

INVESTIGATION OF ANTI-INFLAMMATORY ACTIVITY OF OINTMENTS CONTAINING FENUGREEK EXTRACT

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ABSTRACT

Objective: To formulate and investigate the anti-inflammatory activity of herbal ointments formulated with ethanolic extract of Fenugreek (*Trigonella foenum-graecum*).

Methods: Herbal Ointments were prepared by incorporating ethanolic extract of Fenugreek (5% w/w) into emulsifying ointment and simple ointment bases by fusion method and subjected to physical evaluations. Ointments were evaluated for anti-inflammatory activity by Carrageenan-induced paw edema method in rats. Phytochemical analysis of extracts and stability studies of ointment formulations were also carried out.

Results: Ointment formulation of fenugreek showed brown colour, aromatic odour and good homogeneity. Simple and emulsifying ointment formulations showed pH of 6.35 and 6.97 respectively which lies in normal skin pH range. Viscosity for simple ointment and emulsifying ointment was found to be 9050 ± 12.1 , 9664 ± 16.7 cps respectively.

Emulsifying ointment base containing fenugreek extract exhibited significant anti-inflammatory activity with percent inhibition 41.77% where as simple ointment formulation showed 40.0% inhibition of paw edema. Phytochemical screening of ethanolic extracts of fenugreek seeds showed the presence of alkaloids, phenols, flavonoids, tannins, steroids, carbohydrates, proteins. Stability studies showed that there was no variation in physical parameters of formulations after 3 months which indicate good stability.

Conclusion: This study shows that fenugreek has high potential as anti-inflammatory agent when formulated as ointment for topical use and results of the present study, therefore, support the traditional uses of this plant for inflammations.

Keywords: : Fenugreek, ointments, ethanolic extract, anti-inflammatory activity.

INTRODUCTION

Inflammation is considered as a primary physiologic defense mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for many of the chronic illnesses [1]. Although, currently used anti-inflammatory drugs are associated with some severe side effects, herbal products are often perceived as safe, because they are natural therefore, the development of potent anti-inflammatory and wound healer drugs with fewer side effects is necessary [2].

The delivery of drugs through the skin has long been a promising concept because of the ease of access, large surface area, vast exposure to the circulatory and lymphatic networks and non-invasive nature of the treatment [3]. Formulations suitable for skin delivery are ointment, cream, gels. Ointments are homogeneous, semisolid preparations intended for external application to the skin or mucous membranes. Ointment bases are mainly anhydrous and generally contain one or more medicaments in suspension or solution or dispersion. The type of ointment bases and formulation also affects the activity of the preparation.

Fenugreek (*Trigonella foenum-graecum*; Fabaceae) is a plant whose seeds and leaves are used in traditional medicine. *Trigonella foenum-graecum* is a plant that has different alkaloids, glycosides, saponin, minerals, flavonoids, nicotinic acid and tannin [4]. Fenugreek seed and leaf is known to have several pharmacological effects such as for analgesic [5], and anti-inflammatory activity [6], Plant's seed reduces fever [7], increases milk secretion [8], improves breathing disorder, digestion and is also used as laxative and diuretic. Plant tea can be used as gargle in tonsillitis and sore throat, as enema in

gastrointestinal inflammation and swelling, simple diarrhea, hemorrhoids, rectal prolapsed and as lotion in aphthous and as hot compress in lip fissure. Seed and its extract decrease blood sugar and cholesterol [9, 10].

Even though fenugreek has been used for the treatment of inflammation no report exists on development of topical dosage forms from extract of fenugreek. The new era of medicine prerequisites the formulation containing herbal constituents for better therapeutic activity and treatment. Hence the present study is aimed at formulating and investigating the effective anti-inflammatory ointment from ethanolic extract of Fenugreek.

MATERIALS AND METHODS

Collection and Authentication of Plant material

Seeds of *Trigonella foenum-graecum* were collected from local market and botanical identity was confirmed by Dr. Nagalaxmi, Botany Department, St. Agnes College, Mangalore.

Preparation of Extract

Fenugreek seeds were coarsely powdered using mechanical grinder and then subjected to exhaustive soxhlation using ethanol as solvent for several cycles. After completion of the extraction, solution was filtered and concentrated using rotary vacuum evaporator (Rotavap, Bangalore) below 50°C to get brown coloured dry extract.

