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## Design and characterization of *Moringa oleifera* seed oil impregnated anti-inflammatory topical micro-dispersion

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### ABSTRACT

Rheumatoid arthritis is one of the most serious non-fatal conditions with significant prevalence around the globe. Objective: The objective of present work was to design and characterize *Moringa oleifera* seed oil impregnated anti-inflammatory topical micro-dispersion. The ternary phase diagrams for microemulsion regions were constructed using *Moringa oleifera* seed oil as oil phase, Tween 80 and Span 80 as surfactants. The microemulsion was characterized for its percentage transmittance, refractive index, pH, globule size, zeta potential, viscosity, in-vitro drug release, skin irritancy and anti-inflammatory activity. The batch F3 with 25.30% of oil, 59.02 % of  $S_{mix}$  and 15.68% of water; was found to be more stable over other batches. The percentage transmittance and refractive index (RI) of formulation was found to be 99.60 and 1.469 respectively; whereas pH and average globule size was 7.1 and 167nm respectively. zeta potential and viscosity of optimized microemulsion was found to be -35.8 and 88.7 cps respectively. The permeability of *Moringa oleifera* seed oil microemulsion was 76.86% as compared to 54.06% of pure oil. The prepared microemulsion was found to be non-irritant and showed significant ( $p < 0.05$ ) anti-inflammatory effect. The *Moringa oleifera* seed oil can be used to develop stable micro-dispersion system with better skin permeation and promising anti-inflammatory activity without any skin irritation.

**Keywords:** Rheumatoid arthritis, *Moringa oleifera* seed oil, micro-dispersion, ternary phase diagram, anti-inflammatory activity.

### INTRODUCTION

Inflammatory conditions are the common afflictions irrespective of ages. Rheumatoid arthritis (RA) is a systemic auto-immune disease beginning in the small joints of the hands and the feet, spreading later to the larger joints involving severe inflammation. The inflamed joint lining or synovium extends and then erodes the articular cartilage and bone, causing joint deformity and progressive physical disability [1]. It is 31<sup>st</sup> leading cause of disabilities; Years Lived with Disability (YLD) at global level, accounting for 0.8% of total global YLDs [2]. Numbers of NSAIDs are commonly used to treat rheumatic conditions and the most widely cited side-effect of NSAIDs includes, gastrointestinal ulcer, accompanied by anemia due to the bleeding. Hence it makes it desperate to look for other ways for the management of rheumatism. In order to avoid the gastric irritation, minimize the systemic toxicity and achieve a better therapeutic effect, one promising method is to administer the drug via skin [3]. Green alternatives could be the best answer to this quest as anti-inflammatory herbal preparations cause much less adverse effects. *Moringa oleifera* Lam widely distributed in tropical and sub-tropical regions and has nutritional and therapeutic values. *Moringa oleifera* is traditional medicine used in rheumatoid arthritis[4]. *Moringa* is rich dietary source of Omega 3 poly unsaturated fatty acids (PUFA) [5]. A number of studies showed that Omega 3 fatty acid helps to reduce inflammation and pain related to rheumatoid arthritis[6].

The concept of micro-dispersions(microemulsions) was first introduced by Hoar and Schulman during 1940s. It is defined as a system of water, oil and amphiphile which is an optically isotropic and thermodynamically stable liquid

micro-dispersion with 10-100nm droplet size. Microemulsion is composed of oil, water, surfactants and co-surfactants[7].Microemulsions offer several advantages such as transparency, low viscosity, enhanced drug solubility, good thermodynamic stability, long shelf life, ease of manufacturing and enhancing effect on transdermal delivery of drug compared to conventional formulations [8].Recently, more attention has been focused on microemulsions for transdermal delivery of drugs. This is due to their ability to incorporate large amount of drug, microemulsion components combined synergistically to increase drug flux and enhanced rate of permeation [9].The use of novel approaches for herbal drugs is need of hour for their overall better performance. In this study, we tried to develop a new microemulsion formulation using *Moringa oleifera* seed oil for topical application which may lead to an improvement permeation and better therapeutic effect. The anti-inflammatory activity was evaluated using vascular permeability test and carrageenan induced paw edema in rats.

