and 3 h after carrageenan injection. [19]

Cotton pellet induced granuloma formation in rats

After 16h of fasting, the rats of 150-200 gm were divided into eight groups of six each. Group I served as control group and received distilled water (DW), orally. Group II received Diclofenac as standard at a dose of 5 mg/Kg. Group III, IV and V animals received Pet Ether extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg ; Group VI, VII, VIII received methanol extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg; orally for consecutive six days [20,21]. The cotton pellet weighing 50 ± 1 mg was sterilized in an autoclave (Lab hosp, Mumbai, India) handled with sterile instrument. The pellet was inserted in each animal on the back. Control group received vehicle. The animals were sacrificed on seventh day and cotton pellet along with granuloma mass was collected, it was weighted and dried at 60°C. Results of the assay were calculated as % inhibition of dry weight of granuloma formation by using the formula: $100(A-B)/A$, where, A= gain in dry weight of control pellet (mg), B= gain in dry weight of drug treated (mg).

ANALGESIC ACTIVITY:

Tail flick latency period in rats

Male rats of 150-200 g. rats were divided into eight groups containing six animals in each group. Group I served as control group and received distilled water (DW), orally. Group II

received Diclofenac as standard at a dose of 5 mg/Kg. Group III, IV and V animals received Pet Ether extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg ; Group VI, VII, VIII received methanol extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg orally; A tail flick response was evoked by placing each rat tail over the wire heated electrically, using Analgesiometer (Space Scientific, Nashik, India). The intensity of heat was adjusted so that baseline tail flick latency averaged 3-4 sec in all animals. Cut off time was 15 sec in order to avoid injury to tail. The extracts and reference standard Diclofenac were administered orally in their respective doses 1 hr prior to the test [22].

Acetic acid-induced writhing in mice

Male mice of 20-40 g. were divided into eight groups containing six animals in each group. Group I served as control group and received distilled water (DW), orally. Group II received Diclofenac as standard at a dose of 5 mg/Kg. Group III, IV and V animals received Pet Ether extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg ; Group VI, VII, VIII received methanol extract of *Moringa oleifera* at a dose of 100, 200 and 400 mg/kg orally. The writhing syndrome was elicited by intraperitoneal injection of acetic acid (0.1ml of 0.6% solution) and numbers of writhes displayed from 5 to 20 min were recorded [22]. The extracts and reference standard Diclofenac were administered orally in their respective doses 30 min prior to the test.

STATISTICAL ANALYSIS

Results of all the above estimations have been indicated in terms of mean \pm SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) with Dunnett's test multiple comparisons test using GraphPad InStat version 5.00, GraphPad Software, CA, USA. The level of significance was set at $P < 0.05$.

Results

Effect *Moringa oleifera* extracts on rat paw edema induced by carrageenan

In the present study two different extracts were evaluated for anti-inflammatory activity using carrageenan-induced rat paw edema and the data was compared with that of control.

[Table-1]

Vehicle treated rats and Diclofenac (5 mg/kg p.o.) treated rats showed increase of paw volume as 2.4 ± 0.01 ml and 1.45 ± 0.002 ml respectively after 3h. Treatment with Petroleum Ether extract of *Moringa oleifera* (100, 200, and 400 mg/kg, p.o.) showed a significant inhibition of paw volume after 1h, 2 h and 3 h. ($p < 0.01$). Treatment with Methanol extract (100, 200, and 400 mg/kg, p.o.) showed significant ($p < 0.01$) inhibition of carrageenan induced rat paw edema. Maximum inhibition was observed at 400 mg/kg dose as compared to the control. It was observed that the methanolic extracts of *Moringa oleifera* (400 mg/kg, p.o.) exhibits maximum anti inflammatory activity against carrageenan induced hind paw edema. The inhibition obtained with *Moringa oleifera* and was 61.33% .

Table-1: Effect of various extracts of *Moringa oleifera* on Carrageenan induced rat paw edema

Group	Treatment (mg/kg)	Mean increase in paw volume (ml)				% Decrease in paw volume at 3 h
		0 h	1 h	2 h	3 h	
I	Control	0.90± 0.024*	1.49± 0.014*	1.90±0.013*	2.40±0.014*	-
II	Diclofenac (05)	0.89 ± 0.008*	1.01±0.01*	1.29±0.01*	1.45±0.028**	62.66
III	P.E(100)	0.87 ± 0.033**	1.49± 0.021*	1.77 ± 0.028**	1.89± 0.022*	32.09
IV	PE (200)	0.88 ±0.026*	1.33±0.032**	1.55±0.038**	1.75±0.031**	42.05
V	PE (400)	0.89 ± 0.029**	1.39± 0.019*	1.49± 0.017*	1.70± 0.021*	46.12
VI	ME(100)	0.90± 0.038**	1.56± 0.039**	1.79± 0.022*	1.80± 0.027*	40.08
X	ME(200)	0.91± 0.037**	1.28± 0.045**	1.39± 0.018*	1.65± 0.037**	50.66
XI	ME(400)	0.87± 0.031**	1.21± 0.023*	1.35± 0.029**	1.61± 0.019*	61.33

