ORIGINAL ARTICLE

Preparation and Evaluation of Hydrogel Formulation Containing *Ocimum sanctum* Leaves Extract for Anti-inflammatory Activity

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Abstract

Aim: The aim of the present study was to evaluate the anti-inflammatory effect of *Ocimum sanctum* leaves in the formulation of hydrogel using different types of animal models. **Materials and Methods**: Ethanolic extract of *O. sanctum* leaves was fractionated with methanol to get methanolic fraction. Methanolic fraction (1%vw/w) was used for hydrogel formulation with different combinations of Carbopol 940 and sodium CMC. Prepared hydrogel was characterized for optimum physical characters, pH, spreadability, homogeneity, viscosity, release profile, and irritation effect. Optimized hydrogel was evaluated for anti-inflammatory effect using xylene-induced, croton oil-induced ear edema, and cotton pellet-induced granuloma model in animals. Effect was monitored by the measurement of percent inhibition, nitric oxide (NO), and myeloperoxidase (MPO) levels in mice ears after acute inflammation induced by croton oil. **Results and Discussion**: Physical evaluation confirmed that the color of the prepared hydrogels was brownish and appearance was homogeneous and smooth on application. All other evaluation parameters, for example, pH, viscosity, spreadability, and consistency were found suitable in F3 hydrogel formulation combination. Hence, the optimize composition of F3 formulation was observed as 1.5 g of Carbopol 940 and 1% of sodium CMC. Percent inhibition of edema in xylene-induced ear edema in mice was found comparable to standard group of treatment (65.59%). In the present study, result indicates that prepared hydrogel OSMFH possesses inhibitory effects against acute inflammation. The results showing the ability of croton oil to induce neutrophil influx into mice ear tissue. This may be reduced by treatment with extract hydrogels, as well as Voltaren Emulgel, reduced significantly ($P < 0.01$) the MPO levels and NO levels in mice ears. **Conclusion**: The methanol extracts were exhibits its anti-inflammatory action significantly may be due to the presence of flavonoids in *O. sanctum* leaves.

Key words: *Ocimum sanctum*, Anti-inflammatory, Hydrogel, Voltaren Emulgel, Carbopol 940

INTRODUCTION

Inflammation is one of the disorders which have affecting 25% of world population. The inflammatory disorders that result in the immune system attacking the body's own cells or tissues may cause abnormal inflammation, which results in chronic pain, redness, swelling, stiffness, and damage to normal tissues. The inflammatory process works quickly to destroy and eliminate foreign and damaged cells, and to isolate the infected or injured tissues from the rest of the body.[1]

Ocimum sanctum, commonly known as holy basil, tulasi, or tulsi, is an aromatic perennial plant in the family Lamiaceae. Different parts of tulsi plant, for example, leaves, flowers, stem, root, seeds, etc., have been used traditionally as expectorant, analgesic, anticancer, antiasthmatic, antiemetic, diaphoretic, antidiabetic, antifertility, hepatoprotective, hypotensive, hypolipidemic, and antistress agents. Tulsi has also been used in the treatment of fever, bronchitis, arthritis, convulsions, etc., aqueous decoction of tulsi leaves is given to patients suffering from gastric and hepatic disorders.

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The leaf volatile oil[2] contains eugenol (1-hydroxy-2-methoxy-4-allylbenzene), eugenol (also called eugenic acid), ursolic acid, carvacrol (5-isopropyl-2-methylphenol), linalool (3,7-dimethylocta-1,6-dien-3-ol), limatrol, caryophyllene (4,11,11-trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene), and methyl carvicol (also called Estragole: 1-allyl-4-methoxybenzene) while the seed volatile oil have fatty acids and sitosterol; in addition, the seed mucilage contains some levels of sugars and the anthocyans are present in green leaves. The sugars are composed of xylose and polysaccharides.[3] On the basis of previous research and literature, the present study was aimed to evaluate the anti-inflammatory effect of *O. sanctum* leaves in the formulation of hydrogel using different types of animal models. *O. sanctum* leaves were selected on the basis of traditional knowledge and chemical constituents reported for inflammatory disorders in the available literature.

MATERIALS AND METHODS

Chemicals and reagents

Carbopol 940, sodium carboxy methyl cellulose, methyl paraben, and propyl paraben were purchased from Otto Chemie Pvt. Ltd., Mumbai, India. Triethanolamine, myeloperoxidase (MPO), nitric oxide (NO), naphthylethylenediamine dihydrochloride, sulfanilamide, and hexadecyltrimethylammonium bromide were purchased from Sigma-Aldrich Mumbai, India. Xylene and croton oil were purchased from HiMedia Lab, Mumbai, India. All other general reagents and solvents were used of analytical grade.