Phytochemical Analysis

Prepared extract was qualitatively tested for the presence of tannins, alkaloids, flavonoids, saponins, glycosides, tannins, steroids. The test

was carried out by the method described by Harborne and Sazada *et al* [11, 12].

Preparation of Ointment Formulations

Two topical ointment bases of varying degrees of aqueous/anhydrous character namely: Simple ointment BP (T1), emulsifying ointment BP (T2) was prepared by fusion method [13]. In this method the constituents of the base were placed together in a melting pan and allowed to melt together at 70°C. After melting, the ingredients were stirred gently maintaining temperature of 70°C for about 5 minutes and then cooled with continuous stirring. Formulation of ointment was done by incorporating 5g of the semisolid ethanolic extract of *Trigonella foenum-graecum* into the various bases by triturating in a ceramic mortar with a pestle to obtain 100 g of herbal ointments containing 5% w/w of *Trigonella foenum-graecum* extract and formulation ingredients are shown in Table 1. The prepared herbal ointments were put in ointment jars, labelled and were stored at room temperature.

Table 1: Composition of ointment formulations containing fenugreek extract

Formulations	Ingredients	Concentration(%w/w)
T1	Fenugreek extract	5
	Wool fat	5
	Cetostearyl alcohol	5
	Hard paraffin	85
	White soft paraffin	
T2	Fenugreek extract	5
	Liquid paraffin	20
	Emulsifying wax	30
	White soft paraffin	50

T1: Simple ointment; T2: Emulsifying ointment

Physical Evaluations of Formulated Ointments

Physical assessments were carried out on the ointments for the parameters such as appearance, odour, Colour, pH, Spreadability, homogeneity and viscosity measurement.

Measurement of pH

Measurement of pH of the gel was done using digital pH meter (digital pH meter, 335, systronics, Noroda, Ahmedabad) in accordance with method described previously with minor modifications [14]. Accurately weighed 5 gm of gel was dispersed in 45ml of water to determine the pH of the suspension using digital in pH meter. The determinations were carried out in triplicate and the averages of three readings were noted.

Spreadability

Spreadability of the formulation was determined by an apparatus suggested by Mutimer *et al* with some modifications [15]. It consists of a wooden block having a pulley at one end with fixed glass slide on block. An excess of ointment (3gm) placed on ground plate. The ointment was sandwiched between this plate and another glass plate having the dimension of fixed ground plate and provided with the hook. A 1kg weight was placed on the top of the two plates for 5 min to expel air and to provide a uniform film of the ointment between the plates. Excess of ointment was scrapped off from the edges. The top plate was then subjected to pull of 240gms. With the help of string attached to the hook and time required by the top plate to cover a distance of 10cm. was noted. A shorter interval indicates better spreadability [16]. Spreadability was calculated using the following formula:

$$S = M \times L / T$$

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.

Measurement of Viscosity

Determination of viscosity of prepared ointment was carried out with Brookfield viscometer (model LV-DV-II, helipath spindle S-96) as method described by Kim JY and co-workers [17]. The value of each formulation were done in triplicate and the viscosity values are expressed as Mean \pm Standard deviation

Homogeneity

All developed ointments were tested for homogeneity by visual inspection after the ointments have been set in the container. They were tested for their appearance and presence of any aggregates.

Stability testing

The developed ointment formulations were subjected to stability study as per ICH guidelines

The formulated ointment were filled in the collapsible tubes and stored at different temperatures and humidity conditions, viz. 25°C \pm 2°C / 60% \pm 5% RH, 30°C \pm 2°C / 65% \pm 5% RH, 40°C \pm 2°C / 75% \pm 5% RH for a period of three months and studied for appearance, pH, viscosity and spreadability [18].

Animals

Healthy Wister albino rats of either sex weighing 250-300g were obtained from the Experimental Animal Care Center, Kshema medical college, Nitte University, Deralakatte, Mangalore. Rats were maintained under controlled condition at temperature (22 \pm 20°C), humidity (55%) and light (12 h light/dark condition). The animals were provided with standard diet and drinking water *ad libitum*. The experiments and procedure used in this study were approved by the Ethical Committee of the Nitte University, Deralakatte, Mangalore.

