In -Vitro Assessment Of Anti-Inflammatory Activity Of Ethanolic Extract Of Fenugreek Seeds And Demonstration Of Effectiveness In Animal Models (In- Vivo) Through Characterization Of Gel Formulation Containing The Extract

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Abstract- This present investigation deals with evaluation of anti-inflammatory potential of ethanolic extract of *Trigonella Foenum graecum L.* (Fenugreek) seeds and demonstration of its effectiveness by formulation of a gel containing *the* extract. The extract was first analyzed for potential anti-inflammatory phytochemicals and then gel formulations weredesigned by using ethanolic extract of fenugreek seeds extract in moderate concentration (5%) and evaluated using physiochemical and physiological measurements. The chief constituents of the successfully prepared gel were Carbopol 934, HPMC K4M, Fenugreek seeds extract, Poly ethylene glycol (PEG), Alcohol, Methyl Paraben, Propyl Paraben and Distilled water. The natural skin pH (6.8-7) was adjusted by drop -wise addition of Tri-ethanolamine. The physiochemical parameters of formulations (pH, viscosity, spread ability etc.) were determined. Macroscopic Analysis of the formulation was done by visual inspection for color, homogeneity and clarity on natural light. Acute skin irritation test and feel on application was noted. The drug content was determined and the in-vitro release study was done spectrophotometrically.

Further the formulations were tested for anti-inflammatory activity in- vitro by protein denaturation inhibition assay method using BSA and in-vivo animal models. The results favored the anti- inflammatory potential of the formulation and supported further optimization for commercial prospects.

Index Terms- Anti-inflammatory, BSA, Carbopol, Ethanolic , Extract, Gel , Trigonella Foenum graecum L.

I. BACKGROUND

NNatural products have been a major source of new drugs (Vuorelaa P, 2004). Plants possess medicinal and drug activities. Medicinal plants are used by 80% of the world population and in developing countries (Hasim H, 2010). Current study on natural molecules and products primarily focuses on plants since they can be sourced more easily and be selected based on their ethno-medicinal uses (Arora DS, 2007). They can be extracted and used for chronic and infectious diseases. Clinical microbiologists have great interest in screening of medicinal plants for new therapeutics (Periyasamy A, 2010). The active drugs which play role are secondary metabolites.

In recent times, the medicinal compounds are being widely used and are suggested by doctors to be used in a number of ailments due to their minimal side effects and numerous positive effects on human health. Out of many such medicinal plants, fenugreek [Trigonella foenum-graecum Linn (Fabaceae)] has recently attracted the attention of scientists from across the globe (Bacquer, 2014).

II. INFLAMMATION

Inflammation can be classified as either acute or chronic.

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue.

Prolonged inflammation, known as **chronic inflammation**, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. (G. Leelaprakash, 2011).

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1. Plant Profile

Trigonella foenum- graecum is a plant that has different alkaloids, glycosides, sapogenin, minerals, flavonoids, nicotinic acid and tannin (Hasanzadeh, Rezazadeh, Shamsa, Dolatabadi, & Zarringhalam, 2010).Fenugreek seed and leaf is known to have several pharmacological effects such as for analgesic (S. Biswal, 2003) and anti- inflammatory (Alireza Parvizpur, 2004).

Fig 1: Fenugreek seeds and green plant

Trigonella foenum-graecum is a flowering plant that belongs to Rosaceae order, Legouminosae family, subfamily of Papilonaceae and Trigonela L. genus of the Trifolia group (M, 2006). The name of this plant comes from the Greek word trigonous meaning triangle, because of the triangular shape of its leaflets. The term foenum- graecum means ''Greek hay'' or Greek grass because of its extensive use in ancient Greece. Fenugreek is an herbaceous and annual plant and its height reaches to 50 cm.

Species	Geographical Origin
T coerulecscens	Iran
T striata	Iran
T moresshina	Iran, India, Africa, Egypt
T foenum-graecum	Iran, Turkey
T coerulescens	Iran
T aphanoneora	Iran
T tehranica	Iran
T elliptica	Iran
T monantha	Iran
T astroites	Iran
T uncata	Iran
T anguina	Iran, Sudi Arabia
T stellate	Iran, Sudi Arabia
T fischeriana	Turkey
T velutina	Turkey
T cretica	Turkey
T hamosa	Sudi Arabia
T corniculata	India

This plant has a single stem that is frequently crooked, glabrous, or with distributed tomentums. Leaves are oval, serrated, consisting of 3 small obovate to oblong leaflets and leaflets distributed from one point. Flowers are pale yellow or whitey purple 0.8 to 1.8 cm in diameter and pollination is done by insects. Fruits are curved pods 3- to 11-cm long and containing 5 to 20 angled seeds 4- to 6-mm long. Seeds have bitter and aromatic taste and their color varies from fawn yellow to brown. Its names and scientific classifications are as follows.