Identification and collection of plant material

O. sanctum leaves were collected from the surrounding of SRK University Campus, Bhopal, Madhya Pradesh, India, during August–September month of 2018. Plant specimen identified and authenticated in Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur. Collected plant leaves were shade dried for powdering and then store for extraction and other studies.

Phytochemical screening

Dried powdered leaves of *O. sanctum* (150 g each) were extracted with petroleum ether for defatting and then dried the mark for further extraction with ethanol, repeatedly. After completed extraction process, the ethanol was evaporated completely and further treated with methanol to obtain methanol soluble fraction. Finally, methanol extract fraction dried under reduced pressure, for further use. Phytochemical studies consisting initially qualitative chemical screening of chemical constituents using different chemical tests to detect the presence or absence of different chemical constituents and to identify them qualitatively.[4-6]

Formulation preparation

Five different hydrogel formulations containing extract and one control without extract were prepared according to the modified method of Chirayath *et al*. (2019).[7] Briefly, different proportions of Carbopol 940 and sodium CMC were dispersed in 50 ml of distilled water with continuous stirring. About 5 ml of distilled water was taken and required quantity of methyl paraben and propyl paraben was dissolved by heating on water bath and then cooled. Glycerin was added to this solution. Accurately 1%w/w of extract was taken to get optimized formulation and was added to the above swollen polymer under continuous stirring at 700 rpm in close vessel and maintaining the temperature 30°C until homogeneous gel was obtained and volume made up to 100 ml by adding remaining distilled water. At the end finally, required amount of 98% triethanolamine was added dropwise to the formulation for adjustment of required skin pH (6.8–7) and stirred slowly to mix uniformly [Table 1]. The similar method was followed for the preparation of control formulation without adding any plant extract.

Characterization of prepared hydrogel formulation

All prepared hydrogel formulations were characterized using the following parameters:

Physical evaluation

Physical parameters such as color and appearance of the herbal gel were observed visually.

Measurement of pH

The pH of various gel formulations was determined using digital pH meter. One gram of gel was dissolved in 100 ml distilled water and stored for 2 h. The measurement of pH of each formulation was done in triplicate and average value was calculated.

Table 1: Formulation composition of hydrogel containing methanol fraction of *Ocimum sanctum* leaves (OCMFH)

Ingredients	F1	F2	F3	F4	F5
Carbopol 940 (gm)	0.5	1	1.5	2	3
Sodium CMC	3	2	1	1	0.5
Extract (%w/w)	1	1	1	1	1
Propylene glycol 400 (5%)	5	5	5	5	5
Methyl paraben (0.5%) (ml)	0.2	0.2	0.2	0.2	0.2
Propyl paraben (0.2%) (ml)	5	5	5	5	5
Triethanolamine (ml)	q. s.	q. s.	q. s.	q. s.	q. s.
Distilled water (ml) q.s.	100	100	100	100	100

Spreadability

Spreadability was determined by the apparatus which consists of a wooden block, provided with pulley at one end. By this method, spreadability was measured on the basis of slip and drag characteristics of gels. An excess of gel (about 2 g) under study was placed on the ground slide. The gel was then sandwiched between this slide and another glass slide having the dimension of fixed ground slide and provided with the hook. One kilogram weight was placed on the top of the two slides for 5 min to expel air and to provide a uniform film of the gel between the slides. Excess of the gel was scrapped of from the edges. The top plate was then subjected to pull of 80 g weight with the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5 cm was noted (Phad *et al*.)[8] A shorter interval indicates better spreadability. Spreadability was calculated using the formula given below:

$S = M \times L/T$

Where, S = Spreadability, M = Weight in the pan (tied to the upper slide), L = Length moved by the glass slide, and T = Time (in s) taken to separate the slide completely each other.

Homogeneity and viscosity

All the developed hydrogels were tested for homogeneity by visual inspection after setting the gels in the container. They were observed for their appearance and presence of any aggregates.

Viscosity of gel was measured using Brookfield viscometer with spindle No. 7 at 50 rpm at room temperature. The corresponding dial reading was noted. The viscosity of the gel was obtained by multiplication of the dial reading with factor given in the Brookfield viscometer manual.

Drug content determination

One gram of the prepared gel was mixed with 100 ml of suitable solvent ethyl alcohol. Aliquots of different concentration were prepared by suitable dilutions after filtering the stock solution and the drug content was determined measuring the absorbance at 253 nm using UV/Vis spectrophotometer (Shimadzu UV 1700).

