

Formulation and Evaluation of Anti-Inflammatory Cream by Using *Moringa oleifera* Seed Oil

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ABSTRACT

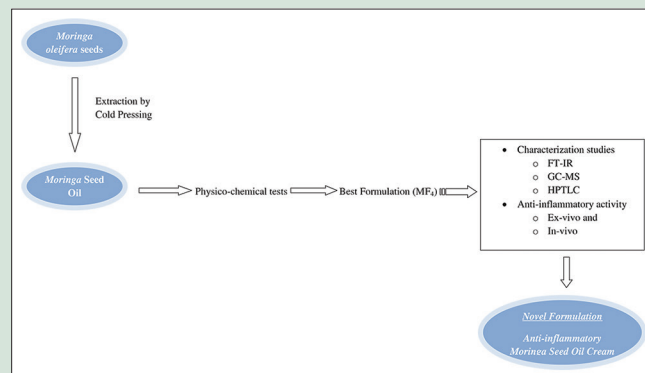
Background: Natural oils have a variety of pharmaceutical applications. They can be used in the preparation of a formulation which is beneficial as additives and also pharmacological agents. One of such medicinally important plants is *Moringa oleifera*. **Objective:** The current investigation mainly focuses on the development of anti-inflammatory cream using *Moringa* seed oil and its pharmacological evaluation. **Materials and Methods:** The oil was extracted from *Moringa* seeds using cold pressing technique and then subjected to phytochemical screening which revealed the presence of alkaloids, glycosides, tannins, and flavonoids. Various creams were prepared with alkali saponification of free fatty acids present in the oil to get o/w type of emulsion type cream. Various physicochemical tests such as the determination of viscosity, pH, irritancy, dye test, and accelerated stability studies were performed for the prepared creams. **Results:** Of all the creams, the formulation MF₄ prepared with 500 mg potassium hydroxide was suitable for all acceptable characteristics of o/w type emulsion type of cream. The *in vitro* diffusion studies were carried out using Franz diffusion cell. The extracted oil was also subjected to various characterization studies such as Fourier transform infrared spectroscopy, gas chromatography-mass spectrometry, and high-performance thin-layer chromatography. The *ex vivo* anti-inflammatory activity was carried out using heat-induced hemolysis and protein denaturation techniques. Whereas *in vivo* anti-inflammatory activity was performed on male Albino rats using paw edema technique. A significant 70% reduction in paw edema was observed. **Conclusion:** Thus, the current research reveals the novel formulation with traditional *Moringa* oil having anti-inflammatory potency.

Key words: Anti-inflammatory, behenic acid, creams, *Moringa oleifera* oil, paw edema

SUMMARY

Oil was extracted from *Moringa oleifera* seeds by cold press technique and an anti-inflammatory cream formulation was made using alkali

saponification method. The prepared cream was evaluated for *ex vivo* and *in vivo* anti-inflammatory activities which revealed the anti-inflammatory potency of *Moringa* seed oil.



Abbreviations Used: FT-IR: Fourier Transform Infra Red; GC-MS: Gas Chromatography–Mass Spectroscopy; HPTLC: High Performance Thin Layer Chromatography; MF4 - *Moringa oleifera* formulated cream.

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INTRODUCTION

Medicinal plants are believed to have therapeutically important as they are rich in various phytochemical constituents which treat many diseases.^[1] Many traditional folklore studies revealed the potential benefit of plants.^[2] Inflammation is a general mechanism in which the body reacts to any injury, infection, or irritation which can be identified by some key features such as redness, warmth, and swelling at the site. It is considered to have different mechanisms for each type of pathogen.^[3] Drugs presently in use for the management of inflammation are associated with well-known side and toxic effects.^[4] Purified natural compounds from plants can serve as a template for the synthesis of new generation anti-inflammatory drugs with low toxicity and higher therapeutic value.^[5] Topically applied plant preparations in the form of cream or liniment are made available.^[6]

Moringa oleifera belonging to the family Moringaceae is a tree distributed throughout India. It is commonly called as drumstick tree which is widely used in culinary and herbal medicine preparations. Leaves applied as a poultice to sores, rubbed on the temples for headaches, and said to have purgative properties. Bark, leaves, and roots are acrid

and pungent and are taken to promote digestion.^[7] Due to its extensive medicinal properties, it is extensively used in Ayurvedic medicine. *Moringa* oil which is extracted from seeds of *M. oleifera* is also called as Ben oil, as it has high amounts of Behenic acid. It is great for topical use on the skin and the hair. *Moringa* oil has many therapeutic benefits such as antioxidant, anti-inflammatory, hepatoprotective, and exfoliant. The present study was aimed to formulate the *M. oleifera* oil-based cream and to evaluate its anti-inflammatory effect.

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MATERIALS AND METHODS

Ibuprofen was gift sample from M/s. Life Line Formulations, Vijayawada. Carrageenan was procured from Ozone International, Mumbai. All other substances used were of analytical grade and commercially procured.

Plant material

The fruits of *M. oleifera* were collected from the farmers of Chandrajupalem, Guntur district and were identified and authenticated by Dr. P. Ammani, Acharya Nagarjuna University, Guntur and a voucher specimen is preserved in the Department of Pharmacology, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur, India. The collected fruits were sun-dried. After the moisture in it was removed, the seeds were separated and dried under ambient conditions. The external coat present on the seed was removed manually. The seeds were then crushed into powder for further use.

Extraction procedure

The dried seeds of *M. oleifera* were crushed to obtain a homogeneous powder. The powder was steamed using a pressure cooker. Subsequently, the steamed powder was mechanically pressed using the cold press to extract the edible oil. The obtained oil was filtered and stored in an amber colored bottle with an airtight lid under room temperature for further use.

Physicochemical screening

The physicochemical screening of *Moringa* seed oil was done by free fatty acid (FFA) test, acid value and saponification value.

Chemical analysis of free fatty acid in *Moringa* seed oil

FFA is defined as the number of milligrams of potassium hydroxide required to neutralize the FFAs in 1 g of the oil sample. FFA content of the oil sample was determined according to the standard protocol of the International Union of Pure and Applied Chemistry. The acid value is a measure of the extent to which the triglycerides in the oil have been decomposed. A total of 2 g oil sample was weighed; 30 ml of methanol was added and heated. A drop of phenolphthalein indicator was added to the mixture and titrated against 0.1N potassium hydroxide until the appearance of a persistent pink color.

Determination of acid value

To 10 g of oil, 50 ml mixture of an equal volume of alcohol and solvent ether was added and subjected to reflux. To this 1 ml of phenolphthalein added and titrated with 0.1N sodium hydroxide, until faint pink color appears.