Taxonomy

Kingdom: Plant
Super division : Angiosperm
Division: Eudicots
Class: Rosids
Order: Fabales
Family: Fabaceae
Sub family : Faboidae
Tribe : Trifoliae

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Genus: Trigonella
Species: foenum-graecum
General name: Fenugreek
English name: Fenugreek
Arabic name: Hhulbah, Hhelbah
French name: Trigonelle, Senegrain, Foingrec German name: Gemeiner, Hornklee, Bockshornklee
Indian name: Sagmethi, Methi, Kasurimethi Italian name: Fienogreco, Erbamedica

Traditional Uses

According to writings obtained from ancient times, fenugreek is one of those oldest medicinal plants used in Rome and Egypt to ease childbirth and to increase milk flow. Even today, Egyptian women use this plant as Hilba tea to alleviate menstrual pains and sedating tummy problems. (Morcos SR, 1981)

In the traditional medicine of China also this plant has been used to boost physique, to treat weakness of body, and gout. People with very slender physique in East used this plant to have strong and well-developed physique. This plant has been used in traditional medicine of India as a tonic and also as breast milk stimulant and as a spice. In non-medicinal applications this plant has been burned and fumed with incense by Egyptians in religious rites and also used to mummify bodies. (Yoshikawa M, 1997)

2. Topical Delivery

Topical formulations for pharmaceutical delivery are becoming popular interestingly. Topical delivery has a number of advantages; the ability to deliver drug at specific site of action, avoiding fluctuations in drug level, inter-intra patient variations. Improved compliance and an enhanced suitability for self – medication. Skin provides an ideal site for delivery of drug substances for both local and systemic effects (Nalamothu, 2015). Drug delivery through the skin has been a promising concept for a long time because the skin is easy to access, has a large surface area with vast exposure to the circulatory and lymphatic networks and the route is non-invasive.

3. Formulation excipients

The effects of formulation excipients on the rate and extent of drug absorption are greater with topical drug delivery than with any other route of drug administration. For example, comparing alternative formulations of the same drug, differences in extent of penetration of 10 to 50 fold and higher have been reported. To put this into perspective, 50-100% (up to one fold) differences in extent of absorption by the oral route are rare. The potential for large differences in the extent of absorption between topical formulations is due to the complex interactions between the drug, the vehicle, and the skin which check partitioning through the stratum corneum.(Sandhu Premjeet, 2012)

4. Drug-excipient permeation relationship

The stratum corneum barrier has been modelled as bricks and mortar structure of coenocytes bricks held together with an extracellular lipid mortar. Most agents are believed to permeate the stratum corneum by the extracellular lipid route. The intrinsic permeation of a drug through the stratum corneum lipid, is dependent on several physicochemical parameters , and it is useful classify these into those factors that affect solubility in the stratum corneum lipids, such as partition coefficient and drug-melting point, and those that affect diffusivity through the barrier, such as molecuIar volume and hydrogen bonding potential. Small, low melting-point hydrophobic compounds such as nicotinates, salicylates and nitroglycerin are, thus, relatively well absorbed accordingly; they are less likely to suffer from problems of bioavailability and bioequivalence. (Sandhu Premjeet, 2012)

6.1 Gel

Gels are transparent or translucent semisolid preparations consisting of solution or dispersion of one or more active ingredients in suitable hydrophilic or hydrophobic bases. Gels may be clear or opaque, and be polar hydro alcoholic or nonpolar. Gels are prepared by either a fusion process or a special procedure necessitated by the gelling characteristics of the gellant.

Gels are better formulated than other semi solid topical dosage forms to administer drug topically because they are non-sticky, requires low energy during formulation (Norris, 2005). Gel consists of a natural or synthetic polymer forming a three-dimensional matrix throughout a dispersion medium or hydrophilic liquid. After application, the liquid evaporates leaving the drug entrapped in a thin film of the gel-forming matrix physically covering the skin (Yasir et al, 2010).