In vitro drug release study of hydrogel

Franz diffusion cell with a diameter 3.7 cm was used in *in vitro* release studies. A glass tube with both ends open, 10 cm height and 3.7 cm outer diameter, was used as a permeation cell. Accurately, 1 g sample was weighed and placed on a semipermeable cellophane membrane to occupy a circle of 3.7 cm diameter. The loaded membrane was stretched over the lower open end of a glass tube of 3.7 cm diameter and made watertight by rubber band. The tube (donor compartment) was immersed in a beaker containing 100 ml of phosphate buffer pH 6.8 (receptor compartment). The cell was immersed to a depth of 1 cm below the surface of buffer. The system temperature was maintained at 37°C ± 1°C and speed was maintained at 30 rpm throughout the experiment by magnetic stirrer. Samples 3 ml were withdrawn at intervals of 15, 30, 45, 60, 90, 120, 180, and 240 min, the volume of each sample was replaced by the same volume of fresh buffer to maintain constant volume. Samples were analyzed without dilution or filtration for luteolin content spectrophotometrically at 253 nm.[9,10] Calibration curve of luteolin was prepared of known concentrations in the appropriate range, to determine the amount of drug released.

Dermal irritation study

A primary skin irritation test was conducted on rabbits to determine the potential of hydrogel to produce an irritation after a single topical application. Three healthy young adult albino rabbits of either sex were allows to free access to laboratory and fed commercial pellets diet with water *ad libitum*. Animals were acclimated to laboratories conditions for a period of 9 days before initiation of dosing. Animal room was kept at a constant temperature (19–24°C).

On the day before application, hairs of rabbits were removed from the dorsal and trunk area using a small animal clipper. On the day of dosing, but before application, the animals were examined for health and the skin checked for any abnormalities. No preexisting skin irritation was observed. 2–3 g of the hydrogel was applied to 6 cm^2 intact area on each animal and caged. After 4 h of exposure to hydrogel, the test sites were gently cleaned from any residual substance. Individual evaluation of test dose was scored according to Draize scoring system at approximately 1, 24, 48, and 72 h after removal of hydrogel.[11] The degree of irritancy was obtained by calculating the primary dermal irritation index (PDII).

$$PDII = \frac{(\text{PDI for 1,24, 48 and 72 h})}{4}$$

Stability studies of prepared hydrogel formulations

All prepared hydrogel formulations were subjected to a stability testing for 1–6 months as per ICH norms at a temperature and RH of 40°C ± 2°C/75% RH ± 5% RH, respectively.

Stability of prepared hydrogel was evaluated in terms of physical changes, which would affect the stability and acceptability of the formulations.

Hydrogel formulations were evaluated in terms of physical changes such as phase separation and color changes, odor, and consistency of the formulations, thereby affecting their stability and other desired formulation properties. Test samples

of the hydrogel formulation were kept at different temperature conditions like at 40°C and room temperature for 30 days. Samples were periodically observed for physical changes such as consistency and development of objectionable color and odor.

Anti-inflammatory activity of prepared hydrogel

Animal protocol

Healthy Swiss albino mice of either sex weighing between 95 and 100 g were selected for anti-inflammatory studies. They will be kept in the animal house in a controlled room temperature at 25°C ± 2°C, relative humidity 44–56%, and light and dark cycles of 10 and 14 h, respectively, for 1 week before the experiment. The animal care and experimental protocols were in accordance with CPCSEA/IAEC. The animal was randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days before dosing to allow for acclimatization to the laboratory conditions. The animals will be grouped and housed in polyacrylic cages for the experiment. Animals had free excess to water and food. However, they were fasted for 48 h before the operative procedure. The animals were divided into following groups containing five animals each group.

Group I was control and given hydrogel base without extract, topically to each animal in the group

Groups II was given prepared hydrogel of methanol fraction of *O. sanctum* (OSMFH) topically to each animal of group

Group III was referred as standard and given Voltaren Emulgel (1%, diclofenac sodium; Novartis India Ltd.) topically to each animal of group

Xylene-induced ear edema

Swiss albino mice were received topical application (20 mg/ear) of extract hydrogel formulation on the anterior surface of the right ear while xylene (0.05 ml) was simultaneously applied on the posterior surface of the same ear.

Control animals received an equivalent volume of the vehicle plain gel, on the anterior ear surface. The left ear was remains untreated. Three hours after xylene application, both ears were cut and ear lobes were weighed. The difference in the weight between the right treated and left untreated ears was calculated and used as a measure of edema.[12] The level of inhibition (%) of edema was calculated using the following equation:

$$\%\ \text{edema} = \frac{\text{wt of right ear} - \text{wt of left ear}}{\text{wt of left ear}} \times 100$$

Edema was expressed as the percentage difference between the weight of the inflamed right ear and the non-inflamed left ear of each mouse.

