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Investigation of Anti-Inflammatory and Immunomodulatory Effects of Methanolic Extracts of Fenugreek Leaves and Seeds to Justify its Use in Topical Creams for Preventing Inflammation and Joint-Pain

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Abstract

Many plants that we consume on a daily basis are known for their medicinal values since ancient times. Research has now once again picked up, to restore those beliefs on a more scientific basis by rigorous investigation and checking of phytochemicals present in the plants. One such plant of interest is Fenugreek (Trigonella foenum-graecum L.). This study includes preparation of crude extracts of Fenugreek leaves and seeds by Soxhlet, phytochemical detection and preliminary screening of Fenugreek secondary metabolites, such as, Flavonoids, Alkaloids, Phenolics, Steroids, Terpenoids, Saponins, etc., followed by depiction of its anti-inflammatory and antioxidant properties and its eventual use in formulating a medicinal cream.

Keywords

Anti- inflammatory, Biochemical assays, Fenugreek, Medicinal Plant, Methanolic Extraction, Phytochemical Analysis.

INTRODUCTION

Inflammation: mechanisms, key players and treatment options

The complex biological reaction of vascular tissues to damaging stimuli is commonly referred to as inflammation. Additionally, inflammation is linked with redness, heat, swelling, pain, and loss of function. It includes, among other things, an increase in protein denaturation, an increase in vascular permeability, and membrane modification.

Leukocyte migration from venous networks to the site of injury and cytokine release are known to have a significant role in the inflammatory response. Polymorphonuclear leukocytes are particularly skilled at producing and releasing reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide anion (•O_2-), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), nitric oxide (NO), and 1O_2 (singlet oxygen) among inflammatory cells. The overproduction of ROS can damage cellular carbohydrates, and lipids, leading to cellular and tissue damage that worsens the inflammatory state.[1]

Inflammations don't always help the body. In some diseases the immune system fights against the body's own cells by mistake, causing harmful effects. Numerous human diseases and ailments, such as obesity, neurodegenerative diseases, ageing, and cancer, are thought to be significantly influenced by excessive or chronic inflammation. An aberrant inflammatory response is also intimately linked to many autoimmune diseases like rheumatoid arthritis, diabetes (type 1&2), skin diseases like psoriasis, and inflammatory bowel diseases like Crohn's disease according to growing data. [1]

Hence the anti-inflammatory targeted drugs need to be good at eliminating not only the inflammatory enzymes but also the elevated ROS levels.

NSAIDS (nonsteroidal anti-inflammatory drugs) are among the most widely used medications for inflammation in the world.

They (ibuprofen and naproxen) are used to treat orthopedic disorders such as osteoarthritis, soft-tissue injuries, and fractures, among others. Glucocorticoids, such as cortisone and prednisone, are another class of medications. However, in addition to their high prices, they are associated with significant adverse effects and toxicity, including an increased risk of infection in subgroups of patients. Current medicines cause G.I. ulceration and bleeding, renal damage, hypertension, and hyperglycemia. The main disadvantage of

today's potent synthetic medications is their toxicity and recurrence of symptoms after withdrawal.

As a result, screening and development of medications for anti-inflammatory action is critical, and many attempts are being made to find anti-inflammatory pharmaceuticals from indigenous medicinal plants.

Plant as better alternative anti-inflammatory agents

Natural plant products are currently emerging as key alternative therapeutic choices for establishing anti-inflammatory medications due to their low cost, abundance, and low toxicity. Several epidemiological studies give compelling evidence that natural dietary elements, such as polyphenols and flavonoids, which people eat as food, have a wide range of biological activities. [2]

Unlike modern allopathic drugs, which are single active ingredients that target a single pathway, herbal medicines work through a synchronous approach. A plant contains a plethora of different molecules that work together to target specific elements of the complex cellular pathway. For millennia, medicinal plants have been a source of a wide range of biologically active substances, which have been widely used as raw material or as pure compounds to treat a variety of illness situations. Because of the toxicity and side effects of allopathic treatments, the usage of herbal remedies is becoming more widespread. Medicinal plants are crucial in the production of powerful medicinal medicines. [2]