Acute Skin Irritation Study

This test was performed on albino rats weighing between 150-200g. The animals were given standard animal feed and had free access to water *ad libitum*. Animals are divided into four groups, each batch containing five animals. Dorsal hairs at the back of the rats were removed one day prior to the commencement of the study and kept individually in cages to avoid contact with the other rats. Two groups of each were used for control and standard irritant. Other two groups were used as test. The 50mg of each formulation were applied over one square centimeter area of whole and abraded skin of different animals. Aqueous solution of 0.8% formalin was used as standard irritant. The animals were observed for seven days for any signs of edema and erythema.

Investigation of Anti-inflammatory Activity of Ointment Formulations

The inhibitory effect of fenugreek on carrageenan-induced paw edema was evaluated using method described by Niemegeer and his co-workers [19]. White Albino Rats of Wister strain of either sex, 3-4 months of age, 180-220gram of average body weight were selected. Animals were allowed to free access to feed and water before the experiment. Rats were divided into four groups each comprised of six rats. Approximately 50 μ l of a 1% suspension of Carrageenan in saline was prepared 1h before each experiment and was injected into the plantar surface of right hind paw of rat. 0.2g of ointments containing ethanolic extract of fenugreek were applied to the plantar surface of the hind paw by gently rubbing 50 times with the index finger. Rats of the control groups received only the ointment base and 0.1% w/w Diclofenac ointment are (Cipla, Ahmedabad, India) applied in the same way as a reference standard. 1h after the application of ointment base, ointments formulations of fenugreek ethanolic extract and standard ; 50 μ l of a 1% suspension of carrageenan in saline was administered in to plantar surface of right hind paw of rat. Paw volume was measured immediately after carrageenan injection and at 1h, 2h, 3h and 4h after the administration of the noxious agent by using a plethysmometer (Model 7159, Ugo Basile arese, Italy) (Niemegeer et al., 1964). The paw volume was recorded at different time points. The percentage inhibition in paw volume is calculated by using the formula.

$$\% \text{ Inhibition} = \frac{\text{Paw volume (Control)} - \text{Paw volume (Test)} \times 100}{\text{Paw volume (Control)}}$$

Statistical Analysis: The results of various studies were expressed as mean \pm SEM. Data analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test using "Graph pad Instat" version 3.00 for Windows 95, Graph Pad Software. Probability values of 0.05 ($p < 0.05$) or less were considered statistically significant.

RESULTS

Phytochemical Studies

The preliminary phytochemical screening of ethanolic extracts of fenugreek seeds showed the presence of alkaloids, phenols, flavonoids, tannins, steroids, carbohydrates, proteins.

Physical Evaluations of Ointment Formulation

Simple ointment BP (T1) and emulsifying ointment BP (T2) containing ethanolic extract of fenugreek were prepared and ointments were evaluated for colour, odour, homogeneity, pH, viscosity and spreadability and results are shown in Table 2. Ointment formulation of fenugreek showed brown colour, aromatic odour and good homogeneity. The pH of both the formulations lies in the normal pH range of the skin. Viscosity of formulations was found to be acceptable.

Table 2: Physical evaluations of ointment formulations

Formulations	pH	Viscosity cps	Spreadability g.cm/sec	Homogeneity
T1	6.35 \pm 0.02	27.27 \pm 0.11	9050 \pm 12.1	good
T2	6.97 \pm 0.01	20.27 \pm 0.14	9664 \pm 16.7	good

Stability Testing: Developed ointment formulations were subjected to stability study as per ICH guidelines and results are shown in Table 3. During the stability studies the appearance of formulations was clear and no significant variation in pH, spreadability and viscosity was observed after 3 months.

Table 3: Stability studies of ointment formulations after 3 months

Parameter	Conditions for stability testing					
	25°C \pm 2°C / 60% \pm 5% RH		30°C \pm 2°C / 65% \pm 5% RH		40°C \pm 2°C / 75% \pm 5% RH	
	T1	T2	T1	T2	T1	T2
pH	6.30 \pm 0.01	6.90 \pm 0.03	6.21 \pm 0.02	6.86 \pm 0.02	6.15 \pm 0.03	6.80 \pm 0.01
Viscosity cps	27.30 \pm 0.10	20.30 \pm 0.12	27.35 \pm 0.11	21.10 \pm 0.13	27.60 \pm 0.10	21.90 \pm 0.11
Spreadability g.cm/sec	9020 \pm 11.1	9598 \pm 10.1	9010 \pm 10.9	9503 \pm 10.1	9005 \pm 10.2	9358 \pm 10.0

Acute Skin Irritation Study

Prepared Ointment formulations did not show any signs of erythema or edema when applied topically to the skin of animals till seven days.