Determination of Saponification value

Saponification value was calculated according to the standard IUPAC procedure. 2.5 g oil was weighed; mixed with 25 ml (0.5N) Ethanolic potassium hydroxide and refluxed for about an hour. After refluxing, the solution is titrated against hydrochloric acid using phenolphthalein as indicator. The disappearance of pink color is taken as end point. Blank is titrated separately under similar conditions.^[8]

Preparation of o/w type cream formulations

These o/w emulsion based preparations contain aqueous phase and an oil phase. The Oil phase (A) containing suitable quantities of *Moringa* seed oil and Oleic acid at 3: 1 ratio were transferred in to a china dish and heated at 60°C–70°C on a water bath. The aqueous phase (B) having suitable quantities of sodium hydroxide or potassium hydroxide or

aluminum hydroxide or liquid ammonia with sodium benzoate were dissolved in distilled water and heated at 60°C–70°C on a water bath. When both aqueous and oily phases reach the same temperature, the oil phase was poured into a mortar and triturated continuously by adding alkali until the smooth cream is obtained. The obtained cream was packed safely and stored.

Evaluation of creams

The formulated creams were subjected to evaluation of various parameters as per the standard procedures.^[9,10]

pH

The pH meter was calibrated using standard buffer solution. About 0.5 g of the cream was weighed and dissolved in 50 ml of distilled water and its pH was measured.

Viscosity

Viscosity of the formulation was determined using Brookfield Viscometer at 100 rpm, using spindle no 7.

Dye test

The scarlet red dye was mixed with the cream. A drop of cream was placed on a microscopic slide and covered with a coverslip. This was examined under microscope. The dispersed globules appear colorless in the red ground indicating the o/w type formulation.

Homogeneity

The formulations were tested for homogeneity by visual appearance and by touch.

Appearance

The appearance of the cream was judged by its color, pearlescence, roughness, and then graded.

After feel

Emolliency, slipperiness and amount of residue left after the application of fixed amount of cream was checked.

Type of smear

After application of cream, the type of film or smear formed on the skin was checked.

Ease of removal

The ease of removal of the cream applied was examined by washing the applied part with tap water.

Irritancy test

The cream was applied to the specified area and time was noted. Irritancy, erythema, and edema, were checked if any for regular intervals up to 24 h and reported.

Accelerated stability analysis

Accelerated stability testing for two most stable formulations, MF4 and MF5 was conducted for at room temperature and observed for 7 days. Similar observations were made when the formulations were kept at 40°C ± 1°C for 20 days. The formulations were kept both at room and elevated temperature and observed on the 0th, 5th, 10th, 15th, and 20th day for the parameters.

Based on the evaluation parameters for various creams, MF4 was selected and further subjected to *in vitro* diffusion studies.

In vitro Diffusion Studies

In vitro diffusion studies were performed using Franz Cell apparatus. It is an *in vitro* skin permeation assay frequently used in formulation

development. The Franz cell apparatus consists of two primary chambers separated by a membrane. The test product was applied to the membrane through the top chamber. The 10 ml of distilled water was taken in the receiver compartment and egg membrane was placed on it. A total of 2 g equivalent cream was weighed and placed on the membrane. It was covered with the donor compartment and clamped to get undisturbed. Then, the apparatus was placed on a magnetic stirrer with 300 rpm and temperature was maintained at 32°C. Samples were collected at regular intervals. The volume was replaced with fresh solvent. This testing determines the amount of medicament that has permeated the membrane at each time point. Sample withdrawn at 1 h along with *Moringa* oil and *Mustard* oil as standards were further subjected to characterization studies for Fourier transform infrared spectroscopy (FTIR), gas chromatography-mass spectrometry (GCMS), and high-performance thin-layer chromatography (HPTLC) analysis.

Fourier transform-infrared spectroscopy analysis

A drop of extracted sample was placed on the IR crystal port and analyzed. It was compared with the standard sample analysis. Later, the formulated cream and the *in vitro* diffused sample of the cream were analyzed using liquid FTIR. The spectra of all the samples were collected using a Bruker FTIR spectroscopy. Each spectrum was obtained in a range of 4000-600 cm^{-1} by performing 20 scans at a spectral resolution of 4 cm^{-1} . The spectra were collected using OPUS 7.2.139.1294 software as the peak height and area measurement were carried out.

Gas chromatography-mass spectrometry analysis

The samples were analyzed on a JEOL GC MATE II gas chromatography with Quadrapole Double Focusing Mass Analyser fitted with "Photomultiplier tube" detector and HP5MS column. Highly Pure Helium was used as a carrier gas with a flow rate of 1 mL/min and the oven temperature was maintained at 50°C to 250°C by increasing 10°C/min.

High-performance thin-layer chromatography analysis

The oily samples were dissolved in n-hexane quantitatively to strength of 0.1 $\mu\text{l}/1 \mu\text{l}$. 2 μl of the prepared solution was injected on a HPTLC Silica Gel GF₂₅₄ (5 cm × 10 cm) plate. The plate was developed in Toluene: Ethyl acetate (9:1) mobile phase. Plate was derivatized using 5% Methanolic sulfuric acid and scanned at 620 nm under a UV Scanner.

Pharmacological Screening for *Moringa oleifera* seed oil and cream

Experimental animals

Healthy adult male albino rats (Wistar strain) weighing 250–300 g, housed in polypropylene cages, maintained under standardized condition, i.e., 12:12 h light/dark cycle at 25 ± 2 with paddy husk bedding at the animal house, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Guntur, India were provided with standard pellet food and had free access to purified drinking water. The guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India were followed and prior permission was sought from Institutional Animal Ethics Committee for conducting the study.

Ex vivo anti-inflammatory activity

Heat induced hemolysis

A volume of 20 ml of uncoagulated fresh rat blood was added to the vials containing 1 ml of 0.1M phosphate-buffered saline (PBS) of 7.4 pH.