India, having the world's largest reservoir of medicinal plants, may continue to play a significant role in the production of raw materials, either directly for crude medications or as bioactive chemicals in the manufacture of pharmaceuticals and cosmetics, among other things. [2]

Fenugreek: appearance, taxonomy and review of literature describing its benefits

Trigonella foenum-graecum L. (Fenugreek) is a common vegetable supplement in the Indian diet. Vernacularly known as Methi in Hindi and Marathi; both its seeds and especially leaves have a culinary importance in India and other parts of the world like the Arabian, Mediterranean and Western cuisine. Its leaves are herbal and seeds play a major role in spice blends and as flavoring agent. Not only is it enjoyed as a staple, but it is also lauded indigenously for its medicinal properties. [3]

Fenugreek belongs to the subfamily of Papilioacae of the family of Leguminosae bean family, Fabaceae. Carotene, vitamin A, ascorbic acid, calcium, and iron are abundant in the leaves. Protein, carbohydrate, sugar, mucilage, minerals, volatile oil, fixed oil, vitamins, and enzymes are all found in the seeds. Fenugreek green leaves are an old medicinal herb that has more beta-carotene (19 mg/100 g), ascorbate (220 mg/100 g), fiber, iron, calcium, and zinc than conventional meals. [3]

India is a large producer of fenugreek, with Rajasthan accounting for over 80% of the country's output.

Ethanol extract, mucilage, and flavonoids of fenugreek seeds were found to have anti-inflammatory, anti-arthritic, and anti-oxidant activities. [3]

Linolenic acid in fenugreek seed petroleum ether extract had significant anti-inflammatory efficacy in a variety of acute models involving carrageenan, prostaglandin E2, leukotrienes, and arachidonic acid-induced inflammation, indicating its capacity to inhibit both cyclooxygenase and lipoxygenase pathways. Most of the studies with polar fractions of fenugreek seeds point toward a strong anti-inflammatory and anti-arthritic activities mediated through anti-oxidant mechanisms. Trigonella foenum-graecum has been extensively studied and experimental and clinical studies have demonstrated its antidiabetic, antioxidant, anti-inflammatory, antipyretic, antiulcer, hypocholestrolaemic, immunomodulatory, wound-healing, CNS-stimulant, anticancer, gastro protective and chemo preventive effects. [4]

Fenugreek secondary metabolites

Flavonoids. Flavonoids isolated from Fenugreek are: *Naringenin, lilyn, kaempferol, vecenin-1, tricin 7-O-D glucopyranoside, saponaretin, isovitexin, isoorientin. Orientin, vitexin, luteolin and quercetin*. [3]

Alkaloids. Alkaloids isolated from Fenugreek are: *Trimethylamine, Neurin, Trigonelline, Choline, Gentianine, Carpaine and Betain.* [5]

Phenolics. A study shows that fenugreek sprouts had the highest total phenolic content among other controls and plants upon treatment of elicitors [6].

Saponins. Saponins isolated from Fenugreek are: *Fenugrin, foenugracin, glycoside, yamogenin, trigonoesides, smilagenin, gitogenin, sarsasapogenin, yuccagenin, hederagin, diosgenin, tigonenin, neotigogenin.* [7]

Steroids. Fenugreek contains *diosgenin*, a steroidal saponin that has been reported to suppress inflammation and is has been investigated as a potential treatment for rheumatoid arthritis, especially the acute phase. [8]

Terpenoids. Terpenoids have been isolated from both volatile and aqueous extracts of fenugreek seeds. Comparatively leaves show lesser presence of terpenoids. [9]

β-Carotene. In a study, among the vegetables analyzed, fenugreek leaves had the highest content of beta-carotene (9.15 mg/100 g). [10]

Others. Some other phytochemicals in Fenugreek are: *Carotenoids, Coumarin, lipids, Vitamin A, folic acid, ascorbic acid, thiamin, riboflavin, biotin, nicotinic acid, gum* [11].

