ORIGINAL ARTICLE

Preliminary screening of hydrogel containing *Martynia annua* extract for anti-inflammatory activity

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Abstract

Objective: Aim of the present study was to investigate preliminary evaluation of hydrogel formulation of methanol fraction of *Martynia annua* for anti-inflammatory activity using different types of animal models. **Materials and Methods:** Five different hydrogel formulations containing extract and one control without extract were prepared according to standard method using different proportions of Carbopol 940 and sodium carboxymethyl cellulose (CMC). Prepared hydrogel was characterized for optimization and screened for anti-inflammatory activity using Xylene induced ear edema and Croton oil-induced ear edema in mice. Effect was observed by measurement of percent inhibition of ear edema and biochemical parameters, for example, nitric oxide level and myeloperoxidase (MPO) level. **Results:** Phytochemical screening showed that petroleum ether extract of *M. annua* showed the presence of sterols, terpenoids, and fatty oils while ethanol extract of showed the presence of glycosides, phenolic compounds, flavonoids, and amino acids. Results of all other evaluation parameters, for example, pH, viscosity, spreadability, and consistency were found suitable in optimized formulation as 1.5 g of Carbopol 940 and 1% of Sodium CMC for best drug release profile. Effect of MAMFH was observed greater percentage of edema inhibition (66.67%) and was comparable to standard group of treatment (65.59%). Results showed that MPO level of inflamed ears (inflamed control and treated only with gel base) was significantly higher in comparison with the non-inflamed control ($P < 0.01$), and with hydrogel formulations. 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 ($P < 0.05$) the MPO levels in mice ears. **Conclusion:** Methanol fraction of *M. annua* was capable of inhibiting ear edema induced by xylene. It may be due to the ability of extract to either inhibit the synthesis, release or action of xylene involved in the inflammation. Methanol extracts was exhibits its anti-inflammatory action by inhibiting the synthesis, release or action of histamine. Significant activity may be due to presence of flavonoids in methanol extract fraction of *M. annua* leaves.

Key words: Anti-inflammatory, Carbopol 940, hydrogel, *Martynia annua*, Voltaren Emulgel

INTRODUCTION

Inflammatory disorders that cause the immune system to attack the cells or tissues of the body can cause abnormal inflammation, leading to chronic pain, redness, swelling, stiffness, and normal tissue harm. Inflammation also known as coordinated response that protect as well as heals the host tissues after infection or damage to the tissue, involving molecular signals generated by the host or disease agent.[1] Inflammation involves an increase of the blood supply to the affected region by means of vasodilation. The capillaries become more permeable so that fluid, large molecules and white blood cells can cross, leaving the blood, and entering the tissue. White blood cells (particularly neutrophils and monocytes) move by chemotaxis to the injured site.[2,3] Redness, heat, swelling, and pain are associated with the inflammatory process. Redness and heat are caused by the increased blood flow. Swelling is the result of the increased movement of fluid and white blood cells into the area of inflammation. The release of chemical compounds and the compression of nerves in the vicinity of the inflammatory process cause pain.[4]

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The *Martynia annua* Linn is a well-known herb for the treatment of tuberculosis, sore throat, inflammation, scabies, and painful urination. The whole plant also used by Santal tribal (India) for fever, hair loss, scabies, sores, and carbuncles. The leaf paste of *M. annua* Linn is used for healing of wounds in domestic animals and many peoples in rural areas also uses healing of skin injury. The leaves contain chlorogenic acid and fatty acids (such as palmitic acid, stearic acid, and arachidic acid) are present in seeds.[5] P-hydroxy benzoic acid, snapic acid, and fatty acids such as palmitic acid and stearic acid present in leaves.[6]

An aqueous and alcoholic extract of *M. annua* reported the presence of 28 compounds in which oleic acid presents in the high amount. Other major biological compounds include pelargonidin-3-5-diglucoside, cyanidin-3- galactoside, p-hydroxy benzoic acid, gentisic acid, arachidonic acid, linoleic acid, palmitic acid, stearic acid, apigenin, and apigenin-7-oglucuronide.[6,7]

On the basis of the previous research and literature, the present study was aimed to evaluate anti-inflammatory effect of *M. annua* Linn leaves in the form of hydrogel formulation using different types of animal models. *M. annua* was selected on the basis of traditional knowledge and chemical constituents reported for inflammatory disorders in the available literature.