Sample and standard drug were added to the vials (in triplicate), to achieve the final concentrations of 50, 100, 150, and 200 $\mu\text{g}/\text{ml}$. PBS and rat blood were added to the control vials. Then, the solutions were subjected to centrifugation at 3000 rpm in centrifuge for 10 min. No residue was observed after centrifugation indicating complete solubility of the drug. After mixing, the contents in the vials are pre-incubated at 37°C for 15 min. Then, the mixtures were heated for 25 min at 54°C. The precipitate gets settled down and the supernatant was measured at 540 nm in spectrophotometer. The percentage inhibition of hemolysis of test should be compared with respect to control.^[11]

%Inhibition of haemolysis =

$$\frac{\text{Absorbance in control group} - \text{Absorbance in test group} \times 100}{\text{Absorbance in control group}}$$

Protein denaturation

The 0.2 ml of egg albumin was added to vials containing 2.5 ml of 0.1M PBS pH 6.4. 2 ml of sample, and standard drug was added to vials (in triplicate) to achieve the final concentration of 50, 100, 150, and 200 $\mu\text{g}/\text{ml}$. PBS (2.5 ml) and egg albumin was added to control vials. Then, the mixture was incubated at 37°C ± 2°C in a BOD incubator for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm using the vehicle as blank. The percentage inhibition of protein denaturation of test should be compared with control.^[12]

%Inhibition of denaturation =

$$\frac{\text{Absorbance in control group} - \text{Absorbance in test group} \times 100}{\text{Absorbance in control group}}$$

In vivo anti-inflammatory activity

Carrageenan-induced paw edema in rats

The animals were treated into four groups each with five animals. Group 1 animals are treated with normal saline. Group 2 animals were treated with Ibuprofen cream. Group 3 animals were treated with *Moringa* seed oil. Whereas, Group 4 animals were treated with *Moringa* seed oil cream and all the groups of animals were treated with 0.1 ml of 1% Carrageenan. The animals were pre-treated with vehicle/Ibuprofen/extracts 30 min before the injection of Carrageenan on the plantar region to the right hind paw of the rats. Paw volumes were measured using the displacement of the water column in a plethysmometer at 1, 2, 3 and 4 h after the administration of test materials. Reduction in the paw volume compared to the control animals was considered as the anti-inflammatory response.^[13]

%Inhibition of edema =

$$\frac{\text{Absorbance in control group} - \text{Absorbance in test group} \times 100}{\text{Absorbance in control group}}$$

RESULTS AND DISCUSSION

The present study was intended to formulate cream using *Moringa* seed oil and to evaluate its anti-inflammatory potency.

Physico-chemical screening of *Moringa* seed oil

As per "Ayurvedic Materia Medica" published by Nadkarni indicated that *Moringa* seed oil is having similar FFAs as that of *Mustard* oil. Among the FFAs, Behenic acid was found to be more responsible for anti-inflammatory activity. Earlier reports indicated that *Moringa* oil chiefly consisted of Behenic acid as FFA and thus responsible for the anti-inflammatory action. Hence, it was also designated as Ben oil. The physicochemical properties such as FFA, acid value, and saponification value evaluated for *Moringa* seed oil thus indicated the presence of FFA and also highly suitable for saponification process. Based on these values obtained for *Moringa* oil, an attempt was made to prepare o/w

type emulsion type cream by saponifying the FFA present in the oil with a suitable concentration of alkali. The results of physicochemical properties are given in Table 1.

Preparation of o/w type cream formulations

Moringa seed oil creams were prepared using 36% of oil phase, 12% oleic acid as neutralizer and enhances soap formation. While potassium hydroxide, sodium hydroxide, ammonium hydroxide, and liquid ammonia at different proportions were used as alkali in different creams. Sodium benzoate was used as a preservative. Distilled water was used as a continuous aqueous phase. All the creams were prepared by the triturating process and under identical conditions. The composition of various creams is given in Table 2.

Evaluation parameters

The formulated creams of *Moringa* seed oil were further evaluated for pH, viscosity, confirmation of the type of emulsion by dye test, homogeneity, appearance, after feel, type of smear, removal, test for irritancy, and accelerates stability studies. All the results are indicated in Table 3.

pH of the cream

The pH of the creams was found to be in the range of 5.6–7.4 which is good for skin pH. Among those formulations, MF₄ showed pH nearer to skin required

Table 1: Physicochemical properties of *Moringa* seed oil

Tests	Oil sample
FFA test (mg KOH/g oil)	1.2
Acid value (mg KOH/g oil)	0.84
Saponification value	154.27

FFA: Free fatty acid

Table 2: Composition of various *Moringa oleifera* seed oil cream formulations

Ingredients (mg/ml) 10 g cream	MF ₁	MF ₂	MF ₃	MF ₄	MF ₅	MF ₆	MF ₇	MF ₈	MF ₉	MF ₁₀	MF ₁₁	MF ₁₂
<i>Moringa</i> seed oil (ml)	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Oleic acid (ml)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Sodium hydroxide (mg)	500.0	750.0	1000.0	-	-	-	-	-	-	-	-	-
Potassium hydroxide (mg)	-	-	-	500.0	750.0	1000.0	-	-	-	-	-	-
Ammonium hydroxide (mg)	-	-	-	-	-	-	500.0	750.0	1000.0	-	-	-
Liquid ammonia (mg)	-	-	-	-	-	-	-	-	-	500.0	750.0	1000.0
Sodium benzoate (mg)	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Distilled water	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Total weight (g)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

Table 3: Physicochemical evaluation of various *Moringa* seed oil creams

Evaluation tests	MF1	MF2	MF3	MF4	MF5	MF6	MF7	MF8	MF9	MF10	MF11	MF12
pH	6.0	6.2	6.5	5.6	5.7	5.9	6.2	6.3	6.4	6.8	7.2	7.4
Viscosity (cps)	2.6	2.8	3.0	2.0	2.2	2.3	2.4	2.8	3.1	3.4	3.6	3.7
Emulsion type	o/w type	o/w type	o/w type	o/w type	o/w type	o/w type	o/w type	o/w type	o/w type	o/w type	o/w type	o/w type
Homogeneity	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform
Appearance	White	White	White	White	White	White	White	White	Smooth	Yellowish white	Yellowish white	Yellowish white
After feel	Emollient	Emollient	Emollient	Emollient	Emollient	Slippery	Slippery	Slippery	Slippery	Slippery	Slippery	Slippery
Type of smear	Nongreasy	Greasy	Greasy	Nongreasy	Greasy	Greasy	Greasy	Greasy	Greasy	Greasy	Greasy	Greasy
Removal	Easy	Easy	Peel off	peel off	Peel off	Peel off	Peel off	peel off	Peel off	Peel off	Peel off	peel off
Irritancy	No	No	No	No	No	No	No	No	No	No	No	No
Accelerated stability	Stable	Stable	Stable	Highly stable	Stable	Stable	Stable	Stable	Stable	Stable	Unstable	Unstable

o/w: Oil/water

Viscosity

The viscosity of creams was in the range of 2.6–3.7 cps which indicates that the cream is easily spreadable by small amounts of shear. However, MF₄ shows good spreadable property than other formulations.