5. Additives in Gel

5.1 Gelling agent

Gels are transparent or semisolid preparation of solution or dispersion of one or more active ingredients in suitable hydro colloidal substances known as gelling agent. Gels are non-greasy exhibit pseudo plastic property due to gelling agent. Gelling agent are hydrocolloids substance which gives thixotropic consistency to the gel. Gelling agents are organic in nature. Gelling agents are also known as solidifiers or stabilizer and thickening agent. Gelling agents are more soluble in cold water than hot water. Gelling agents like methylcellulose and polaxamers have better solubility in cold water while

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bentonite, gelatin and sodium carboxymethylcellulose are more water soluble in hot water. Gelling agents require a neutralizer or pH adjusting chemical to create the gel after the gelling agent has been wetted in the plant-derive dispersing medium. Gelling agents are used in concentration of 0.5 up to 10% depending on the agent most gelling agents require 24-48 hours to completely hydrate and reach maximum viscosity and clarity. It is easier to add the active drug before the gel is formed if the drug does not interfere with the gel formation. The viscosity of the gelling agents in the gelling layer be within range of about 1000 cps to about 100,000 cps.

III. MATERIALS AND METHODOLOGY

1. Plant Material

1.1. Collection and Authentication

The fenugreek seeds were purchased from the local market, cleaned, dried and authentified by some taxonomist from the National Herbarium Centre and Plant Laboratories, Godawari, Government of Nepal as *Trigonellafoenum-graecum L.*

2. Extraction

2.1. Preparation of the ethanolic extract of the Fenugreek seeds

The cleaned and dried seeds were grinded to fine powder in the mechanical mixer-grinder.About 100 gm of powdered seeds were extracted with ethanol by hot extraction method using Soxhlet apparatus. The extraction was continued till the solvent in the thimble became clear. After completion of extraction, the extract was heated for evaporation in a pre-heated water bath.

Fig 2: Appearance of the extract

3. Preliminary Phytochemical investigation

The following approaches were used to test for the presence of various phytochemical constituents in the extract. Phytochemicals are used as templates for lead optimization programs, which are intended to make safe and effective drugs. The following procedures were adopted to test for the presence of various chemical constituents in extract.(Mohsin J,2017)

1. Test for Alkaloids

Mayer's Test: 2 ml of the prepared extract was dissolved in dil. HCL and treated with the Mayer's reagent. Formation of yellow precipitate indicates the positive test of the alkaloid.

Wagner's Test: 2 ml of the extract was treated with Wagner's reagent. The presence of alkaloid is inferred from the reddish brown precipitate formed.

Hager's Test: 3 ml of the extract was treated with the Hager's reagent. Formation of yellow precipitate indicates the positive test of alkaloids.

2. Test for Saponin Glycosides

Foam Test: 1 ml of the extract was diluted to 20 ml and taken in a graduated cylinder and shaken for about 15 minutes. 1 cm layer of persistent foam indicates the presence of saponin glycosides.

3. Test for Flavanoid Glycosides

Alkaline Reagent Test : To the extract , few drops of Sodium Hydroxide was added , formation of intense yellow color which fades on adding dilute acid indicates the positive test of flavonoid .

Lead Acetate Test: Lead acetate solution was added to 2 ml of the extract. Formation of yellow colored precipitate indicates the presence of flavonoids.

4. **Test for phenolic:** 1 ml of the extract was taken in a test tube and 2 dropsof freshly prepared 5 % Fecl3 was added. Formation of a blue, green, red or purple color indicates the presence of phenolic compounds.

5. Test for Tannins

Fecl3 Test: 2ml of extract was treated with 0.1% of Ferric chloride. Brownish green layer indicates the positive test of tannins.

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6. Test for Glycosides

Keller-killiani test: The test solution was treated with 3 drops of glacial acetic acid in 2 ml of ferric chloride solution and conc H2So4 is added from the sides of test tube, if it shows a ring formation between two layers or a separation, distinguished by lower reddish brown and upper bluish green layer : indicates the positive test for glycosides.

Legal's test: The extract was treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red color indicates the presence of glycosides.

Test for steroids

Salkowski reaction: 2 ml chloroform and 2 ml of conc. H2SO4 was added to test tube containing 2 ml of the extract and shaken well. Formation of red color indicates the presence of steroids.

7. Gums and Mucilage

Extract was treated with distilled water and filtered. The filtrate was then treated with few ml of absolute alcohol with constant stirring. A characteristic white or cloudy precipitate indicates the presence of gums and mucilage.