Croton oil-induced ear edema

The acute anti-inflammatory potential assay was conducted according to previously reported method, with slight modifications.[13] In each mouse, 20 µL of a fresh solution of 2.5% croton oil were topically applied on the inner surface of the right ear, while in the left ear, an equal volume of gel base was applied as control. Six hours after induction of inflammation, the animals were euthanized and 6 mm diameter ear punch biopsies were collected. For edema quantification, ear punch biopsies were individually weighed using an analytic balance immediately after collection and measure percent edema inhibition as similar to the previous method. Then, mouse ears were stored at –80°C for posterior quantification of inflammatory parameters and histological studies.

Collected ear biopsies were chopped and homogenated in 500 µL of phosphate-buffered saline pH 7.4 (137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 10 mM Na_2HPO_4). Then, samples were centrifuged at 10,000 g at 4°C for 10 min. The supernatants were used for quantification of nitrite, as an indirect marker of NO production. The pellets were further employed for MPO enzyme estimation, as an indirect marker of neutrophil migration.

Determination of NO

In the inflammatory process, release of NO and reactive oxygen species occurs, which together causes tissue damage, through an inflammatory reaction to increase the synthesis and release of inflammatory mediators, such as cytokines, reactive oxygen species (ROS), and arachidonic acid derivatives including prostaglandins E2. Besides this, NO is a potent vasodilator, which is involved in the inflammation process, leading to edema formation.[14-16]

NO levels in mice ears were quantified indirectly by Griess reaction, which quantifies nitrite, a product of the reaction of NO with oxygen.[17] Briefly, 50 µL of each ear supernatant was mixed with 50 µL of 1% sulfanilamide in 5% phosphoric acid and incubated in dark at 22°C for 5 min. Then, 50 µL of 0.1% naphthylethylenediamine dihydrochloride was added and the absorbance at 540 nm was read on a microplate reader (ELISA, Micro Lab, Ahmedabad, India). The amount of nitrite was calculated from a sodium nitrite standard curve and expressed as nmol of nitrite per ear.

MPO enzyme estimation

The activity of tissue MPO was assessed 24 h after croton oil application to the mouse ear according to the method described by Krawisz *et al*.[18] In brief, 6 mm ear tissue was punched and minced in 10 mL of ice cold 50 mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyltrimethylammonium bromide and homogenized.

The homogenate was sonicated and centrifuged at 4°C for 20 min at 12,000 g (Remi Centrifuge, India). The MPO activity was determined spectrophotometrically by adding 2.9 mL of 50 mM phosphate buffer in 0.0005% hydrogen peroxide to 0.1 mL of the supernatant. The enzyme activity was determined at 460 nm. One unit of MPO activity was defined as the change in absorbance per min at room temperature. The activity was presented as the percentage of MPO relative to vehicle-treated rat.

Histological study

Ear biopsies from control, test, and standard group were taken and were fixed in 10% buffered formaldehyde solution, dehydrated, and embedded in paraffin. Sections of 5 µm of thickness were obtained for hematoxylin-eosin staining and examined by light microscopy (100×) for the evaluation of edema intensity and leukocyte infiltration.[19]

Cotton pellet-induced granuloma in rats

This model is based on the foreign body granuloma which is provoked in animals by subcutaneous implantation of pellets of compressed cotton. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal.[20]

The animals were divided into five groups, fasted overnight, and allowed free access to water. Animals were kept under aseptic conditions for the entire duration of the study. They were administered with vehicle, standard drug, and test drugs. One hour after the first dosing, the animals were anesthetized with anesthetic ether and 20 mg of the sterile cotton pellet was inserted one in each axilla and groin of rats by making small subcutaneous incision. The incisions were sutured by sterile catgut.[21] Hydrogel/vehicle treatment was continued for the duration of 6 more days. The animals were sacrificed by excess anesthesia on the 8th day and cotton pellets were removed surgically. Pellets were separated from extraneous tissue and dried at 60°C until weight become constant. The net dry weight, that is, after subtracting the initial weight of the cotton pellet was determined. The average weight of the pellet of the control group as well as of the test groups was calculated. The percent change of the granuloma weight relatively with vehicle control was determined and statistically evaluated.

The percentage inhibition increase in the weight of the cotton pellet was calculated using the following formula.

% Inhibition = {Wc–Wd/Wc} × 100

Wd = pellet weight of the drug-treated group.
Wc = pellet weight of the control group

On day 8, cotton pellets were excised. The pellets were dried overnight at 60°C until a constant weight was recorded at two consecutive recordings. The difference between the initial and post-implantation weight was considered to be the dry weight of the granuloma tissue.

Statistical analysis

The data were expressed as standard error mean. The statistical significance of the difference in each parameter among the groups was evaluated using one-way analysis of variance followed by the followed by the multiple comparison test of Tukey–Kramer tests. Criterion for statistically significant difference was chosen to be at $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

The dried powder of *O. sanctum* leaves was subjected to defatting with petroleum ether and extraction with ethyl alcohol to obtain ethanol extracts. The moderately coarse powder of the leaves of *O. sanctum* was subjected to extraction with petroleum ether and ethanol, and yield of both extracts was found to be 2.1 and 2.7% w/w, respectively. Petroleum ether extract of *O. sanctum* showed the presence of steroids and fatty acids while *O. sanctum* ethanol extract showed the presence of alkaloids, flavonoids, and glycosides.