Dye test

This dye test confirms that all formulations were o/w type emulsion cream. However, formulation (MF₄) shows more stable in o/w type emulsion.

Homogeneity

All formulations produce a uniform distribution of extracts in cream. This was confirmed by visual appearance and by touch.

Appearance

When formulation was kept for a long time, it was found that there was no change in color of cream.

After feel

Emolliency, slipperiness, and amount of residue left after the application of the fixed amount of cream were found.

Type of smear

After application of creams MF₁ and MF₄, the type of smear formed on the skin was non greasy.

Removal

The creams of MF₁ and MF₄ applied on the skin were easily removed by washing with tap water.

Irritancy test

The formulations MF₁ to MF₁₂ showed no redness, edema, inflammation, and irritation during irritancy studies. These formulations are safe to use for skin.

Accelerated stability studies

All the formulations remained stable even after 3 months of storage. Among those formulations, MF₄ showed more acceptability and high stability.

These tests indicated that formulation MF₄ prepared by using 500 mg potassium hydroxide was found to be suitable with all acceptable characteristics of o/w type emulsion type of cream. Based on the tests performed, formulation MF₄ was further subjected to *in vitro* diffusion studies.

Characterization studies

The extracted oil, cream, and diffusion sample at 1 h were further evaluated for FTIR, GCMS and HPTLC.

Fourier transform-infrared spectroscopy analysis

FTIR data of *Moringa* oil and its cream formulation diffused with the standard Mustard oil sample showed a characteristic peak for O-H stretching at 3339.39 cm⁻¹ and C-H stretching at 2921.7 cm⁻¹. IR peaks at 1742.68 and 1641.99 cm⁻¹ were assigned to C=O and O-C=O stretching, respectively. The C-C-bending vibration peak was attributed to peak at 1461.99 cm⁻¹. The absorption peak at 1158.49 was assigned to C-O-C bending. Further, absorption peaks at 1265-1074, 949-969 and 864 cm⁻¹ were assigned to C-O stretching, C-H bending, respectively. Both the oils and cream formulation appeared identical in their characteristic bands that can be used for quantitative determination. Usually, the oils get converted when reacted with the alkali, but in the case of FTIR spectra of *Moringa seed oil* and the cream not much difference was observed in the absorption band of the compound. This indicates that the oil in the cream got diffused as such making the complete diffusion of FFA s without wastage. Thus, the data indicated that the formulated cream (MF₄) was an optimized formulation with high diffusibility. The fingerprint of the oils and formulation along with the diffused sample by FTIR were depicted in Figures 1-4, and the data are given in Table 4.

Gas chromatography-mass spectrometry analysis

The GC data of pure oil sample showed the peak data at retention time (Rt) of 15.97, 17.78, 18.8, 19.68, 20.68, 22.12, 23.15, and 24.53 min. The mass spectral data of these peaks were measured using electron impact ionization mass spectrometry and their fragmentation pattern was observed. The corresponding molecular ion peak (M+) for gas chromatographic peaks were found to be at 228 (Myristic acid), 256 (Palmitic acid), 296 (Oleic acid), 282 (oleic acid), 284 (Stearic acid), 312 (3-ortho, methyl ester of oleic acid), 340 (Behenic acid), and 382 (2-Butoxyethylester of oleic acid), respectively.

The fragmentation pattern also reveals the structural arrangement of fatty acids present in the given sample. This indicates that oleic acid and Palmitic acid were the good percentage in the given oil. However, the

presence of Behenic acid makes their oil highly selective and specific compared to other available oils, as it is absent in other oils except Mustard oil. The data are given in Table 5, and the chromatograms are depicted in Figure 5.

The GC analysis for diffused sample shows the peaks at 17.25, 17.95, 18.88, 19.45, 21.83, 22.3, and 24.63 as shown in the figure 6 which were of away from the noise. The corresponding mass spectral data showed the same fatty acids as of pure sample were differed. All the contents of oil formulation were differed. This indicates that the active constituents were differed from the formulation developed, this pertains the good activity. The retention time of the FFAs in the oil and the diffused sample was found to be similar as shown in Table 6. As the literature reveals the anti-inflammatory activity of Behenic acid, the present GCMS data correlating the presence of Behenic acid

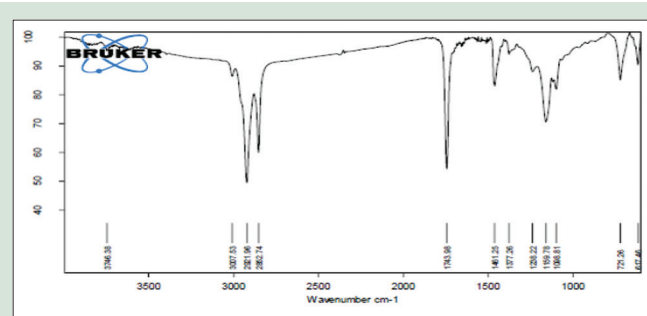


Figure 1: Fourier transform-infrared spectroscopy spectrum of Mustard oil. Fourier transform-infrared spectroscopy analysis

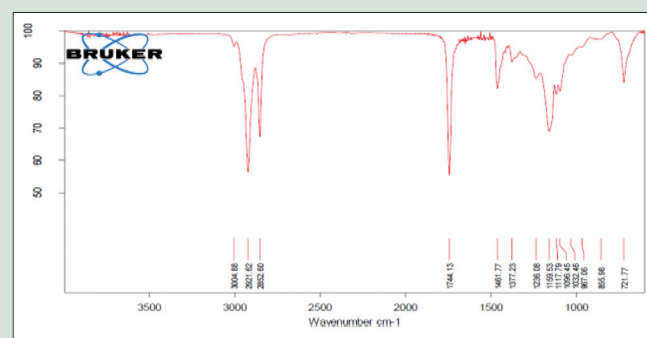


Figure 2: Fourier transform-infrared spectroscopy spectrum of Moringa oil

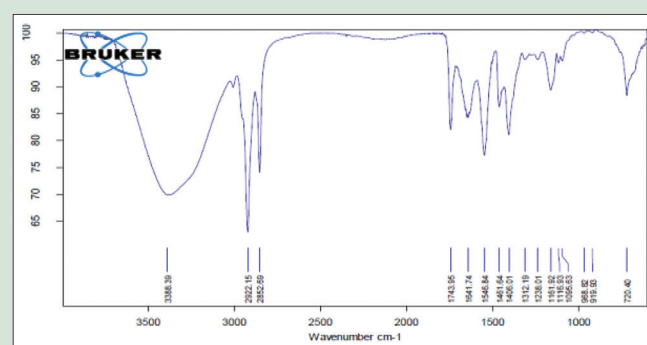


Figure 3: Fourier transform-infrared spectroscopy spectrum of Moringa oil-based cream formulation