## MATERIALS AND METHODS

### Materials

*Moringa oleifera* seeds were procured from local market. Span 80, Tween 80 and other materials used were of analytical grade and purchased from SD Fine Chemicals, Pvt. Ltd, Mumbai.

### Methods

#### Collection and authentication

Fresh *Moringa oleifera* seeds were authenticated by Prof. (Dr.) Sandanshiv, Dept. of Botany, S. S. G. M. College, Kopergaon Dist: Ahmednagar (M.S.)India.

#### Extraction of oil

*Moringa oleifera* seeds were air dried for a week and placed in hot air oven at 40°C (Lab Star, Mumbai), ground and sieved through #40 and #60 to get coarse powder. Extraction of seeds was carried out by Soxhlet extraction using petroleum ether (60-80 °C) as solvent [10].

#### Preliminary physicochemical study[11]

##### 1. Organoleptic properties

*Moringa oleifera* seed oil was analyzed for organoleptic properties like color, odor, and appearance.

##### 2. Saponification value

About 2g of oil and 25ml of alcoholic potassium hydroxide was taken in flask. The resultant mixture was heated for 1h; to which 1ml of 1% phenolphthalein was added and titrated with 0.5N HCl.

##### 3. Iodine value

About 2g of oil and 10ml of carbontetrachloride were taken in flask and 20ml of Wij's solution (1.5% iodine monochloride in 98% acetic acid) was added and kept in dark for 30min. Fifteen ml of potassium iodide and 100ml of water added in above solution. The resultant solution was titrated with 0.1M sodium thiosulphate solution using starch as an indicator.

##### 4. Peroxide value

About 1g of oil, 20ml acetic acid-chloroform (2:3), 1g potassium iodide was taken in test tube and boiled. This mixture was transferred to 20ml of 5% potassium iodide solution. The resultant solution was titrated with 0.1N sodium thiosulphate solution using starch as an indicator.

##### 5. Identification of oil actives

*Moringa oleifera* seed oil was run over TLC plate using silica gel-G as stationary phase. After the sample has been applied on the plate, solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. The qualitative evaluation of the plate was done by determining the migrating behavior of the separated substances given in the form of R<sub>f</sub> value[12].

### Analytical methods

#### 1. UV spectroscopy

The ultra-violet absorption spectrum of *Moringa oleifera* seed oil in n-hexane was obtained using UV-Visible spectrophotometer (Shimadzu 1650) in the range of 400-200 nm[13].

## 1. Infrared spectroscopy

### Oil-excipients interaction study

FT-IR spectroscopy used to determine the molecular interaction between oil and excipients. FT-IR measurement of oil was taken at ambient temperature. The blend of *Moringa oleifera* seed oil and other excipients (Tween 80 & Span - 80) used for final formulation were analyzed by FT-IR spectrophotometer (FT-IR 8400-S; Shimadzu, Japan). Drop of oil and blend placed on the thin polymeric film were scanned over a wave number range of 4000 to 400 cm<sup>-1</sup> in FT-IR instrument and spectral analysis was done[14].

### Formulation of Moringa seed oil microemulsion

#### 1. Construction of ternary phase diagram

The ternary phase diagrams were constructed using water titration method[15] to determine the microemulsion region and to detect the possibility of making microemulsions with different possible compositions of oil, surfactant/s and water. Various ratios (1:9, 2:8, 3:7... to 9:1) of Tween 80 and Span 80 mixed with oil phase. These mixtures were titrated with double distilled water by drop wise addition using micropipette and stirred on magnetic stirrer at room temperature. After each addition, the system was examined for the appearance and flow properties. The end point of the titration was the point where the solution becomes cloudy or turbid. The quantity of the aqueous phase required to make the mixture turbid was noted. The microemulsion region was identified as transparent and isotropic mixture. All these value substituted in CHEMIX School Software and ternary phase diagram was constructed by plotting amount of oil, surfactant, and water phase combination.