Data were analyzed using ANOVA and expressed as Mean ± SEM ($N=5$) followed by Dunnett's test and differences between means were regarded significant at * ($P < 0.05$), ** ($P < 0.01$)

Effect of *Moringa oleifera* extracts on cotton pellet granuloma formation in rats

The Effect of *Moringa oleifera* extracts on cotton pellet granuloma formation is shown in Table 2. . The extracts significantly inhibited cotton pellet granuloma. The percent inhibition for diclofenac Standard was found to be 44%. The percent inhibition for Petroleum ether extract was 19% ,28% , 32 % at doses of 100,200, and 400 mg/kg, respectively. The percent inhibition for Methanol extract was 17% ,29.6% , 36.8 % at doses of 100,200 and 400 mg/kg, respectively.

Table 2: Effect of various extracts of *Moringa oleifera* in cotton pellet induced granuloma formation

Treatment (mg/kg)	Average weight of cotton pellet	Average weight of cotton pellet with granuloma	%Inhibition
Control	50 ± 0.01	125.10 ± 5.91**	
Diclofenac (05)	50 ± 0.01	70.52 ± 3.21*	44.13
Pet. Ether (100)	50 ± 0.01	101.38 ± 6.18**	19.07
Pet. Ether (200)	50 ± 0.01	90.23 ± 5.41*	28.11
Pet. Ether (400)	50 ± 0.01	85.16 ± 2.16*	32.19
Chloroform (100)	50 ± 0.01	103.58 ± 5.71**	17.25
Chloroform (200)	50 ± 0.01	88.08 ± 6.75**	29.68
Chloroform (400)	50 ± 0.01	79.67 ± 3.69*	36.87

Data were analyzed using ANOVA and expressed as Mean ± SEM ($N = 5$) followed by Dunnett's test and differences between means were regarded significant at * ($P < 0.05$), ** ($P < 0.01$).

Effect of *Moringa oleifera* extracts on tail flick latency period

Treatment of methanolic extract of *Moringa oleifera* 100, 200 and 400 mg/kg, p.o. significantly inhibited nociception in rats by 18 %, 22% and 25% respectively. [table- 3]

Table 3: Effect of various extracts of *Moringa oleifera* in tail flick latency period

Treatment (mg/kg)	Tail flick latency (in sec)	% analgesia
Control	4.21 ± 0.34	
Diclofenac (05)	8.36 ± 0.88**	39.38
Pet. Ether (100)	6.90 ± 0.28*	24.93
Pet. Ether (200)	6.05 ± 0.36**	17.05
Pet. Ether (400)	5.61 ± 0.39**	12.97
Chloroform (100)	6.08 ± 0.31*	17.05
Chloroform (200)	5.36 ± 0.27*	10.65
Chloroform (400)	4.97 ± 0.25*	7.04

Data were analyzed using ANOVA and expressed as Mean ± SEM ($N = 5$) followed by Dunnett's test and differences between means were regarded significant at * ($P < 0.05$), ** ($P < 0.01$)

Effect of *Moringa oleifera* extracts in acetic acid induced writhing in mice

The effect of different extracts of *Moringa oleifera* against acetic acid induced writhing in mice. It was observed that mice treated with Petroleum ether extract of *Moringa oleifera* was shown protection against 10% ,21% , 45 % at doses of 100,200 and 400 mg/kg, respectively, shows significant ($P < 0.01$) protection compared to control group, however methanol extract of *Caesalpinia pulcherrima* was shown protection against 25% ,32% , 52 % at doses of 100,200 and 400 mg/kg, respectively, was found to be more significant ($P < 0.01$) in protecting acetic acid induced writhing compared to control group. Diclofenac shown 58.18% protection against acetic acid induced writhing in mice.