Table 4: Fourier transform infrared spectroscopy analysis of mustard oil, *Moringa seed oil*, *Moringa cream* and diffused sample of formulation (MF₄)

Functional group	Wave number (cm ⁻¹)			
	Mustard oil	Moringa oil	Moringa cream	Diffused sample (MF ₄)
O-H	-	-	3388.39	3339.14
C-H	2921.96	2921.82	2922.15	2921.70
C=O	1743.88	1744.13	1743.95	1742.57
O-C=O	-	-	1641.74	1633.80
-C-C-	1461.25	1461.77	1461.63	1461.99
-C-O-C-	1238.22	1236.08	1238.01	1238.29
-C-O- and -C-H	<1000.0	<1000.0	<1000.0	<1000.0

Table 5: Gas chromatography-mass spectrometry analysis of *Moringa* seed oil and diffused sample of formulation (MF₄)

Fatty acid	<i>Moringa</i> oil		Diffused sample of formulation (MF ₄)	
	Rt – Retention time	Molecular weight	Rt	Molecular weight
Oleic acid (C18)	19.68	282.0	19.45	282.0
Stearic acid (C18)	20.68	284.0	21.83	284.0
Palmitic acid (C16)	17.78	256.0	17.95	256.0
Behenic acid (C22)	23.15	340.0	24.63	340.0
Iso-oleic acid (C18)	18.88	296.0	17.25	296.0
16 octadecanoic acid (C19)	18.0	296.0	18.88	296.0
Myristic acid (C14)	16.1	228.0	17.27	228.0
Octadecanoic acid 3 oxo methyl ester	22.12	312.0	22.3	312.0
9 octadecanoic acid	24.53	382.0	-	-

Table 6: High-performance thin-layer chromatography data of *Moringa* seed oil

Peak	Maximum R _f	Area (%)
1	0.04	1120.9 (4.43)
2	0.08	487.7 (1.93)
3	0.35	8980.8 (35.53)
4	0.46	6763.7 (26.76)
5	0.56	6701.8 (26.51)
6	0.61	330.2 (1.31)
7	0.79	797.3 (3.15)
8	0.95	93.7 (0.37)

Table 7: High-performance thin-layer chromatography data of standard sample (mustard oil)

Peak	Maximum R _f	Area (%)
1	0.04	2563.8 (10.26)
2	0.08	287.5 (1.15)
3	0.33	7815.8 (31.28)
4	0.40	925.2 (3.70)
5	0.45	5861.2 (23.45)
6	0.56	5348.4 (21.40)
7	0.74	1102.2 (4.41)
8	0.79	1086.1 (4.35)

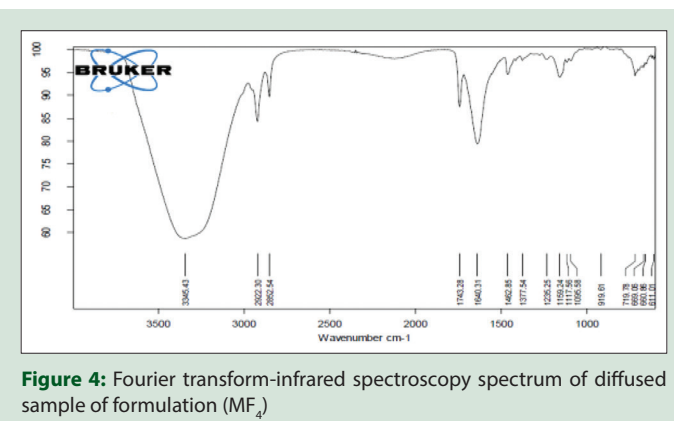
range diffused studies showed at 24.63 min and 23.15 min indicating the diffusivity of the formulation.

These studies thus revealed that the cream formulation by alkali saponification was done with the lower molecular weight FFA such as stearic acid, palmitic acid, oleic acid, and myristic acid, in the presence of additional oleic acid was added to the cream. Whereas the Behenic acid which is known as anti-inflammatory activity was remained in the unsaponifiable form in the cream was released through the membrane for producing anti-inflammatory activity.

High-performance thin-layer chromatography analysis

Mustard oil as standard showed 8 peaks, first 2 being very close to the sample application cannot be considered. Major peaks with the good surface area are R_f 0.33, 0.45, and 0.56 with a peak area of 31%, 23%, and 21% respectively. Others are minor peaks at R_f 0.4, 0.74, 0.79 with a peak area between 4% to 5%. The values obtained are given in Table 7 and graph is depicted in Figures 7 and 8.

Moringa seed oil also showed 8 peaks, first 2 being the same peaks as that of MST very close to the sample application which cannot be considered. The major peaks with good surface area present in the MST were also seen in MOR; R_f 0.35 (~0.33), 0.46 (~0.45), and 0.56 with a peak area of 35%, 27%, and 26%, respectively. Other were minor

**Figure 4:** Fourier transform-infrared spectroscopy spectrum of diffused sample of formulation (MF₄)

peaks at R_f 0.61, 0.79, 0.95 with a peak area between 1% to 3%. R_f 0.61 is a different peak. While R_f 0.95 is close to solvent front cannot be considered. The values obtained are given in Table 6 and graph is depicted in Figures 7 and 8.

A diffused sample of the formulated cream was not soluble completely, and the soluble portion of the oil showed 3 peaks. All the 3 peaks were major peaks with R_f 0.45 that corresponds to both MST and MOR with a peak area of 43.6%; R_f 0.5 a newer peak may be a shoulder peak as result of merging and the third peak is at R_f 0.58 which corresponds to 0.56 of MST and MOR with a surface area of 23%. The values obtained are given in Table 8 and graph is depicted in Figures 7 and 8.

From the analytical data, it is observed that, the band in HPTLC at 0.45 R_f is commonly observed in both pure sample and diffused sample of the formulated cream. Hence, it is predicted to be Behenic acid. This HPTLC data were supported by GCMS data were the retention times of 23.15 and 24.63 min of GC for pure sample and diffused sample of the formulated cream, respectively, shown the mass of 340, which implies that Behenic acid was present in both pure diffused sample of the cream. Studies were proved to be successful for Behenic acid. This confirmation of the compound was also correlated with pharmacological studies tested further.