#### 2. Formulation of microemulsion

Appropriate quantities of Tween 80, Span 80 and *Moringa oleifera* seed oil were weighed mixed in glass beaker to make transparent mixture and vortexed for 15min. The appropriate amount of double distilled water was added with help of micropipette followed by stirring on magnetic stirrer at room temperature to obtain microemulsion system. While formulating the microemulsion the surfactant blend of 83% Span 80 and 17% of Tween 80 (S<sub>mix</sub>) was taken by considering the required HLB for the preparation. Different compositions as shown in Table 1; were used to develop microemulsion formulation using *Moringa oleifera* seed oil.

Table 1: Formulation of microemulsion using *Moringa oleifera* seed oil

Sr. No.	Batch No.	Oil (%)	S <sub>mix</sub> (%)	Water (%)
1	F1	8.57	77.12	14.31
2	E2	17.16	68.66	14.16
3	<b>F3</b>	<b>25.30</b>	<b>59.02</b>	<b>15.68</b>
4	E4	33.81	50.71	15.46
5	E5	44.17	44.17	11.66
6	F6	57.58	38.38	4.03
7	E7	65.54	28.08	6.37
8	E8	73.87	18.47	7.66
9	F9	82.72	9.19	8.09

### Characterization of prepared herbal microemulsion

#### 1. Thermodynamic stability

To overcome the problem of metastable formulation, thermodynamic stability tests were performed in following manner.

**a. Heating-Cooling Cycle:** Six cycles were carried out between temperature 4°C and 45°C for 48h and the formulations were observed for phase separation. Formulations that did not show any phase separation were taken for centrifugation test[16].

**b. Centrifugation Test:** Formulations were centrifuged at 3000 rpm for 30 min. The formulations with no phase separation were used in a Freeze–Thaw Cycle Stress Test[17].

**c. Freeze–Thaw Cycle Stress Test:** The formulations were subjected to a total of three complete freeze–thaw cycles, each cycle consisting of 24 h at 25°C followed by 24 h at –5°C[18].

The formulations that survived thermodynamic stability tests as described earlier were selected for the further characterization.

#### 2. Globule size and Zeta Potential

Average globule size, Zeta Potential and Polydispersity Index of microemulsion were determined by using dynamic light scattering Malvern Zetasizer Nano-ZS90 (Malvern Instruments, UK). Sample was prepared by dissolving 1g of sample in 10ml of dispersion medium i.e. distilled water in glass test tube. The sample was loaded into transparent cuvette having volume of 1cm<sup>3</sup> in the thermostatic chamber at 25°C. Laser light scattering was monitored at fixed angle 90°.

### 3. Transparency and Refractive Index

Transparency and homogeneity of microemulsion was determined by measuring the percentage transmittance at 650nm by UV spectrophotometer against water as reference. The Refractive Index of pure oil and microemulsion was determined using an Abbe's refractometer at  $25 \pm 0.5^\circ\text{C}$ . One to two drops of sample applied and reading was recorded.

### 4. Determination of viscosity

The viscosity of optimized microemulsion was determined using Brookfield viscometer (LVII, Brookfield Inc., USA) using spindle no.4 with shear rate 100 rev/ min. The measurement was done at room temperature[19].

### 5. pH measurement

The pH of optimized formulation was determined by pH meter (PICO+ Lab India); which was calibrated by using phosphate buffer solutions i.e. pH 4, 7 and 9 (triple point calibration). The electrode was inserted into the sample 10 min prior to study at room temperature[20].

### 6. *In-vitro* permeation study

*In-vitro* drug release study of microemulsion containing *Moringa oleifera* seed oil was carried out using modified Franz diffusion cell with cellulose acetate membrane having  $0.45\mu$  pore size and 1mm thickness. The cellulose acetate membrane was hydrated for 24h prior to study. The membrane was placed between donor and receptor compartment. The receptor compartment was filled with methanolic phosphate buffer (pH 6.8) and maintained temperature at  $37 \pm 0.5^\circ\text{C}$  using a circulating water bath. The receptor cell was stirred with a small magnetic bar at a 500 rpm. The donor compartment was filled with accurately measured 1g optimized microemulsion and pure oil separately. The amount of drug released from microemulsion and pure oil was analyzed using UV spectrophotometer (Shimadzu 1650, Japan) at 240 nm[21].