4. Assessment of In-vitro anti-inflammatory activity of extract Inhibition of albumin denaturation

The study of anti-inflammatory potential of the fenugreek seeds extract was carried out by employing albumin denaturation inhibitory technique according to Mizushima et al and Saket et al followed with slight modifications. 1% aqueous solution of Bovine Serum Albumin (BSA) fraction was prepared and 1ml of it was added to 5ml of the extract, then pH of the mixture was adjusted using 1ml of diluted HCL. The mixtures were incubated at 37°C for 15 min and then heated to 55 °C for 25 min. After cooling the test mixtures, the turbidity so formed in the process was measured at 665 nm using UV-spectrophotometer. The same experiment was performed thrice. The Percentage inhibition of protein denaturation was calculated by formula:

Percentage inhibition of Protein Denaturation = $\frac{(\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100}{\text{Absorbance of Control}}$

5. Drug-Excipients compatibility study

Study of interaction of the drug with excipients by physical compatibility study

Each excipients used in the formulations was mixed separately with the extract at same concentration as the final dosage form to increase drug-excipients molecular contacts and notice for the physical incompatibility. After 20 days of storage at room condition temperature, samples were observed for physical incompatibilities such as caking, change of color (discoloration) and liquefaction.

6. Formulation of the Gel

A. Preparation of gel [F1] with Carbopol 934:

Firstly, Carbopol 934 was weighted accurately and taken in a beaker and dispersed in 75 ml of distilled water. The beaker was kept aside to swell the Carbopol for 24 hour. On next day, the swelled carbopol was stirred carefully with a lab stirrer. Weighed quantity of Propyl Paraben and Methyl Paraben was added to 5ml of Propylene glycol and stirred properly. 10 ml of ethanol was taken in another beaker and 5 gm of the semi-dried extract was dissolved. The dissolved extract and the preservatives dissolved in PEG were also added with constant stirring to the carbopol base. Final volume was made up to 100 ml by adding distilled water. Triethanolamine was added drop by drop to the formulations for adjustment of desired skin pH (6.8-7) and to obtain proper consistency.

B. Preparation of gel [F2] with Carbopol 934 and HPMC K4M

The above procedure was followed for this formulation too. At first, Carbopol 934 and HPMC K4M was weighted accurately and taken in a beaker and dispersed in 75 ml of distilled water. The beaker was kept aside to swell the Carbopol and HPMC mixture for 24 hour. On next day, the swelled polymers were stirred carefully with a lab stirrer. Weighed quantity of Propyl Paraben and Methyl Paraben was added to 5ml of Propylene glycol and stirred properly. 10 ml of ethanol was taken in another beaker and 5 gm of the semi-dried extract was dissolved. The dissolved extract and the preservatives dissolved in PEG were also added with constant stirring to the carbopol base. Final volume was made up to 100 ml by adding distilled water. Triethanolamine was added drop by drop to the formulations for adjustment of desired skin pH (6.8-7) and to obtain proper consistency.

7. Optimization of the gels

Both the preparations F1 and F2 were formulated successfully and subjected for further analysis.

8. Physicochemical evaluations

8.1. Macroscopic Analysis of the formulation

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Visual inspection was done for the color, clarity, and homogeneity and phase separation using natural light for both F1 and F2.

Fig 3: Physical appearance of the gels

8.2. Measurement of pH

The pH of gel formulations was determined using a digital pH meter. 2. 5 gm of gel was dissolved in 50 ml distilled water and kept aside for half an hour. The measurement of pH of each formulation was triplicated and average values of pH were calculated.

8.3. Spreadability

A glass plate was fixed on a smooth surfaced table. A circle of about 3 cm diameter was marked on the fixed glass plate .A distance of about 5 cm was marked on the table. The glass plate was fixed on the end mark. 2 gm of gel was weighed and placed on this ground plate in the marked circle. The gel was then pressed between this glass plate and another movable glass plate which was provided with a pulley like system. 500 gm weight was placed on the top of the two glass plates for 5 min to provide a uniform film of the gel between the glass plates. Then 100 gm weight was attached to the top plate. A thread attached to the hook could provide drag to the top glass plate and the time (in sec.) required by it to cover a distance of 5cm was noted. Spreadability was calculated using the following formula where a short interval indicates better spreadability:

$S = W \times D / T$

Where, S= Spreadability,

W= weight (tied to upper movable glass plate), D= Distance moved by the glass plate

T= Time (in sec.)

Fig 5: Determining spreadability

8.4 Rheological Study

The viscosity of the developed gel formulations was determined by using Brookfield viscometer (Brookfield viscometer DV2T) at QC lab of Lomus Pharmaceuticals Pvt ltd using spindle no.7 at different speeds (2.5 rpm , 5 rpm and 10 rpm) . All values were taken in triplicate and average was calculated.

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Fig 6: Determining viscosity using Brookfield Viscometer DV2T

8.5. Drug content determination assay

Drug content was determined by dissolving accurately weighed 1 g of gel in phosphate buffer of pH 6.8. After suitable dilution, total phenolic content were determined spectrophotometrically following Folin-Ciocalteau method described previously with minor modification.