MATERIALS AND METHODS

Identification and Collection of Plant Materials

The plant material *M. annua* leaves were collected from the surrounding of SRK University Campus, Bhopal (M.P), India during August-September month of 2018. Plant specimens were identified and authenticated in Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur. Collected plant leaves were should be shade dried for powdering and then store for further processing such as extraction and other studies.

Extraction and Phytochemical Screening

The dried powdered leaves of *M. annua* were taken for extraction (150 g each). Plant materials were initially extracted with petroleum ether for defatting and then dried the mark for further extraction with ethanol using Soxhlet apparatus method. After completed extraction process, the ethanol was evaporated completely using vacuum evaporator under reduced pressure, to obtained completely dried extract for further use. Petroleum ether and ethanol extracts obtained from *M. annua* leaves were proceed for qualitative chemical tests for the detection of the presence or absence of different chemical constituents.[8-10]

Fractionation of Ethanol Extract of *M. annua* Leaves

The filtrate ethanol extract was concentrated and completely dried and treated with chloroform repeatedly to obtained chloroform soluble fraction and chloroform insoluble fraction. The chloroform insoluble fraction was dissolved in methanol repeatedly to obtained methanol insoluble and methanol soluble fraction. The methanol soluble fraction has greater yield than methanol insoluble fraction. The methanol soluble fraction was concentrated and dried to subject for formulation preparation and other study.

Formulation Preparation and Characterization

Five different hydrogel formulations containing extract and one control without extract were prepared according to the modified method of Chirayath *et al*.[11] Briefly, different proportions of Carbopol 940 and sodium carboxymethyl cellulose (CMC) were dispersed in 50 ml of distilled water with continuous stirring. About 5 ml of distilled water was taken and required quantity of methylparaben and propylparaben were 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 drop wise 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 preparation of control formulation without adding any plant extract.

Characterization of Prepared Hydrogel Containing Methanol Extract of *M. annua* Leaves (MAMFH)

Physical characters

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

Table 1: Formulations of hydrogel containing methanol extract of *Martynia annua leaves* (MAMFH)

Ingredients	F1	F2	F3	F4	F5
Carbopol 940 (g)	0.5	1	1.5	2	3
Sodium carboxymethyl cellulose	3	2	1	1	0.5
Extract (%w/w)	1	1	1	1	1
Propylene glycol 400 (5%)	5	5	5	5	5
Methylparaben (0.5%) (ml)	0.2	0.2	0.2	0.2	0.2
Propylparaben (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

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.

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.[12] 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 sec.) taken to separate the slide completely each other.

Viscosity

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 Brookefield 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 end 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 water tight 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±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.[13,14] Calibration curve of luteolin was prepared of known concentrations in the appropriate range, to determine the amount of drug released.

Dermal irritation study of hydrogel formulation

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 lab 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.[15] The degree of irritancy was obtained by calculating the primary dermal irritation index (PDII).

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

Stability studies of hydrogel formulation

All prepared hydrogel formulations were subjected to a stability testing for 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, 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 such as 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.

Stability of formulated hydrogel to centrifugation was determined in 10 ml-graduated cylinders at 10,000 rpm for 10 min using a centrifuge (Remi). The formulation, which was resistant toward centrifugation, was further selected for evaluation.

Anti-inflammatory Activity

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±2°C, relative humidity 44–56%, 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 in to 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 topically prepared hydrogel of methanol fraction of *M. annua* (MAMFH) to each rat 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 received topical application (20 mg/ear) of extract hydrogel formulation and 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. 3 h 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.[16] The level of inhibition (%) of edema was calculated using 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.[17] 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 previous method. Then, mouse ears were stored for posterior quantification of inflammatory parameters.