### Anti-inflammatory activity

#### 1. Vascular permeability test

Vascular permeability test [22] was performed to find out anti-inflammatory effect of prepared microemulsion formulation. The Institutional Animal Ethics Committee had approved the experimental protocols for animal study (IAEC No. 1093/A/07/CPCSEA) and care of animals was taken according to CPCSEA guidelines. Wistar albino rats of either sex weighing 180–200g were used for this study. The animals were grouped in polyacrylic cages and maintained under standard laboratory conditions (temperature  $25 \pm 2^\circ\text{C}$ ) and relative humidity ( $50 \pm 5\%$ ) with dark and light cycle (14/10 h). They were allowed free access to standard dry pellet diet and water ad libitum. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. The five groups of animal were categorized as follows

- Group 1: Vehicle (Inflammation control)
- Group 2: Piroxicam gel 0.5% topically
- Group 3: *Moringa oleifera* seed oil 0.5% topically
- Group 4: Microemulsion 0.5% topically
- Group 5: Microemulsion 1.62%, topically

Animals were anaesthetized using 0.1ml of 0.1% chloral hydrate solution given orally. The effect on blood vessels was observed under motic microscope at 10min time interval for 1h.

#### 2. Carrageenan induced paw edema in rats

Carrageenan induced paw edema method [23] was performed to confirm anti-inflammatory effect of prepared microemulsion formulation. The Institutional Animal Ethics Committee had approved the experimental protocols ((IAEC No. 1093/A/07/CPCSEA) and care of animals was taken according to CPCSEA guidelines. Anti-inflammatory activity of prepared formulation was evaluated and compared with marketed preparation. Healthy Wistar albino rats weighing 180-200g were used in this experiment and animals were starved overnight. Four groups consisting of five animals were employed in the present study.

- Group 1: Vehicle
- Group 2: Piroxicam gel 0.5% topically
- Group 3: *Moringa oleifera* seed oil 0.5% topically
- Group 4: Microemulsion 1.62% topically

The animals were housed in cage maintain temperature at  $25 \pm 1^\circ\text{C}$  and  $60 \pm 5\%$  relative humidity. 0.1ml of 1% w/v solution of carrageenan in normal saline solution was injected into sub planer tissue of left hind paw of each rat. The

paw is marked with ink at particular level and immersed in mercury up to this mark. Microemulsion, pure oil and standard formulation were applied topically to the paw of the animals and spreaded gently; before and after 1, 2, and 3 h the injection of carrageenan into left hind of paw. Measurement of foot volume was performed using digital Plethysmometer.

The % inhibition of edema was calculated as:

$$\% \text{ inhibition of edema} = (C_t - C_0)/C_0 \times 100$$

Where,

$C_t$  = Paw thickness after injection of carrageenan

$C_0$  = Paw thickness before injection of carrageenan

### Skin irritation study

Three healthy albino rabbits of around 18 months age and weighing 1.5-2kg were selected for this study. Dorsal surface of rabbit was cleaned and hairs were removed by shaving of three different region of dorsal surface prior to study. Shaved skin was cleaned with rectified spirit. Pure oil, microemulsion were applied on shaved surface area. The animals were observed for erythema, edema, and inflammation for 72h after application[24].

### Accelerated stability study

The optimized microemulsion was stored in closed glass vials at 40°C and 75% RH for three months in stability chamber. The physical parameters were determined at the end of study and compared with initial results. The microemulsion was subjected to centrifugation at 3,500 rpm for 30 min at different time intervals to observe phase separation[25].

### Statistical analysis

All data are reported as Mean  $\pm$ SEM and groups were compared using ANOVA with  $p < 0.05$  considered statistically significant.