Investigation of Anti-inflammatory Activity of Ointment Formulations

Anti-inflammatory activity of ointment formulations were investigated by Carrageenan induced paw edema method and results obtained is shown in Table 4.

Ethanolic extract of fenugreek showed significant reduction of paw edema throughout the entire period of observation in comparison to control ($p < 0.05$), although emulsifying ointment base containing fenugreek extract showed higher percentage of inhibition (41.77) of edema compared to simple ointment base (40.0).

Table 4: Evaluation of anti-inflammatory activity of ointment formulations

Treatment	Paw volume(ml) ^a at times after carrageenan administration			
	1h	2h	3h	4h
Control	0.71 \pm 0.02	0.77 \pm 0.02	0.79 \pm 0.01	0.60 \pm 0.02
Standard	0.42 \pm 0.03*	0.43 \pm 0.04*	0.44 \pm 0.04*	0.40 \pm 0.03*
T1	0.47 \pm 0.03*	0.50 \pm 0.04*	0.47 \pm 0.04*	0.46 \pm 0.03*
T2	0.45 \pm 0.02*	0.47 \pm 0.04*	0.46 \pm 0.02*	0.41 \pm 0.04*

^aValues are expressed as mean \pm SEM (Number of animals, n=6) ; one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability values of 0.05 ($p < 0.05$) or less were considered statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Vs control.

DISCUSSION

Two types of ointment formulation were prepared using anhydrous and lipophilic ointment bases and they were found to be stable during the period of stability testing.

Formulations were subjected for investigations of anti-inflammatory activity using carrageenan-induced rat paw edema. Carrageenan-induced paw edema in rat has known as a sensitive method for studying of non steroidal anti-inflammatory agents and show a biphasic event which is attributed to the different mediators. At the first (about 2 h after carrageenan injection), hyperemia mainly induces because of the release of histamine and serotonin, whereas prostaglandins and bradykinin potentiate the second phase of edema by mobilization of leukocytes. The edema was reached its highest thickness 4 h after the application of the stimulus [20]. Investigation anti-inflammatory efficacy of the topical preparations of *T. foenum-graecum* was best demonstrated when oil-in-water emulsion base was employed in its preparation. The compounds responsible for anti-inflammatory activity present in the extracts were released more readily from emulsifying ointment than the simple ointment formulations.

Phytochemical analysis of fenugreek extract showed the presence of flavonoids [21], saponins [22] and alkaloids [23] which are reported to be the major compounds responsible for anti-inflammatory activity. Flavonoids act as potential inhibitors of cyclooxygenase, lipoxygenase, and nitric oxide synthase as well as being antioxidants [24, 25]. Thakur *et al.* reported no significant anti inflammatory activity of the aqueous extract of *T. foenum-graecum* seeds [26], whereas in another report the ethanol extract of the plant obtained in soxhlet method has exerted significant anti inflammatory effect. The latter report shows that the ethanol extract of *T. foenum-graecum* seeds increases the peritoneal exudates as well as macrophage cell counts which indicates that this plant probably acts via activation of macrophages [27]. Gastroprotective effect reported for the seeds of this plant is a valuable factor regarding the gastrointestinal disturbance caused by non steroidal anti inflammatory drugs (NSAIDs) [28].

CONCLUSION

This study concludes that formulated ointments are safe and efficient anti-inflammatory formulations for the topical delivery of the ethanolic extract of *Trigonella foenum-graecum*. Therefore fenugreek has high potential as anti-inflammatory agent when formulated as emulsifying ointment for treating acute inflammatory disorders. The results here support the traditional uses of the seeds of fenugreek for treatment of inflammations. Furthermore regarding the presence of different secondary metabolites in this plant, separation and identification of biological compounds of the plant is valuable for finding new agents with anti inflammatory activity.

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