Formulation preparation

Physical properties of prepared hydrogel such as color, appearance, homogeneity, consistency, phase separation, and odor were observed [Table 2]. From the physical evaluation, the color of the prepared gels was brownish in color and appearance of hydrogel was homogeneous and it was smooth on application. The hydrogel was found to be homogenous and good consistency and did not found any phase separation.

The pH value, viscosity, and spreadability of the prepared hydrogels were studied at room temperature. At initial phase, pH of prepared hydrogel was measured using pH meter (Systronics, India) at room temperature found ranging 6.97–7.01. Therefore, the pH of the gel having neutral value was desirable to skin since they did interfere with the physiology of the skin. The pH 7.01 and viscosity between 193,200 and 196,100 cps were recorded for hydrogel formulations containing methanol fraction of *O. sanctum* leaves (OCMFH). Spreadability of both hydrogel formulations was recorded in the range of 14.27–17.64 g. cm/s [Table 3]. The drug content (luteolin concentration) was determined by spectrophotometric method and found as 0.053 µg/ml, for OCMFH. This content was also basis for drug release study of hydrogel formulations.

Percentage drug release of hydrogel containing methanol fraction of *O. sanctum* leaves (OCMFH) was observed initially to be 52.62% and 50.83% (at 240 min) for F1 formulation. Drug release of all formulations F1, F2, F3, F4,

Table 2: Physical properties of prepared hydrogel formulation containing methanol fraction of *Ocimum sanctum* leaves (OCMFH)

Parameters	F1	F2	F3	F4	F5
Appearance	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Color	Brownish	Brownish	Brownish	Brownish	Brownish
Odor	Odorless	Odorless	Odorless	Odorless	Odorless
Consistency	Good	Good	Good	Good	Good
Phase separation	No phase separation	No phase separation	No phase separation	No phase separation	No phase separation

Table 3: Observation parameters of prepared hydrogel formulations containing methanol fraction of *Ocimum sanctum* leaves (OCMFH)

Parameters	F1	F2	F3	F4	F5
pH	7.02	6.96	7.01	7.02	6.95
Viscosity	193,200	194,600	196,100	194,100	195,100
Spreadability (g.cm/s)	15.75±0.47	16.85±0.76	16.21±0.28	15.67±0.58	16.62±0.77

Value represents mean±SEM (n=5)

and F5 is shown in Figure 1. Out of all different formulations, F3 formulation was showing highest percentage drug release at 240 min as 68.31 compared to other formulations. These results showing that the hydrogel formulations containing 1.5% Carbopol 940 and 1% of sodium CMC having higher percentage of luteolin release in 240 min. All other combinations of hydrogel were showing lower percentage of luteolin release up to 240 min. Results of all other evaluation parameters, for example, pH, viscosity, spreadability, and consistency were found suitable in F3 hydrogel formulation combination. Hence, the optimize composition of F3 formulation was observed as 1.5 g of Carbopol 940 and 1% of sodium CMC for the best drug release profile.

During skin irritation study, all prepared formulations were applied on the rabbit skin and its adverse effects such as change in color and swelling of the skin were noted by visual observation as per scoring which are mentioned in Table 4. The results of skin irritation were showed that no any formulation was showed major undesired effect. F3 formulation was showed PDII as 0.25. The objective of this study was to determine the potential of formulations to produce irritation from a single topical application to the skin of rabbits. Results of this test showed that at 1 h after the application, very slight erythema was observed at all three treated sites. The overall incidence and severity of irritation decreased with time. All animals were free from dermal irritation after 48 h. Under the conditions of this study, the PDII for extract hydrogel was found as 0.25. Apart from the dermal irritation noted, all animals appeared active and healthy and with no other signs of unpleasant toxicity or abnormal behavior.