1ml of the above solution was drawn into thile ' s tube . To it, 5 ml of the distilled water was added, after 2 minutes, 0.5 ml of the FC reagent was added and after 5 minutes 1.5 ml of 7.5 % Na2co3 solution was added. The final volume of the solution was maintained 10 ml with distilled water. The cap of the thile ' s tube was closed and kept aside in dark at room temperature for about 1 hour . After 1 hour, the TPC in the formulation was determined spectrophotometrically at 760 nm using double beam UV-Visible spectrophotometer. The drug content was determine using the formula:

% drug content = CV/ M

Where,

C = concentration of the drug in terms of GAE V= volume of the solution

M= mass of the gel taken

8.6. Gallic Acid Calibration Curve

0.05 gm. of the Gallic acid monohydrate was dissolved in 50 ml of ethanol. Different concentration of the Gallic acid were prepared by dilution (40 µg /ml , 80 µg /ml , 120 µg/ml , 160 µg/ml and 200µg/ml) . 1 ml of the Gallic acid prepared by above dilution was taken in thile ' s tube , 5 ml of distilled water was added and after 2 minutes , 0.5 ml of the FC reagent was added and after 5 minutes 1.5 ml of 7.5 % Na2Co3 was added to it and the final volume was made 10 ml using distilled water . The cap of the thile ' s tube was closed and incubated at room temperature for 1 hour . After 1 hour, the absorbance was determined at 760 nm using double beam UV-visible spectrophotometer. Then the standard calibration curve was drawn.

8.7. In vitro drug release study

Diffusion studies of the all the formulations were carried out in Franz diffusion cell through egg membrane. In diffusion cell, sample (0.1 g) was applied on egg membrane in donor compartment. The entire surface of membrane kept in contact with the receptor compartment containing phosphate buffer (pH 6.8) as the dissolution medium. Magnetic stirrer was used for stirring the receptor compartment. The temperature maintained was 37±2 °C. The study was carried out for 4 h with samples removed at 0.5, 1, 2, 4 hours. The sample was withdrawn at a predetermined period of time and the same volume was replaced with fresh phosphate buffer (pH 6.8). Samples were analyzed for total phenolic content according to the Folin-Ciocalteu method and the absorbance was measured at 760 nm.

8.8. Acute skin irritation test

For each gel 5 volunteers were selected. 0.5 gm of the gel was applied on an area of a sq. inch to back of the hand of each volunteer. After 24 hours, the test area was observed for any sign of redness, irritation or lesions or edema.

9. Assessment of in vivo anti-inflammatory effect

9.1. Experimental animals

Wister Albino rats weighing 200-275 gms were used for the study. Both sexes of the animals were inclusive for this study. The healthy animals were procured from the animal husbandry of Department of Plant Resources, Thapathali, Kathmandu. The animals were allowed for free commercial diet and water ad libitum prior to the study. Animals were divided into three groups’ viz. control, standard and test containing six animals in each group.

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Fig 7: Experimental Animals

9.2. Preparation of 1 % carrageenan suspension

For the preparation of 1 % carrageenan suspension, 0.02 gm of the carrageenan powder was weighed accurately and 2 ml of normal saline was added to it drop wise in a mortar and pestle so that it is dissolved by continuous trituration.

9.3. Standard drug

1% Dicofenac sodium gel which was commercially available served as standard.

9.4. Control drug

The gel base used for the formulation served as the control.

9.5. Test drug

The gel formulation coded as F2 was used as the test drug for the study.

9.6. Carrageenan induced rat paw edema

1% carrageenan suspension was prepared 1 hour prior to the conduction of the study. At first, the left hind paw diameter of the animals were measured using digital vernier caliper as noted as the initial diameter. About 0.2 gm of the standard drug was applied to the left hind paw of the experimental animals initially grouped as standard and rubbed gently with the index finger for 50 times. Similarly same amount of the control drug and test drug were applied to the animals grouped under the respective category. After 30 minutes, 0.1 ml of the carrageenan suspension was injected into theleft hind paw of the experimental animals. And after each hour interval i.e. 1 hr., 2 hr and 3 hr interval, the paw diameter was measured using digital vernier caliper. The difference in the initial and the final paw diameters denotes the extent of inflammation. The edema inhibitory activity was calculated according to the following formula: Percentage inhibition = {(D.control-D.test) / D.control} × 100 %

Where,

D.control = Mean edema diameter of the control And

D.test = Mean edema diameter of the test group

10. Statistical Analysis

All the values of the result were expressed statistically as Mean ± Standard Deviation, while the values for the in vitro anti-inflammatory effect were expressed as Mean ± SEM .The significance of the study was determined by estimating p values by using one – way ANOVA followed by Dunnett test via Minitab 18 statistical Software. $P < 0.05$, $P < 0.01$, $P < 0.001$ were considered statistically significant.