Discussion

Moringa oleifera (Moringaceae) is a bush of African savannah, commonly known as Drum stick used in folk Medicine for the treatment of rheumatic and articular pain. Its seeds shown analgesic activity [7], Antipyretic activity [8]. Analgesic and anti-inflammatory effects of flavonoids, steroids and tannins have been reported [23] hence the analgesic and anti-inflammatory effects produced by these extracts may be attributed due to the flavonoids and steroids. However, its pharmacological actions and mechanisms have not been precisely documented in spite of its increasing usage recently. Present work reported the potential effects of the stem bark of *Moringa oleifera*, as an anti-inflammatory and analgesic agent using both *in vivo* and *in vitro* models. Carrageenan-induced paw edema and cotton pellet granuloma formation in rats reflect the edematous stages during acute and chronic inflammation. [24,25] In the present study, two different extracts of stem bark of *Moringa oleifera* were tested. Carrageenan induced rat paw edema has been a popular inflammatory model to investigate nonsteroidal anti-inflammatory effect of compounds [26] Serotonin, histamine, bradykinin, and prostaglandin have been identified as a mediators for carrageenan induced rat paw edema. [27]

Petroleum ether and methanolic extracts were found to possess a prominent anti-inflammatory activity, showing inhibition to the paw edema induced by carrageenan during the three time points from 1 to 3 h. In cotton pellet granuloma model Petroleum ether and methanolic extracts showed significant inhibition. The effectiveness of these extracts at 1 and 3 h in carrageenan induced paw edema indicates their antagonist effect at Serotonin, histamine, bradykinin and prostaglandin. Because the release of serotonin and histamine occurs 1 h after carrageenan whereas bradykinin and prostaglandin are released 2 and 3 h, respectively, after carrageenan injection. [28] The cotton pellet granuloma is a model of chronic inflammation, and dry weight has been shown to correlate with the amount of granulomatous tissue formed. [29] In the present study animals treated with Petroleum ether and methanolic extracts showed significant inhibition of granuloma formation. Diclofenac was found to be more effective in preventing granuloma formation compared to extracts respectively. Since inflammation is also associated with pain, majority of anti-inflammatory drug possess analgesic activity. The peripheral analgesic effect of drugs may be mediated through inhibition of cyclo-oxygenases and/or lipoxygenases (and other inflammatory mediators), while the central analgesic action may be mediated through inhibition of central pain receptors. This hypothesis is in line with previous reports of Eddy *et al.* and Williamson *et al.* [30,31] who have postulated that acetic acid-induced writhing and tail flick methods are useful techniques for evaluation of peripherally and centrally acting analgesic drugs, respectively. Present study also showed the effects of *Moringa oleifera* extracts on acetic acid-induced writhing and tail flick latency test. Treatment of *Moringa oleifera* extracts (400 mg/kg, p.o.) significantly inhibited nociception. Whereas petroleum ether and

methanolic extract (400 mg/kg, p.o.) inhibited pain perception respectively in tail flick latency test and acetic acid-induced writhing. These results indicated extracts might produce the analgesic effect peripherally as well as centrally.

Flavonoids isolated from some medicinal plants have been proven to possess antinociceptive and/or anti-inflammatory effects. [32] It has been shown by Meli *et al* and Dicarlo *et al*. [33,34] that flavonoids also inhibit gastric motility in a dose-dependent manner. It is therefore possible that the inhibitory effects on anti-nociceptive and anti-inflammatory effects observed in these extracts may be attributed in part to its flavonoid content. Flavonoids also inhibit the phosphodiesterases involved in cell activation. [33] Much of this effect is upon the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Flavonoids inhibit biosynthesis of prostaglandins, which are involved in various immunologic responses and are the end products of the cyclooxygenase and lipoxygenase pathways. [35] Protein Kinases are another class of regulatory enzymes affected by flavonoids. Inhibition of these enzymes provides the mechanism by which flavonoids inhibit inflammatory processes. [36,37] Flavonoids [36] potently inhibit prostaglandins, a group of powerful pro-inflammatory signaling molecules. Studies have shown that this effect is due to flavonoid inhibition of key enzymes involved in prostaglandin biosynthesis (i.e., lipoxygenase, phospholipase, and cyclooxygenase). Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of this effect is upon the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Protein kinases are another class of regulatory enzymes affected by flavonoids. Inhibition of these key enzymes provides the mechanism by which flavonoids inhibit inflammatory processes [36]. Analgesic and anti-inflammatory effects have already been observed in flavonoids as well as in tannins. [38,39].

Conclusion

From the present study, it was concluded that extracts of stem bark of *Moringa oleifera* is capable of inhibiting inflammatory reactions as well as pain. The results provided experimental evidence for its traditional use in treating various diseases associated with inflammation and pain.

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