Table 4: Draize scoring system for dermal reactions

Value	Erythema	Value	Edema formation
0	No erythema	0	No edema
1	Very slight erythema	1	Very slight edema
2	Slight erythema (edges of area well defined)	2	Slight edema (edges of area well defined)
3	Moderate severe erythema (defined color and area)	3	Moderate severe edema (area raised approx. 1 mm)
4	Total possible erythema score	4	Total possible edema score

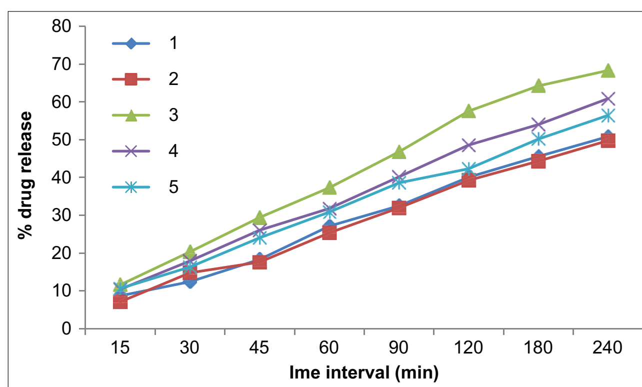

Figure 1: Percentage drug release of different formulations containing methanol fraction of *Ocimum sanctum* leaves (OCMFH)

The stability study of all prepared hydrogel was performed according to the ICH guideline (2013) by keeping at room temperature (27 ± 1°C) for about 30 days and again physical properties are shown in Table 5. After 30 days, it was observed that all properties were same except color. The color of the gel was little faint bluish. There were no phase separation

Table 5: Visual observation of prepared hydrogels containing methanol fraction of *Ocimum sanctum* leaves (OCMFH) at various temperatures

Formulations	Room temperature (RT)		40±2°C	
	15 days	**30 days**	**15 days**	**30 days**
F1	No change in color, odor	No change in color, odor	No change in color, odor	No change in color, odor but slight separation of oil phase
F2	No change in color, odor	No change in color, odor	No change in color, odor	No change in color, odor
F3	No change in color, odor	No change in color, odor	No change in color, odor	No change in color, odor
F4	No change in color, odor	No change in color, odor	No change in color, odor	slight separation of oil phase
F5	No change in color, odor	No change in color, odor	No change in color, odor	No change in color, odor
Hydrogel base	No change in color, odor	No change in color, odor	No change in color, odor	No change in color, odor

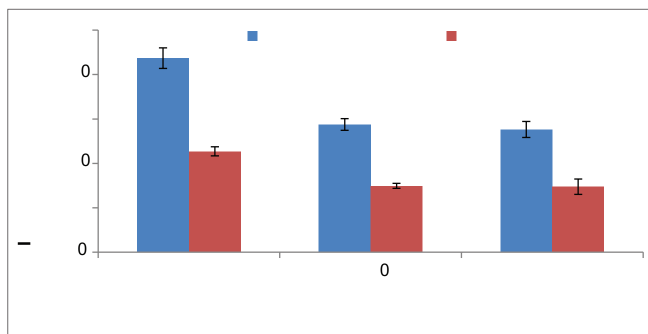

Figure 2: Effect of prepared hydrogels containing flavonoid-rich fractions of both plants on nitric oxide and myeloperoxidase levels in mice ears after acute inflammation induced by croton oil. **P* < 0.01 when compared to the control group and compared by one-way ANOVA followed by Tukey's test. Data showed as mean ± SEM

and liquefaction of the gel in the period of 30 days. Other parameters were evaluated after 30 days. After 30 days, slight changes were recorded in pH in the range of 6.97–7.03.

The spreadability of formulation depends on its viscosity. The spreadability of formulations was recorded for optimized gels and found as 16.21 ± 0.28 g.cm/s for OCMFH. These observations have indicated that the gel was easily spreadable in response to the little force applied. These assured that the formulation could maintain a good wet contact time when applied at the target site.[22]

The pH of the prepared formulations was found in the range of 6.9–7.02 which was observed almost near to the pH of skin. Prepared hydrogel was found to be stable even at room temperatures, and no any separation of oil phase was observed at elevated temperature, 40°C ± 2 °C. There was no evidence of phase separation, development of objectionable odor, or any other evidence of physical instability, and effect on storage at varying temperature of hydrogel was recorded [Table 5].

Natural products have recently fascinated industrial applications not only as the source of pharmaceutical products but also for their advantageous effects on human health. The studies revealed that the hydrogel formulation F3 containing methanol extract of *O. sanctum* leaves (OCMFH) was comparatively better than that of other formulation combination and base. All formulations were found non-irritant and did not show any skin toxicity when applied topically to rabbit skin. The conclusion of the present study has forthcoming therapeutic importance, particularly for patients who are susceptible to develop skin diseases.