IV. RESULTS AND DISCUSSION

Table 1: Extractive value of the fenugreek seeds

Sample	Extraction Method	Solvent used	Weight of sample taken	Extractive value
Fenugreek seeds coarse powder	Hot continuous method using soxhlet apparatus	Ethanol	100 gm	25.75%

Table 2: Solubility of the extract

Medium	Extract
Water	Soluble
NaoH	Soluble

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HCL Phosphate Buffer Methanol Chloroform	Soluble Soluble Soluble In Soluble

Table 3: Preliminary Phytochemical Investigations

SN	Test	Observation	Inference
1	**Test for Alkaloids** Mayers Test Wagners Test Hagers Test	Cream yellow precipitate Brown precipitate Yellow precipitate	++ ++ ++
2	**Test for Flavanoid Glycosides** Alkaline Reagent Test Lead Acetate Test	Formation of yellow ppt that fades upon addition of dilute acid Formation of Yellow colored ppt	++ ++
3	**Test for saponin Glycosides**	Formation of persistent foam	++
4	**Test for Phenolics**	Blue green color	++
5	**Test for steroids** Salkowski Test	Red color develops	++
6	**Test for Gums and mucilage**	Characteristics cloudy white color	++
7	**Test For Tannin** FeCl3 Test	Deep blue color	++

5.1. Drug-Excipients compatibility study:

After 20 days storage of drug extract with excipients at room temperature, the samples were observed for physical incompatibilities however, no physical changes were observed in the mixture of extract and polymer combination.

Table 4: Physical compatibility study

Batch	Caking	After 20 days	Discoloration	After 20 days	Liquefaction	After 20 days
Extract	No	No	No	No	No	No
Extract + Carbopol 934	No	No	No	Slight	No	No
Extract + HPMC K4M	No	No	No	Slight	Yes	Yes
Extract + Carbopol 934 + HPMC K 4M	No	No	No	slight	No	No

5.2. In-vitro Anti-inflammatory study

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Table 5: Effect of Albumin Denaturation

Sample	Concentration	Absorbance	% Denaturation
Control	-	0.342±0.001	-
EEFS	100	0.2177±0.0021**	36.35
EEFS	200	0.1707±0.0015**	50.09
EEFS EEFS	400 500	0.1187±0.0012** 0.1097±0.0015**	65.29 67.92
Aspirin	100	0.1013±0.0015**	70.38
Aspirin	200	0.091±0.001**	73.39

Values above are expressed as Mean ± SD and analyzed by ANOVA followed by dunett test (comparisons, experimental group vs. control) $p < 0.001$** were considered statistically significant.

5.3. Quantitative composition of the formulations

Table 6: Batch formula of the formulated gels

Composition	F1	F2
Fenugreek seeds extract	5 %	5 %
Carbopol 934	1 %	1 %
HPMC K4M	-	1 %
Propyl Paraben	0.3 gm	0.3 gm
Methyl Paraben	0.1 gm	0.1 gm
Triethanolamine	Qs	Qs
Propylene Glycol	10 ml	10 ml
Ethanol	5 ml	5 ml
Distilled water	100 ml	100 ml

5.4. Physicochemical Evaluation of the formulated gels

Table 7: Physiochemical data

Formulation code	Color	Homogeneity	pH	Spreadability
F1	Light yellow	Good	6.78±0.18	17.05±0.44
F2	Light yellow	Good	7.0±0.12	33.96±0.87

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Above values are expressed as Mean ± SD. n=3

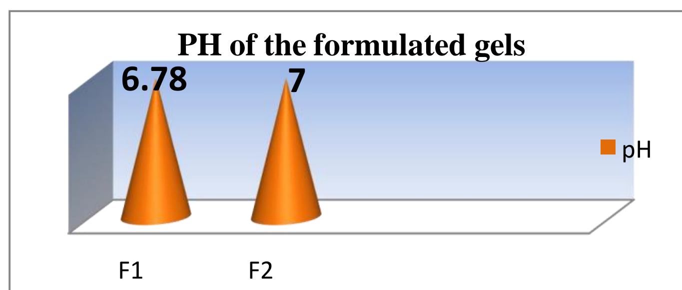


Fig: Graphical representation of the pH

5.5. Measurement of viscosity

The flow characteristics of the formulations were determined by the measurement of viscosity by using Brookfield viscometer DV2T at the QC laboratory of Lomus Pharmaceuticals Pvt ltd. Formulation f1 showed the greatest viscosity.