Collected ear biopsies were chopped and homogenated in 500 µL of phosphate buffered saline pH 7.4 (137 mMNaCl, 3 mMKCl, 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 nitric oxide (NO) production. The pellets were further employed for myeloperoxidase (MPO) enzyme estimation, as an indirect marker of neutrophil migration.

Determination of NO

In the inflammatory process, release of NO and reactive oxygen species (ROS) occurs, which together cause tissue damage, through an inflammatory reaction to increase the synthesis and release of inflammatory mediators, such as cytokines, 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.[18-20]

NO levels in mice ears were quantified indirectly by Griess reaction, which quantifies nitrite, a product of reaction of NO with oxygen.[21] 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*.[22] 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 12000 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 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 5µm of thickness were obtained for hematoxylin-eosin staining and examined by light microscopy (×100) for the evaluation of edema intensity and leukocyte infiltration.[23,24]

Statistical Analysis

Values were represented as mean ±SD all statistical data. The number of animals in each group was six, and considered statistically significant, if $P < 0.05$. The difference between mean values that were calculated by a one-way variance analysis (ANOVA) followed by the Tukey's test for multiple comparisons. The results were statistically analyzed by GraphPad Instat Software.

RESULTS

The dried powder of *M. annua* was subjected to defatting with petroleum ether and extraction with ethanol to obtain ethanol extracts. The yields of petroleum ether and ethanol extract of *M. annua* were found as 2.6% and 3.4% w/w, respectively. The ethanol extract was concentrated under vacuum and store for further uses.

The phytochemical analysis of petroleum ether and ethanol extracts of *M. annua* leaves using various chemical tests was performed to find out presence of various classes of compounds. Petroleum ether extract of *M. annua* showed the presence of sterols, terpenoids, and fatty oils while ethanol extract of *M. annua* leaves showed the presence of glycosides, phenolic compounds, flavonoids, and amino acids.

Formulations Preparation and Characterization

Carbopol-940 was used as gel former in order to select leading gelling agent. Preliminary, less than 1% of carbopol-940 with 1.3 ml of triethanolamine was tried for the hydrogel preparation [Table 1]. In this concentration, we found that, it forms very thin gel that liquefies within 6 h of preparation. Somewhat better gel was formed with >1% of carbopol-940.

Physical properties of prepared hydrogel such as color, appearance, homogeneity, consistency, phase separation, and odor were observed and are shown in Table 2.

The pH value, viscosity, and spreadability of the prepared hydrogels were studied at room temperature [Table 3]. 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 viscosity of gel was 193600–197500 cps and was recorded at initial phase for hydrogel formulations containing methanol extract of *M. annua* leaves (MAMFH). Spreadability of hydrogel formulations was recorded in the range of 14.27–17.64 g.cm/s.

The drug content (Luteolin concentration) was determines by spectrophotometric method and found as 0.084 µg/ml for MAMFH. This content was also basis for drug release study of hydrogel formulations.

Percentage drug release of hydrogel containing methanol extract of *M. annua* leaves (MAMFH) was observed initially to be 8.68% (at 15 min) and 52.62% (at 240 min), respectively, for F1 formulation. Out of all different formulations, F3 formulation of extract was showing highest percentage drug release at 240 min as 66.07 compared to other formulations [Figure 1]. 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.

The skin irritation study was performed on Swiss albino mice and so as to assess gentleness of the prepared formulations against the skin irritation [Table 4]. The optimized formulation F3 was not showing any adverse effect on rabbit skin.

The stability study of all prepared hydrogel was performed according to the ICH guideline (2013) by keeping at 27±1°C for about 30 days and again physical properties were observed.