Anti-inflammatory activity

Inflammation constitutes body's response to injury and is characterized by a series of events that mainly occur in three distinct phases. The first phase is caused by an increase in vascular permeability resulting in exudation of fluids from the blood into the interstitial space; the second phase involves the infiltration of leukocytes from the blood into the tissue; and the third phase is characterized by granuloma formation and tissue repair.[23] Therefore, it is vital to estimate the activities of the test substance in different phases of inflammation, while evaluating the anti-inflammatory effect. Accordingly, methanol fraction of *O. sanctum* was used for hydrogel formulation and investigated for anti-inflammatory potential using xylene-induced ear edema, croton-induced ear edema, and cotton pellet-induced granuloma inflammation models.[24]

Effect on xylene-induced ear edema

Xylene-induced ear edema in mice is a simple animal model for evaluating potential anti-inflammatory agents, especially in case of fluid accumulation and edema that are characteristic of the acute inflammatory response. In this model, the application of xylene induces neurogenous edema. It is partially associated with the substance P. It

is an undecapeptide, which is widely distributed in the central and peripheral nervous system and it functions as a neurotransmitter or neuromodulator in variety of physiological processes. Substance P is released from the neurons in the midbrain in response to stress, where it facilitates dopaminergic neurotransmission from sensory neurons in the spinal cord in response to noxious stimuli where it excites dorsal neurons. In the periphery, release of substance P from sensory neurons causes vasodilatation and plasma extravasations suggesting its role in neurogenous inflammation. Thus, it can cause the swelling of ear in the mice.

In the present study, hydrogel formulations, OSMFH (1%) was applied topically to observe percent inhibition of xylene-induced ear edema in mice [Table 6]. Effect of OSMFH was observed greater percentage of edema inhibition (63.44%) that was calculated by measurement of ear weight after xylene treatment. Percent inhibition of edema was found comparable to standard group of treatment (65.59%). In the present study, result indicates that prepared hydrogel OSMFH possesses inhibitory effects against acute inflammation.

Table 6: Effect of prepared hydrogel formulations on xylene-induced ear edema in mice

Animal groups	Weight of ear lobe (in gm) Mean±SEM	% inhibition of ear edema
Control (hydrogel base)	0.93±0.06	-
OSMFH (20 mg/day)	0.34±0.07	63.44*
Standard (Voltaren Emulgel)	0.32±0.12	65.59*

Each value is the mean ± S.E.M (n = 5), *P < 0.05 compared with control and standard. OSMFH: Hydrogel containing methanol fraction of *Ocimum sanctum*

Table 7: Effect of extract hydrogel formulations on croton oil-induced ear edema

Animal groups	Weight of ear lobes (in gm) Mean±SEM	% inhibition of ear edema
Control (hydrogel base)	1.07±0.27	-
OSMFH (20 mg/day)	0.54±0.38*	49.53
Standard (Voltaren Emulgel)	0.52±.37*	51.40

Each value is the mean±SEM (n=5), **P*<0.05 compared with control and standard; OSMFH: Hydrogel containing methanol fraction of *Ocimum sanctum*

Effect on croton oil-induced ear edema

In the present study, hydrogel formulations, OSMFH (1%) was applied topically to observe percent inhibition of croton oil-induced ear edema in mice [Table 7]. Effect of OSMFH was observed greater percentage of edema inhibition (49.53%) but less than the standard group of treatment after croton oil treatment. In the present study, result indicates that prepared hydrogel OSMFH possesses inhibitory effects against acute inflammation.

As results was observed that there was an increase in NO levels of inflamed mice ears (inflamed control and treated only with placebo base) in comparison with standard and MAMFH ($P < 0.001$), thus showing the involvement of NO release in acute inflammation induced by croton oil in the model used. The formulations containing different flavonoids components present in extract, reduced significantly ($P < 0.01$), in relation to placebo control shows nitrite concentration in mice ears [Figure 2]. Thus, these results suggest that the acute anti-inflammatory activity of herbal gels tested may be related, at least partially, with their ability to interfere in the pathways involved in the synthesis of NO, since the activation of intracellular signaling pathways dependent on the enzyme inducible NO synthase by croton oil plays a fundamental role in the control of the inflammatory response in the skin, where the NO produced favors vasodilatation that contributes directly to the formation of edema and the inflammatory process.[25]

MPO is another enzymatic substance present in neutrophil and much lower concentration in monocytes and macrophages. It is well known that the level of MPO activity is directly proportional to the neutrophil concentration on the inflamed tissue.[26] Inhibition of MPO activity by the drug preventing the generation of oxidants such as hypochlorous acid, directly correlated to anti-inflammatory potential of prepared herbal hydrogel.

Results showed that MPO level of inflamed ears was significantly higher in comparison with the non-inflamed control (untreated left ear control) ($P < 0.01$) and treated group with hydrogel formulations [Table 8]. The results showing the ability of croton oil to induce neutrophil influx into mice ear tissue [Figure 2]. This may be reduced by treatment with extract hydrogels, as well as Voltaren Emulgel, reduced significantly ($P < 0.01$) the MPO levels in mice ears.

Results of histopathological observations showed that inflammation and leukocytes infiltration were reduced considerably in OSMFH-treated groups as compared to control group of animals. Control group of animals showed accumulation of inflammatory cells and fibroblast cells. These results suggest to acute anti-inflammatory activity of prepared herbal hydrogels tested, may involve to interfere in cell migration at the inflammation site.