Table 8: Viscosity measurements of the gels at different rpm

Formulation code	Avg. Viscosity at 2.5 rpm	Avg. Viscosity at 5 Rpm	Avg. Viscosity at 10 rpm
F1	89,640	70,080	40,500
F2	70.840	54,120	38,190

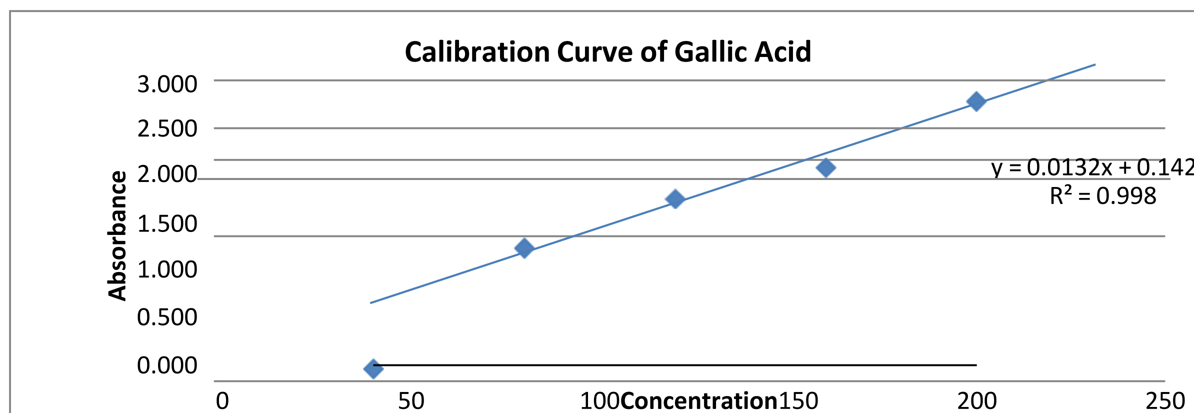
5.6. Acute skin irritation study

Table 9: Acute skin irritancy test results

Volunteer	Redness followed by skin irritation	Skin Lesion	Skin Edema
V1	Not detected	Not detected	Not detected
V2	Not detected	Not detected	Not detected
V3	Not detected	Not detected	Not detected
V4	Not detected	Not detected	Not detected
V5	Not detected	Not detected	Not detected
V6	Not detected	Not detected	Not detected
V7	Not detected	Not detected	Not detected
V8	Not detected	Not detected	Not detected
V9	Not detected	Not detected	Not detected
V10	Not detected	Not detected	Not detected

5.7. Gallic acid calibration curve

The standard Gallic acid calibration curve was plotted to determine the poly-phenolic content (drug content) of the formulated gels. The linear equation and the R-value are mentioned in the curve below.



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5.8. Determination of drug content

The Gallic acid calibration curve is used as reference in order to calculate the net drug content in the formulation.

Table 10: Gallic Acid Calibration data

Formulation code	Absorbance	GAE (mg/ml)	% Drug content
F1	0.736	45.00	90.653±0.682
	0.740	45.30	
	0.745	45.68	
F2	0.760	46.80	94.037±0.383
	0.763	47.05	
	0.765	47.19	

Both the formulations showed the drug content above 90 %.

5.9. In- vitro release of the poly-phenolic component from the gel formulations through semi-permeable membrane

The in-vitro release study was carried out by using franz diffusion cell apparatus .The membrane used for the study was egg membrane .The sample was withdrawn at interval of 30 min , 60 min , 120 min , 180 min and 240 min . The sample was analyzed for the release of poly phenolic content from the formulations spectrophotometrically at 760 nm by Folin – Ciocalthaeu method with minor modification. The formulation coded as F2 showed faster drug release pattern compared to the formulation F1.

Table 11: % Drug Release from the formulations

Time of sample drawn (min)	Absorbance at 760 nm		%Drug release from the formulated gels	
	F1	**F2**	**F1**	**F2**
30	0.208±0.002	0.221±0.002	1o.00	11.97
60	0.230±0.005	0.255±0.013	13.33	17.12
120	0.320±0.007	0.337±0.003	26.96	29.55
180	0.374±0.005	0.410±0.027	35.15	40.60
240	0.465±0.006	0.557±0.004	48.94	62.88

5.13 Evaluation of anti-inflammatory activity of the selected gel formulation on the experimental animals using carrageenan induced rat paw edema

The gel formulation containing Carbopol 934 and HPMC K4M was selected for the study of in vivo anti-inflammatory activity in experimental animals by carrageenan induced rat paw edema method. The extent of inflammation is the difference in the diameter of the paw at different intervals of carrageenan administration and the initial reading of the paw diameter measured by calibrated digital vernier caliper.