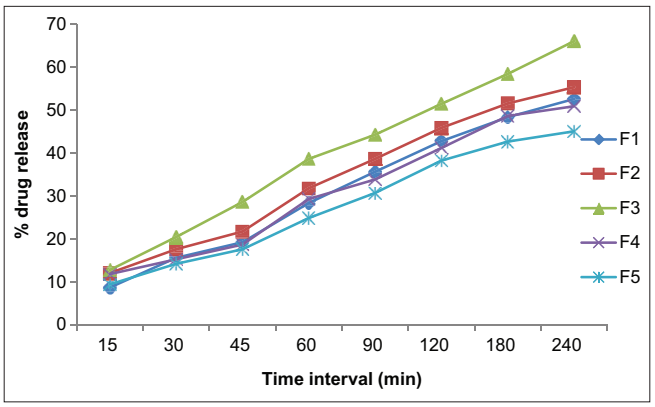

Figure 1: Percentage drug release of different formulations containing methanol extract of *Martynia annua* leaves

Table 2: Physical properties of prepared hydrogel formulation containing methanol fraction of *Martynia annua* (MAMFH)

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: Evaluation of prepared hydrogel formulations containing methanol extract of *Martynia annua* (MAMFH)

Parameters	F1	F2	F3	F4	F5
pH	6.98	6.97	7.01	6.98	6.99
Viscosity (cps)	195200	194400	197500	193600	197200
Spreadability (g.cm/s)	14.27±0.51	17.64±0.75	15.82±0.62	14.37±0.84	16.82±028

n=5

Table 4: Primary dermal irritation scores in albino rabbits after exposure to hydrogels containing methanol extract of *Martynia annua* leaves

Formulations	Time post instillation (h)	Incidence of dermal irritation		Total PDIα (Mean score)	Primary dermal irritation index
		Erythema	Edema		
F1	1	0	0	0	0.5
	24	1	0	1	
	48	1	0	1	
	72	0	0	0	
F2	1	0	0	0	0.25
	24	1	0	1	
	48	0	0	0	
	72	0	0	0	
F3	1	0	0	0	0.25
	24	1	0	1	
	48	0	0	0	
	72	0	0	0	
F4	1	0	0	0	0.25
	24	1	0	1	
	48	0	0	0	
	72	0	0	0	
F5	1	0	0	0	0.25
	24	1	0	1	
	48	0	0	0	
	72	0	0	0	

n=3 albino rabbits; α=Primary dermal irritation=Average erythema + Average edema

After 30 days, it was observed that all properties were same except color [Table 5]. The color of the gel was little faint bluish. There was no phase separation 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 6.97–7.01.

The spreadability of formulation depends on its viscosity. The spreadability of formulations was recorded for optimized gels and found as of 15.82±0.62 for MAMFH.

The pH of the prepared formulations was found in the range of 6.9–7.01 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.

The results indicate they were nearly same in the terms of applicability or spreading capacity. Storage even at accelerated stability conditions does not influence the stability of prepared hydrogel. Thus, it may be concluded that formulations were adequate and satisfactory as far as physical parameters are concerned.

The studies revealed that the hydrogel formulation F3 containing methanol extract of *M. annua* leaves 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.

Table 5: Visual observation of prepared hydrogels containing methanol extract of *Martynia annua* leaves at various temperatures

Formulations	Room temperature		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

Anti-inflammatory Activity

Effect on xylene-induced ear edema

In the present study, hydrogel formulations, MAMFH (1%) was applied topically to observe percent inhibition of xylene induced ear edema in mice [Table 6]. Effect of MAMFH was observed greater percentage of edema inhibition (66.67%) and was comparable to standard group of treatment (65.59%). In the present study, result indicates that prepared hydrogel MAMFH possesses significant inhibitory effects against acute inflammation.

Effect on croton oil induced ear edema

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 [Table 7]. The formulations containing different flavonoids components present in extract, reduced significantly ($P < 0.001$), in relation to placebo control shows nitrite concentration in mice ears.