Ex vivo pharmacological screening

Effect on heat-induced hemolysis

The effect of the formulated cream of *M. oleifera* seed oil on heat-induced hemolysis in Albino rats is given in Table 9 and depicted in Figure 9. The result obtained indicates that the formulation showed significant anti-inflammatory activity in Albino rats by protecting the red blood cell (RBC) from hemolysis. The formulation MF₄ at 200 µg/mL inhibited 69% hemolytic damage which was nearly equal to the standard group treated with Ibuprofen cream. Whereas, the standard drug Ibuprofen at 200 µg/mL, inhibited 74% hemolytic damage.

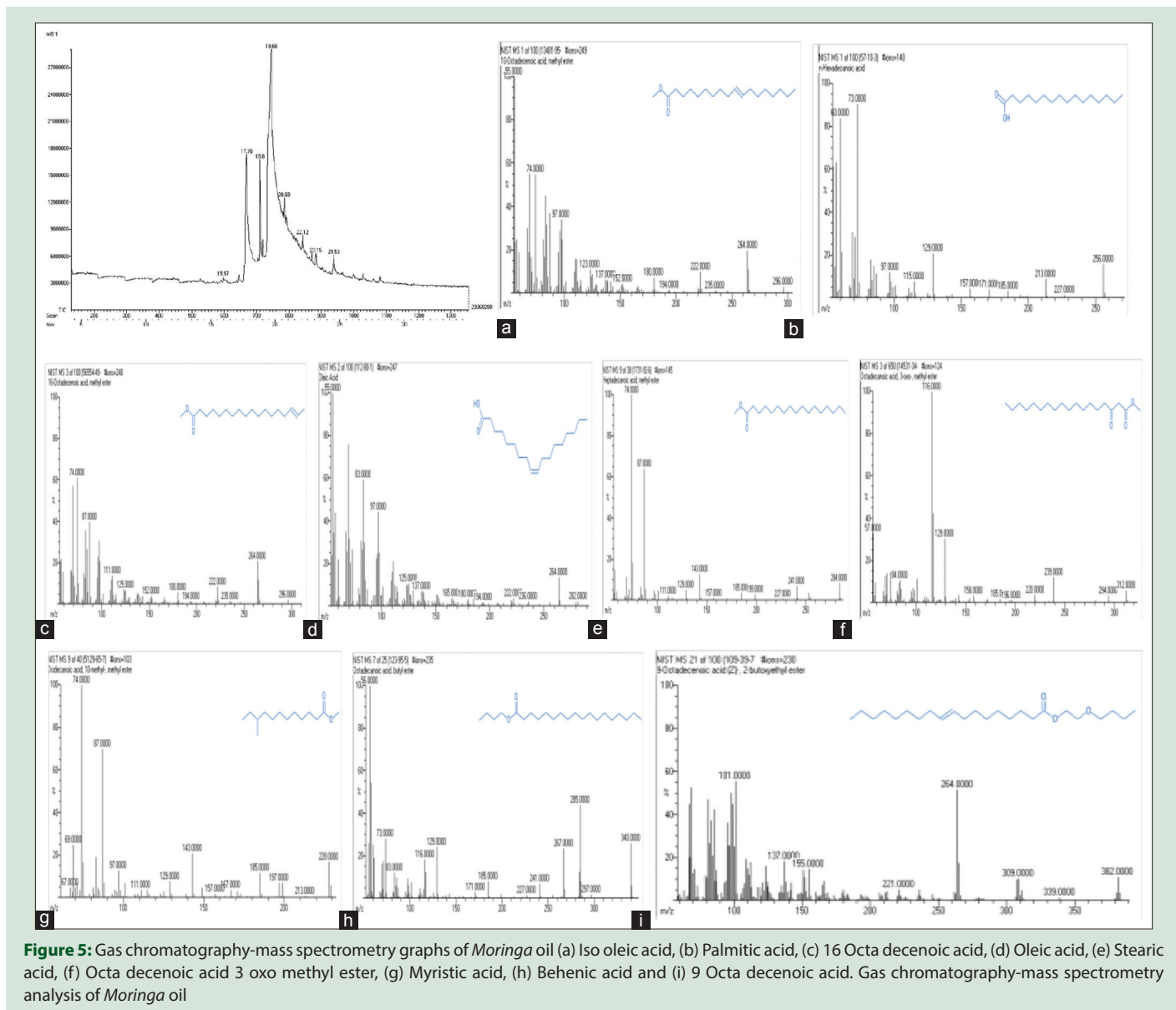


Figure 5: Gas chromatography-mass spectrometry graphs of *Moringa* oil (a) Iso oleic acid, (b) Palmitic acid, (c) 16 Octa decenoic acid, (d) Oleic acid, (e) Stearic acid, (f) Octa decenoic acid 3 oxo methyl ester, (g) Myristic acid, (h) Behenic acid and (i) 9 Octa decenoic acid. Gas chromatography-mass spectrometry analysis of *Moringa* oil

Table 8: High-performance thin-layer chromatography data of diffused sample of formulation (MF₄)

Peak	Maximum Rf	Area (%)
1	0.45	3604.4 (43.64)
2	0.50	2801.8 (33.92)
3	0.58	1852.8 (22.43)

Table 9: Ex-vivo anti-inflammatory activity of formulated cream (MF₄)

Concentration (µg/mL)	Percentage inhibition of heat induced hemolysis	
	Standard ibuprofen (mean±SD)	Test formulation (MF ₄) (mean±SD)
50	33.28±1.188	33.05±1.134
100	50.14±1.210	47.45±1.604
150	62.85±1.291	59.54±1.178
200	74.02±1.246	69.85±1.104

SD: Standard deviation

The heat-induced hemolytic studies were performed on *M. oleifera* cream which significantly decreased the lysis of red blood cells. In

this, lysosomes play a major role in the inflammatory reaction.^[14] The vitality of cells depends on the integrity of their membrane, exposure of RBCs to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by hemolysis and oxidation of hemoglobin.^[15]

Effect on protein denaturation

The effect of the formulated cream of *M. oleifera* seed oil in protein denaturation in egg albumin is given in Table 10 and depicted in Figure 10. The result obtained indicates that the formulation showed significant anti-inflammatory activity by reducing the protein denaturation. The formulation (MF₄) at 200 µg/mL inhibited 60% protein denaturation which is nearly equal to the standard group treated with Ibuprofen cream. Whereas, the standard ibuprofen at 200 µg/mL, inhibited 65% protein denaturation. Compounds with membrane stabilizing properties are well known for their ability to interfere with the release of phospholipases that trigger the formation of inflammatory mediators. It may be inferred that stabilization of lysosomal membrane was one of the mechanisms by which the *Moringa* cream mediates their anti-inflammatory activity.^[16]