Table12:Result of the study

Initial diameter of left hind paw of the experimental animals	Treatment group	Diameter of the paw of the experimental animals at different hours of the drug administration			% Inhibition		
		1 hr	**2hr**	**3 hr**	**1 hr**	**2 hr**	**3hr**
3.16±0.08	Control	4.55±0.34	4.70±0.20	4.53±0.27	-	-	-
3.12±0.16	Standard	4.13±0.10	3.67±0.18*	3.40±0.09**	27.3	64.3	79.5
3.07±0.016	Test	4.14±0.23	3.81±0.14*	3.60±0.07*	23	51.9	61.3

All the values are expressed as Mean ± SEM, n=6, (comparisons, experimental groups vs. control group), p< 0.05 *, p < 0.001 ** were considered statistically significant analyzed by one-way ANOVA using Minitab 18 statistical software.

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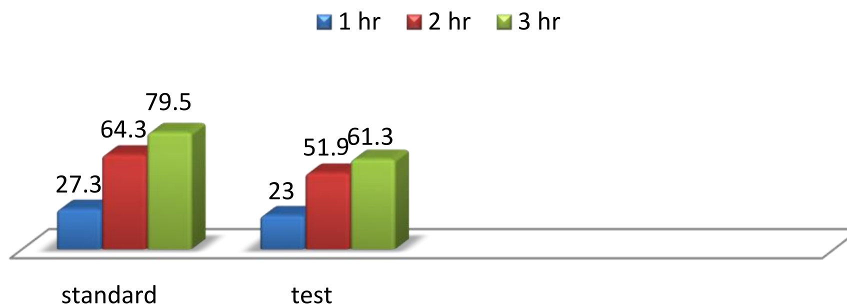

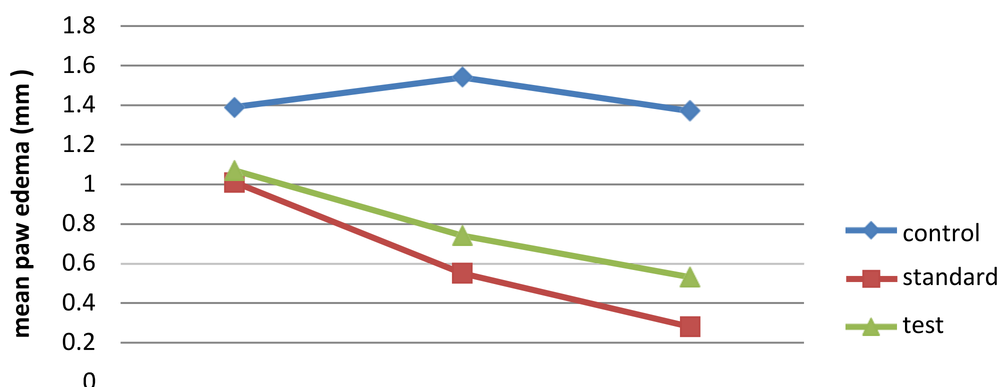

Fig: Graphical representations of the data

	1 hr	2 hr	3 hr
Control	1.39	1.54	1.37
standard	1.01	0.55	0.28
Test	1.07	0.74	0.53

The study revealed topical administration of the selected batch of the gel formulation caused significant inhibition of carrageenan induced rat paw edema. The gel formulation F2 exhibited significant ($p< 0.05$, $p< 0.001$) anti- inflammatory activity with 23 % , 51.9 % and 61.3 % inhibition at interval of 1 hr. , 2 hr. , and 3 hr. after administration of 1 % carrageenan suspension sub-cutaneous at the plantar surface of the left hind paw of the experimental animals .

V. Conclusion & Recommendation

Topical gels containing fenugreek extract can be successfully prepared using carbopol-934 and HPMC K4M as gelling agents. The topical gel prepared from mixture carbopol-934 and HPMC K4M will be better gelling agent for making an ideal topical preparation. *Trigonella foenum greacum* seed extract in the form of gel possess significant topical anti-inflammatory effects as demonstrated from the pharmacological studies in the experimental animal models.
From the present study, based on the result it can be concluded that the seeds of the *Trigonella foenum-graecum*

L. are highly valuable in medicinal usage against treatment of inflammatory responses along with different chemical constituents present in it. Moreover the seeds are important source of potentially useful compounds for the development of new therapeutic agents.

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Further and specific scientific studies are highly recommended for the pharmacological potentials of the fenugreek seeds so that the chemical constituents present in the seeds can be extracted for different formulations and utilized as alternative medicine for the treatment of certain diseases.

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