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	-
MAMFH (20 mg/day)	0.31±0.11	66.67*
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. MAMFH: Hydrogel containing methanol fraction of *M. annua*

Table 7: Effect of extract hydrogel formulations on Croton oil-induced *e*ar edema

Animal groups	Weight of ear lobes (in gm) Mean ± SEM	% inhibition of ear edema
Control (hydrogel base)	1.07±0.27	-
MAMFH (20 mg/day)	0.51±0.06	52.33
Standard (Voltaren Emulgel)	0.52±0.37	51.40

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

Results showed that MPO level of inflamed ears (inflamed control and treated only with gel base) 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. This may be reduced by treatment with extract hydrogels, as well as Voltaren Emulgel, reduced significantly ($P < 0.05$) the MPO levels in mice ears.

Results of histopathological observations showed that inflammation and leukocytes infiltration was reduced considerably in MAMFH as well as OSMFH treated groups as compared to control group of animals. Control group of animals showed accumulation of inflammatory cells and fibroblast cells.

DISCUSSION

Inflammation is the body's protective response to injury and infection; it is a complex process involving many cell types, as well as different components of blood. 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.[25] Inflammatory disorders arise when inflammation becomes uncontrolled, and causes destruction of healthy tissue.

Gelling agent is one of the main ingredients in the hydrogel formulations. Viscosity enhancer is another important ingredient in the hydrogel preparation, which depends on its concentration, will lead to a simple solution or lotion with very poor consistency, while excess concentration may lead to formation of gels with high viscosity leading to non-uniform distribution of drugs and problem with handling of gel.[26] Gel containing 1–3% of carbopol-940 with 1.3 ml tri-ethanolamine was tried, but we found that uniform and smooth gel were formed using carbopol-940 that did not liquefy upon keeping, for a long duration at room temperature.

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

Animal groups	Inflammatory components	
	Nitric oxide level	MPO level
Control (hydrogel base)	21.84±1.16	11.36±0.52
MAMFH (20 mg/day)	12.44±0.84*	6.61±0.37*
Standard (Voltaren Emulgel)	13.82±0.9	7.38±0.86

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

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. 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 best drug release profile.

The 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.[26]

The objective of irritation study was to determine any undesired irritation effect of formulation 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 both 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.

The used models in the present study have given broad spectrum for the evaluation of the anti-inflammatory activity. In different models, the inflammation has produced by different inducers by releasing inflammatory mediators. Each is having different mechanism of action for producing inflammation either by increased in vascular permeability, the infiltrations of leukocytes from the blood into the tissue or granuloma formation and tissue repair.

Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based on the ability of such agents to inhibit the edema produced in ear of the animal after injection of a phlogistic agent. For producing edema, histamine, xylene, arachidonic acid, phorbol myristate acetate, oxazolone, croton oil, and formalin are generally used. Accordingly, methanol fraction of *M. annua* was selected for hydrogel formulation and investigated for anti-inflammatory potential using xylene induced and croton induced ear edema models.

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 is characteristic of the acute inflammatory response. In this model, the application of xylene induces neurogenous edema. It is partially associated with the substance P. Substance P is an

undecapeptide, which is widely distributed in the central and peripheral nervous system and it functions as a neuro-transmitter or neuro-modulator 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.

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.[27]

These results suggest to acute anti-inflammatory activity of prepared herbal hydrogels tested, may involve to interfere in cell migration at the inflammation site. Overall, we can conclude that methanolic fraction of *M. annua* containing hydrogels inhibits the neutrophil migration at inflammatory sites.

Natural products have recently fascinated industrial applications not only as the source of pharmaceutical products but also for their advantageous effects on human health. It was observed that methanol fraction of *M. annua* 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.

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.[28] 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. 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 development of proliferative cells which can either spread or form granuloma.[29]

CONCLUSION

Results were confirmed that the methanol fraction of *M. annua* significantly inhibited the ear edema during the early phase of inflammation, indicating that the extracts hydrogel may be blocks histamine and serotonin release within the early phase. This suggested that the methanol extracts was 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 *M. annua* leaves.

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