## RESULTS AND DISCUSSION

### Physicochemical properties of *Moringa oleifera* seed oil

Table 2: Physicochemical characteristics of *Moringa* seed oil

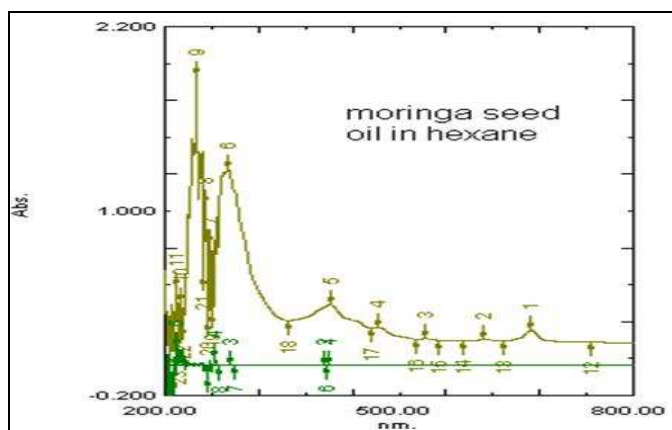
Characteristics	Observation
% yield of oil in petroleum ether	24%
Color	Pale yellow
Odor	Characteristic
Refractive index	1.458
Saponification value	195.1mg KOH/g
Iodine value	86.7 gI <sub>2</sub> /100g
Peroxide value	7.52 mEq/Kg

The color and odor of *Moringa oleifera* seed oil found to be pale yellow and characteristic respectively. The Refractive Index of *M. oleifera* seed oil (1.458) was in close agreement with values reported for conventional oils from soybean (1.466- 1.470) and palm kernel (1.449- 1.451). The high refractive index of this oil seems to confirm the high number of carbon atoms in their fatty acids [26]. Refractive index also increases as the double bond increases [27]. The saponification value indicates average molecular weight of fatty acid contents as glyceride in oil. Generally higher number of saponification value of oil used in manufacturing of soap. Iodine value is reflection of unsaturated degree of fats and oil and therefore, the high value; indicate high number of unsaturated double bonds. Peroxide value is an index of rancidity, thus low peroxide value indicates resistance of the oil to peroxidation during storage. The peroxide value of *Moringa oleifera* seed oil is low (7.52 mEq/Kg) compared to the maximum acceptable value of 10 meq KOH/g set by the Codex Alimentarius Commission for groundnut seed oils [28]. The oil is thus stable and would not easily go rancid.

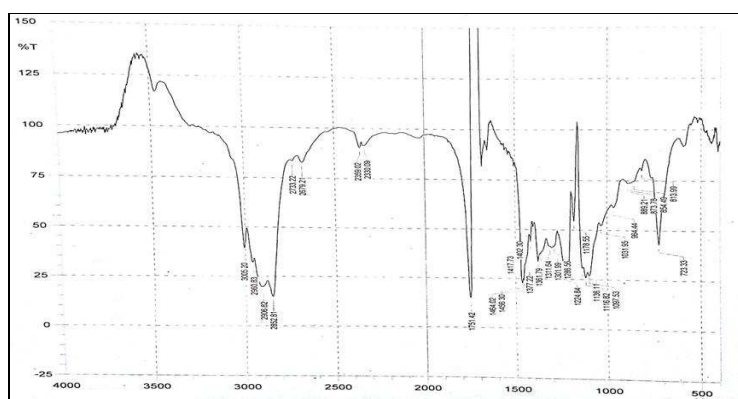
### Analytical methods

#### UV- spectroscopy

As shown in Fig 1 *Moringa oleifera* seed oil containing oleic acid exhibited absorption maxima at 240 nm; whereas palmitic acid and stearic acid showed absorbance at 281 nm and 411 nm respectively in n-Hexane.