These results suggested to acute anti-inflammatory activity of prepared herbal hydrogels tested, may involve to interfere

Table 8: Effect of extract hydrogel formulations on inflammatory components in croton oil-induced ear edema

Animal groups	Inflammatory components	
	NO level (unit/ear)	MPO level (unit/ear)
Control (hydrogel base)	21.84±1.16	11.36±0.52
OSMFH (20 mg/day)	14.38±0.67*	7.47±0.28*
Standard (Voltaren Emulgel)	13.82±0.9*	7.38±0.86*

Each value is the mean±SEM (*n*=5); *$P<0.05$ compared with control group; OSMFH: Hydrogel containing methanol fraction of *Ocimum sanctum*. NO: Nitric oxide, MPO: Myeloperoxidase

Table 9: Effect of extract hydrogel formulations of *Ocimum sanctum* on cotton pellet-induced granuloma in rats

Groups	Granuloma weight (mg)	Percent inhibition
Control (hydrogel base)	61.42±3.67	-
OSMFH (20 mg/day)	25.36±1.47*	58.71
Standard (Voltaren Emulgel)	24.71±1.63*	59.41

Each value is the mean±SEM (*n*=5), *$P<0.05$ compared with control and standard; OSMFH: Hydrogel containing methanol fraction of *Ocimum sanctum*

in cell migration at the inflammation site. Overall, we can conclude that methanolic fraction of *O. sanctum* leaves containing hydrogels inhibits the neutrophil migration at inflammatory sites.

Effect on cotton pellet-induced granuloma formation

Cotton pellet-induced granuloma is widely used to assess the transudative and proliferative components of chronic inflammation. Winter *et al*.[20] have reported that indomethacin inhibits cotton pellet granuloma and is more potent than phenylbutazone and hydrocortisone.

Treatment with OSMFH was significantly reduces ($P < 0.05$) the granuloma formation induced by cotton pellets in rats significantly [Table 9]. OSMFH showed maximum reduction on the granuloma tissue formation on implanted cotton pellets with inhibition of 58.71% which was compared with Voltaren Emulgel (20 mg/day) produced inhibition 59.41%.

We used the rat cotton pellet-induced granuloma model for chronic inflammation to assess the effect of anti-inflammatory drugs on the proliferation phase of inflammation. Extract hydrogel formulations containing 1% flavonoids rich fractions containing various flavonoids compound, markedly inhibited granuloma formation surrounding cotton pellets compared with the vehicle control group. Thus, *O. sanctum* can inhibit granuloma formation in the proliferation phase of the inflammatory process. This leads to the dilation of arterioles and venules and may increase vascular permeability.[27]

Cotton pellet granuloma model has been widely used to evaluate the transudative, exudative, and proliferative components of chronic inflammation. Transudate phase causes increase in the wet weight of the cotton pellet while hosting inflammatory response to the implanted cotton pellet between 3 and 6 days causes granuloma formation. Therefore, increase in dry weight is considered as a measure of proliferative component of inflammation.[28,29] Methanolic fraction of *O. sanctum* was showed anti-inflammatory effects in chronic inflammatory tests with different efficacy. The extract hydrogels reduced cotton pellet-induced granuloma, thereby suggested its activity in the proliferative phase of the inflammation. Other studies have demonstrated that various flavonoids such as quercetin, luteolin, and hesperidin produce significant and anti-inflammatory activities.[30] Therefore, it could be suggested that the anti-inflammatory effects of the methanolic extract fraction of *O. sanctum* may be due to their contents of flavonoids.

It was observed that methanol fraction of *O. sanctum* was capable of inhibiting ear edema induced by xylene. It can be suggested from the study that the effectiveness for suppression of edema is due to the ability of extract to either inhibit the synthesis, release, or action of xylene involved in the inflammation. Chronic inflammation is the reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes proliferation of fibroblasts and infiltration of neutrophils with exudation of fluid. It occurs by means of the development of proliferative cells which can either spread or form granuloma.[31]

The development of edema is a biphasic event: The early phase (0–2.5 h after carrageenan injection) involves the release of inflammatory mediators such as histamine, serotonin, and bradykinin; the late phase (3–6 h post-injection) is associated with the release of prostaglandins.[32]

CONCLUSION

In the present study, methanol fraction of *O. sanctum* significantly inhibited the ear edema during the early phase of inflammation, indicating that the extracts of both plant and standard gel may be blocked histamine and serotonin release within the early phase. This suggested that the methanol extracts were exhibits its anti-inflammatory action by inhibiting the synthesis, release, or action of histamine. The results can be concluded that the significant activity may be

due to the presence of flavonoids in methanol extract fraction of *O. sanctum* leaves.

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