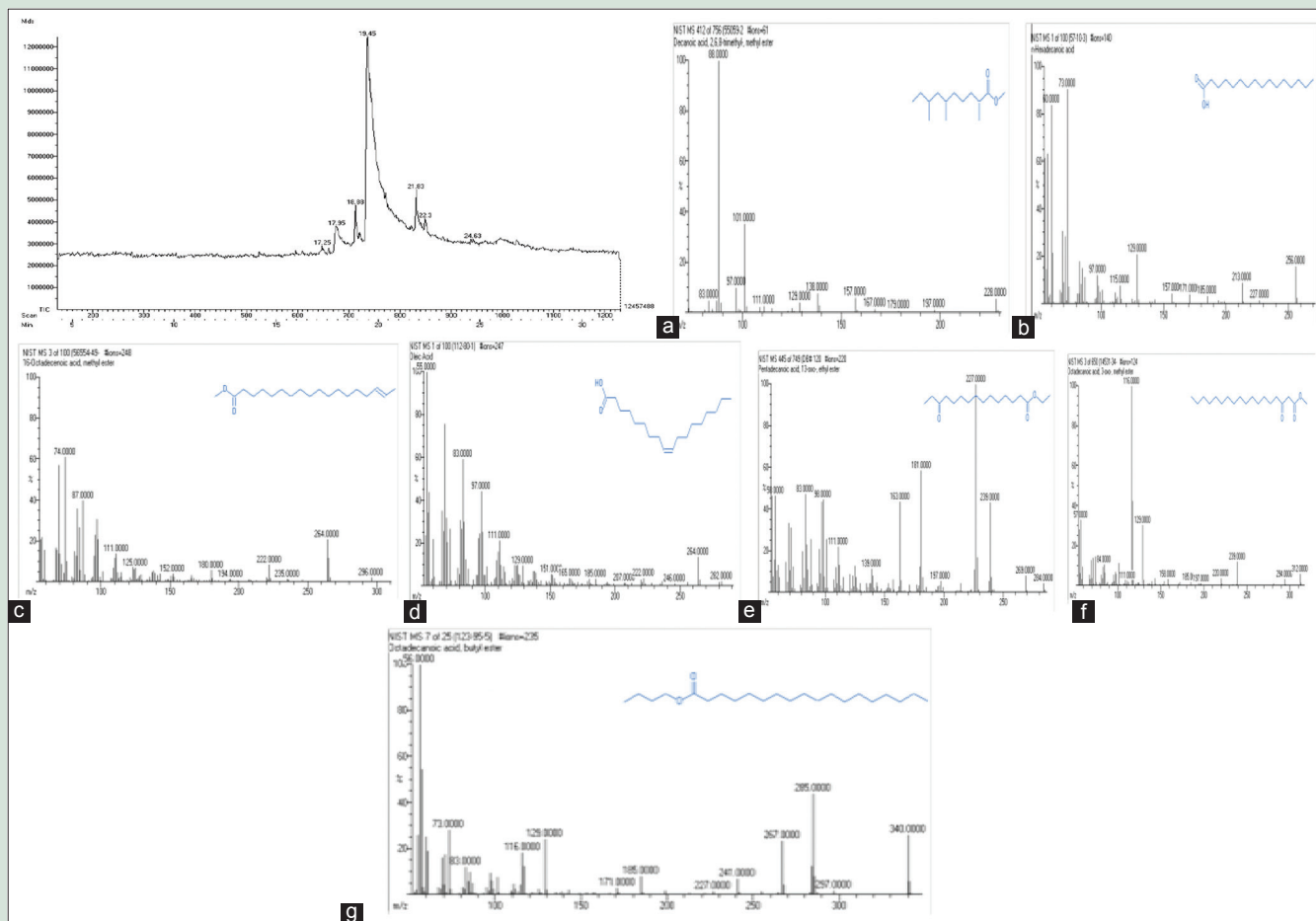


Figure 6: Gas chromatography-mass spectrometry graphs of *Moringa* formulation (cream) diffused sample (a) Iso oleic acid, (b) Palmitic acid, (c) 16 Octa decenoic acid, (d) Oleic acid, (e) Stearic acid, (f) Octa decenoic acid 3 oxo methyl ester and (g) Behenic acid. Gas chromatography-mass spectrometry analysis of diffused sample (MF₄)

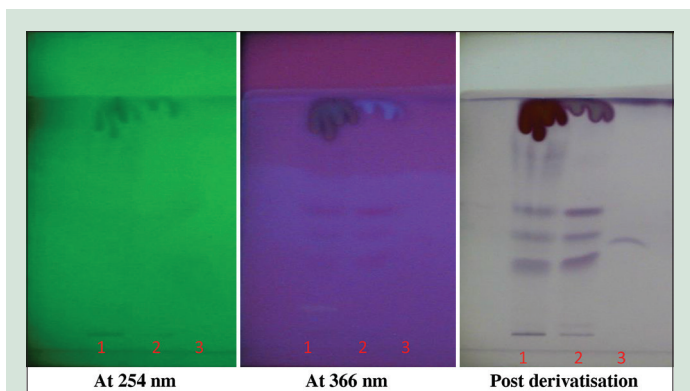


Figure 7: High-performance thin-layer chromatography fingerprint profile of *Moringa* Oil and its formulation, high-performance thin-layer chromatography analysis

In vivo anti-inflammatory activity Effect on carrageenan-induced paw edema

Carrageenan-induced inflammation is most commonly used as an experimental model for evaluating the anti-inflammatory potency of compounds or natural products because it produces reproducible results.^[17] The effect of oil and formulated cream on

Table 10: Ex vivo anti-inflammatory activity of formulated cream (MF₄)

Concentration (µg/mL)	Percentage inhibition of protein denaturation	
	Standard ibuprofen (mean±SD)	Test formulation (MF ₄) (mean±SD)
50	32.68±1.410	30.10±1.218
100	48.12±1.009	45.05±0.998
150	58.24±1.114	54.82±1.011
200	65.778±1.102	60.01±0.912

SD: Standard deviation

carrageenan-induced paw edema in Albino rats are given in Table 11 and depicted in Figure 11. The results obtained indicated that both the oil and formulation had significant anti-inflammatory activity in Albino rats when compared to the control group. The oil reduced the edema by 64%, whereas the formulated cream reduced by 70%. The formulation produced an inhibitory effect equal to that of the standard group which showed 71% inhibition in paw edema. The development of edema in the paw of the rats is due to the release of histamine, serotonin, and prostaglandin-like substance. The knowledge of these mediators involved in different phases is an important step for interpreting the mode of drug action. The extracted oil and formulated cream of *M. oleifera* showed a more significant reduction in paw edema at 2nd h when injected with carrageenan, suggesting that *M. oleifera* produces the anti-edematous effect.