Fig 1: UV spectra of *M. oleifera* seed oil in n-Hexane

### Infrared spectroscopy

Fig 2: IR Spectrum of *Moringa oleifera* seed oil

IR spectral analysis of *Moringa oleifera* seed oil has shown peak  $3200-2800\text{cm}^{-1}$  C-H alkane stretch,  $1450-1375\text{cm}^{-1}$   $\text{CH}_3$  bending,  $1600-1900\text{cm}^{-1}$  C-C alkane,  $1000-650\text{cm}^{-1}$  alkene out of plane bend,  $1850-1650\text{cm}^{-1}$  C=O carbonyl stretch.

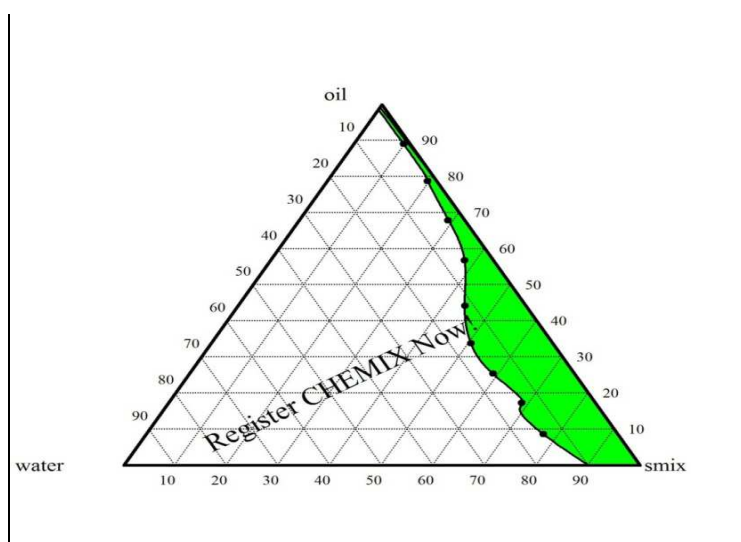


Fig 3: Ternary phase diagram for Moringa seed oil microemulsion

### Construction of ternary phase diagram and microemulsion formulation

Ternary phase diagram constructed using various ratio of Moringa seed oil, Tween 80, Span 80 and water. Microemulsion existence regions were identified from ternary phase diagram. There was no phase inversion from water-in-oil to oil-in-water microemulsion observed. Ternary phase diagram with 83% Span 80 and 17% Tween 80

( $S_{mix}$ ), oil and water with different concentrations is depicted in Fig 3. Batch F3 with maximum water and more stability for a week after formulation was selected for further characterization. It was observed that when  $S_{mix}$  ratio becomes more lipophilic, the microemulsion zone moved to the lipophilic side of the diagram, vice-versa.

#### Thermodynamic stability of microemulsions

Visual examination showed that all microemulsion systems were stable after being subjected to heating-cooling, centrifugation and freeze-thaw cycles.

#### Globule size and polydispersity index and zeta potential analysis

Globule size and polydispersity index are important physical characteristics of microemulsion; as they can affect the stability of microemulsion. Globule size of microemulsion was observed between 10-200 nm ranges. This micro size is useful for topical drug delivery; as it promotes the skin permeation. The mean diameter of globule denoted by Z-Average (d. nm) was found to be 167nm. The globule size distribution graph was found to be bell shaped with even distribution range (Fig 4). The low polydispersity index indicates higher uniformity of particle size in the formulation. Polydispersity index of Moringa microemulsion of was found to be 0.3; indicating higher uniformity of globule size in formulation. Zeta potential of optimized microemulsion was found to be in the range of -35.8 indicating no agglomeration and stability of the microemulsion[29].

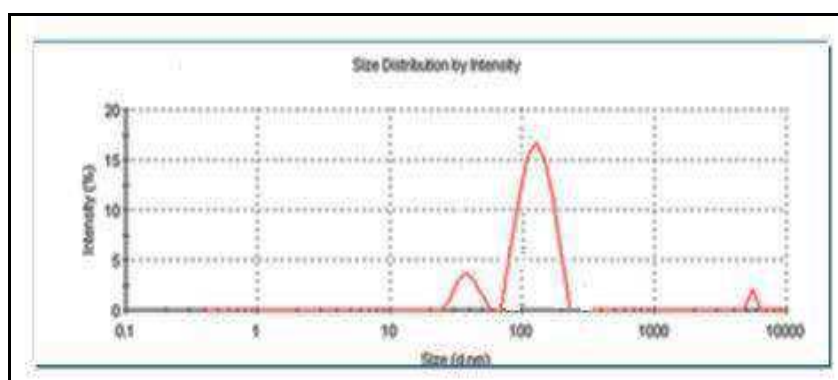


Fig4: Globule size and size distribution of optimized microemulsion

#### Transparency

Microemulsion was found to be transparent and homogeneous phase system. The percentage transmittance of formulation was found to be 99.60. The Refractive Index (RI) of *M. oleifera* seed oil microemulsion was found to be 1.469 suggesting no change RI value of Moringa seed oil (1.458) after formulating into microemulsion system.

#### Viscosity

Viscosity is important parameter for topical delivery. Higher viscosity is preferred as it increases residence time but permeation rate also decreases with increase in viscosity and hence formulation should have moderate viscosity. The viscosity of optimized microemulsion was found to be 88.7 cps.

#### pH measurement

The pH deviations may cause irritation to the patient; hence it should be within range of skin pH. The pH of formulation was found to be 7.1; that is suitable for topical application.

#### In-vitro permeation study

The drug release study of *Moringa oleifera* seed oil through cellulose acetate membrane was observed using Franz diffusion cell. Microemulsion showed higher permeation rate than pure oil. Various factors affecting on percutaneous penetration study such as oily phase nature, globule size, and composition of microemulsion. Small globule size of droplets can interact with number of vesicles on fixed area of stratum corneum and increasing efficacy of percutaneous uptake. The thermodynamic activity in formulation is a significant driving force for the release and penetration of drug into skin. The drug get released from inner phase to outer phase and then further into skin[21]. Tween 80 not only acts as surfactant but also act as penetration enhancer[30]. The percentage release of drug from pure oil and microemulsion was found to be 54.06 and 76.86 respectively.

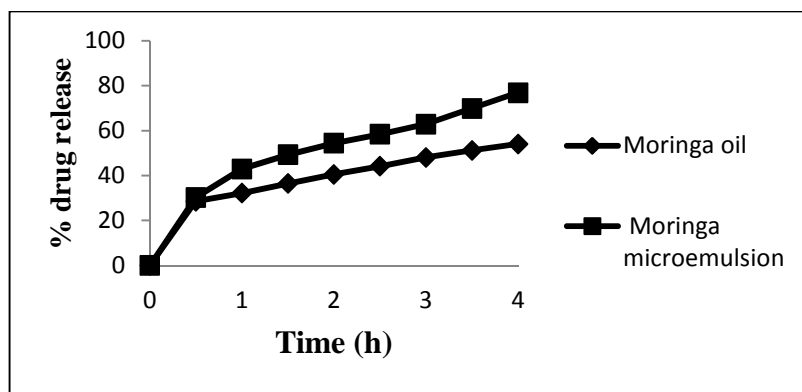


Fig 5: *In vitro* drug release from Moringa oil and Moringa microemulsion Anti-inflammatory activity

### 1. Vascular permeability study

Inhibitory effect of *Moringa oleifera* seed oil, microemulsion, and standard formulation on blood vessels of rat was observed. The vascular permeability indicates acute phase of inflammation where there is increased vascular permeability and migration of leukocytes in to the inflamed area occurs. Constriction of blood vessels indicates reduction in vascular permeability[31]. The microemulsion preparation caused constriction of blood vessels indicating reduction in vascular permeability and thus anti-inflammatory potential. The results in Fig 6; indicate anti-inflammatory potential of Moringa seed oil microemulsion.