Table 11: *In vivo* anti-inflammatory activity of *moringa* seed oil and formulated cream (MF₄)

S. No	Dose	Paw edema (ml), mean±SD (percentage inhibition)			
		1 st h	2 nd h	3 rd h	4 th h
Control	Saline 2 ml p.o	0.60±1.082	0.91±1.124	1.04±1.232	0.78±1.941
Standard	Ibuprofen 1 g	0.20±2.422 (66.6)	0.26±1.672 (71.42)	0.32±2.424 (69.2)	0.26±2.542 (64.10)
Test-I	<i>Moringa</i> oil 1 ml	0.28±2.003 (53.3)	0.32±1.244 (64.8)	0.38±1.843 (63.46)	0.30±1.422 (61.5)
Test-II	Formulated cream (MF ₄) 1 g	0.22±1.962 (63)	0.27±2.142 (70.32)	0.33±2.566 (68.2)	0.29±2.279 (62.82)

SD: Standard deviation

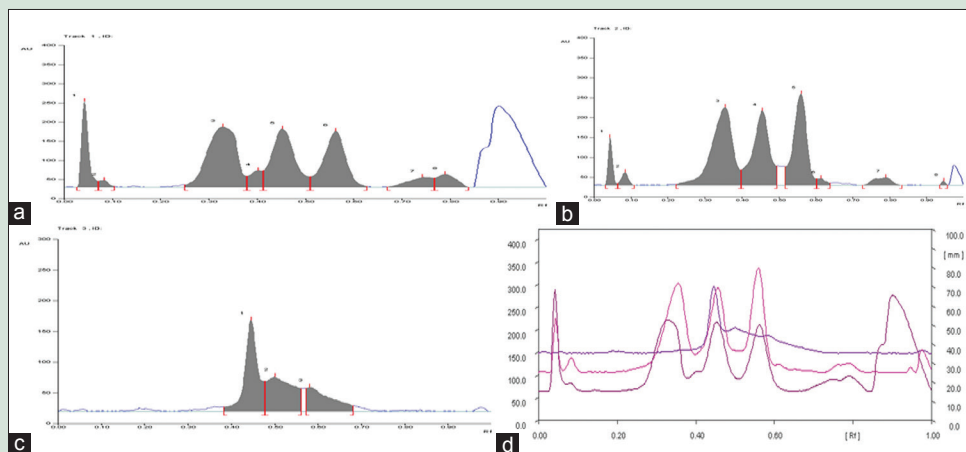


Figure 8: High-performance thin-layer chromatography densitometric scan at 620 nm, representing (a) Mustard oil (standard), (b) *Moringa* oil, (c) Diffused sample of Formulation and (d) Combination of three samples

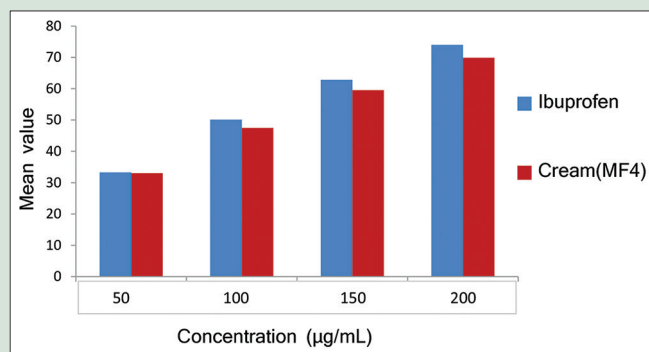


Figure 9: Anti-inflammatory activity of formulated cream (MF₄) by heat induced haemolysis

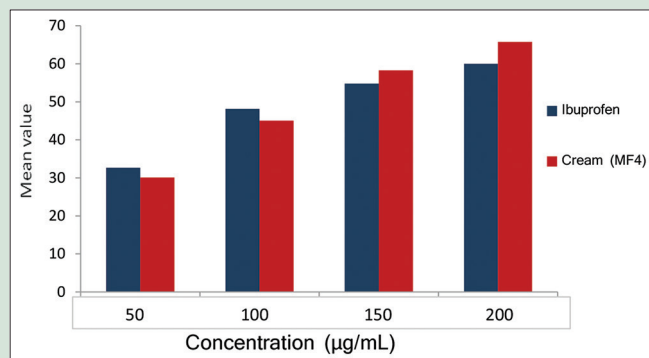


Figure 10: Anti-inflammatory activity of MF₄ cream by protein denaturation

CONCLUSION

The *Moringa* oil-based Cream formulation MF₄ prepared using 500 mg of potassium hydroxide as alkali was found to be a stable formulation after performing suitable physicochemical evaluation studies. This formulation was further subjected to pharmacological evaluation. From the obtained results, it was concluded that MF₄ reduced edema 68.2%, near to the standard group induced by carrageenan on sub-plantar administration when compared to untreated group. There is a significant inhibition of heat-induced hemolysis of 69.85% and inhibition in protein denaturation up to 60.01% when compared to that of the standard. Thus, it was concluded that the extracted oil and formulated cream of *M. oleifera* antagonizes the initial and late phases of inflammation caused due to carrageenan. The formulated cream was more efficacious than oil. The results support the traditional use of this plant in inflammatory conditions and suggest the presence of biologically active compounds which was supported by the analytical data such as GCMS exploring the exact active constituents for the anti-inflammatory activity which in turn may result in the development of a potent anti-inflammatory agent with low toxicity and better therapeutic index.

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Nil.

Conflicts of interest

There are no conflicts of interest.

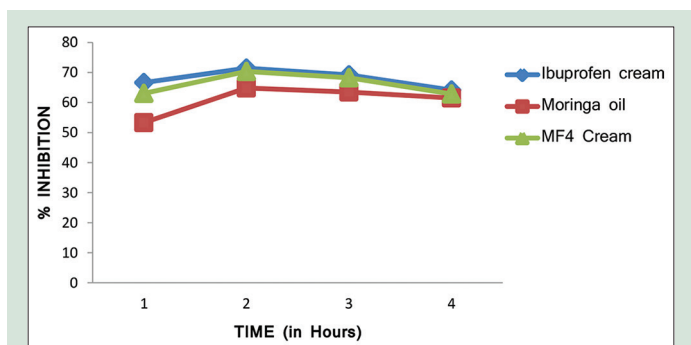


Figure 11: *In vivo* anti-inflammatory activity of formulated cream (MF₄) by carrageenan induced paw Edema method

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