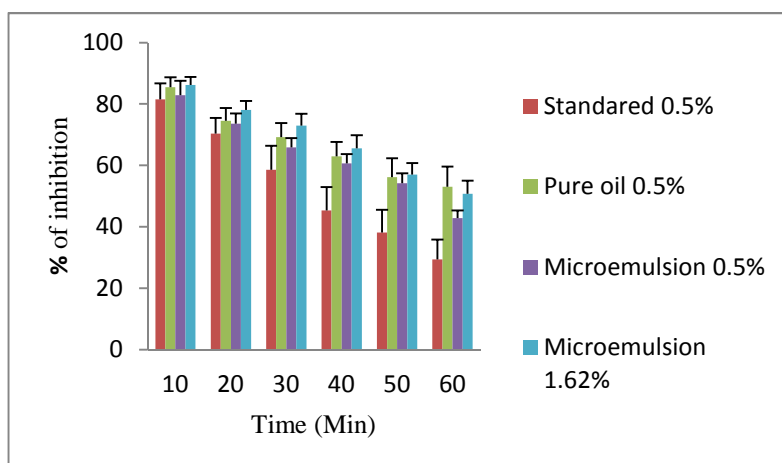


Fig 6: Inhibitory activity of pure oil and microemulsion

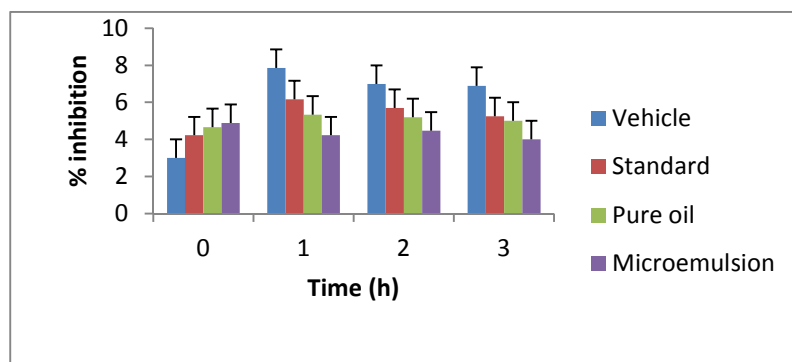


Fig7: % inhibition in carrageenan induced paw edema in rats

### 2. Carrageenan induced paw edema

Carrageenan injected in sub-planter region of hind paw induced inflammation is time dependent. Carrageenan induced paw edema is standard experimental model for acute inflammation and widely used as a model for the evaluation of anti-inflammatory activity of drugs. Carrageenan-induced oedema is a biphasic event, with early hyperemia due to the release of histamine and serotonin and the delayed oedema due to the release of bradykinin and



prostaglandin[24]. Standard formulation (Piroxicam 0.5%), pure oil, microemulsion decreased inflammation about 29.96%, 35.16% and 46.84% respectively. The microemulsion containing *Moringa oleifera* seed oil was found to be superior to standard formulation. Treatment with pure oil and microemulsion significantly inhibited paw edema. The effect of *Moringa* seed oil and *Moringa* microemulsion was compared with standard formulation (Fig 7).

#### Skin irritation study

Skin irritation study did not show erythema, edema and inflammation on rabbit skin after 72h. Skin irritation study revealed that optimized formulation was non-irritant and non-sensitizing to skin.

#### Accelerated stability study

Optimized microemulsion was found to be stable over period of three months as there was no significance change in parameters of microemulsion found (Table 6). There was no phase separation even after centrifuged test.

**Table 3: Observations of stability study**

Sr. No.	Parameter	0 month	1 month	2 months	3 months
1	Appearance	Clear	Clear	Clear	Clear
2	pH	7.1	7.0	6.9	6.8
4	Viscosity (cps)	88.7	88.0	87.8	87.2
5	% Transparency	99.60	99.40	98.50	98.45

### CONCLUSION

Topical microemulsion of *Moringa oleifera* seed oil was successfully developed using widely accepted safe excipients and reproducible methodology. This microemulsion system has shown acceptable parameters with better skin permeation as compared to pure oil. Microemulsion shown promising anti-inflammatory activity up to 3h. This microemulsion proved to be non-irritant to skin and stable over study period.

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