MATERIALS AND METHODS

Plant Material: Leaves and Seeds

Fresh fenugreek was collected from local market in Thane, Maharashtra, surface sterilized by carefully washing under running tap water and made sure that there was no sign of decay or contamination. Shade drying was carried out for a week.

A packet of fenugreek seeds was bought from local market in Thane, Maharashtra. 40 gm of seeds from the packet, were

weighed out, washed and grinded fine.

Reagents

Industrial grade methanol, NaOH, dil. HCl, 5% FeCl, Chloroform puriss, dil. H2SO4, Trichloroacetic acid.

Preparation of Crude Extracts

15gm of leaves were weighed and submerged in 150 mL (90% *v/v*) methanol, in a volumetric flask, and left on a rotary shaker overnight and then filtered, first by a muslin cloth, and then by Whatman filter paper No.40. It was oven dried in petri dishes at 50°C for two days to get a concentrated crude extract.

Crude extract of 40gm seed was obtained in 400mL methanol via Soxhlet hot- continuous method of extraction for 5-6 hrs. at 50°C and then a Rotary evaporator (Buchi Rotavapor R-200) was used to get a concentrate of the extract at 70°C.

The dried extracts were weighed, their %yield was calculated, Using formula:

(wt. of dry crude extract/ wt. of crushedplant material used) ×100

And stored in a sealed bag for easy use in assays within two weeks.

Preliminary Phytochemical Analysis

The Trease and Evans (1983) method was used to conduct a qualitative phytochemical test to determine the presence of flavonoids, alkaloids, phenolics, steroids, terpenoids, and saponins. The amount of the component present was determined by the intensity of the colouring.

Anti-inflammatory Analysis

The anti-inflammatory activity of methanolic extracts of fenugreek was evaluated using two in vitro-based assays: **preliminary hemolysis test and hyposaline induced hemolysis inhibition.**

Standard used for experiments was NSAID Ibuprofen.

Preliminary Hemolysis Test

Erythrocyte suspension was prepared according to the method described in Shin de *et al* with appropriate modifications. 4mL of fresh blood was drawn and collected in heparinized vacutainers containing EDTA. 500 µL of blood was transferred into four Eppendorf tubes and another 500 µL of freshly prepared 0.85% Isosaline was added to each, to make a 1:1 dilution. The 4 tubes were centrifuged at 2700 rpm for 10 mins at 27°C in an EMTEK refrigerated centrifuge. The supernatant was discarded in a prepared Dettol solution. And the pellet was pooled into a single Eppendorf tube.

Simultaneously various dilutions of the two plant extracts (1, 2, 3 and 4 mg/mL) were prepared in Isosaline from a 10mg/mL stock solution.

For Ibuprofen, the 200mg tablet was dissolved in 1 mL saline and from this stock the following concentration of 1, 2, 3 and 4mg/mL were prepared.

Table 1. Reaction mixtures were prepared as follows in Eppendorf tubes and then kept for incubation at 37°C for 10 min.

Scheme	Plant Extract	Blood	Saline	Dist. Water
Test	480µL	20µL	-	-
Positive Control	-	20µL	-	480µL
Negative Control	-	20µL	480µL	-

Following incubation, 200µL from each reaction mixture is transferred into a 96 well plate and absorbance of the plate was read at 530 nm.

$$\text{Formula: \%Hemolysis} = \frac{\textbf{Absorbance (Test-Blank)}}{\text{Absorbance of Positive Control}} \times 100$$ [12]

Hyposaline Induced Hemolysis Inhibition

Blood was prepared similar to the Preliminary Hemolysis test. Moreover a 0.15% Hyposaline (*w/v*) solution was prepared in 100mL distilled water.

Table 2. Reaction mixtures were prepared as follows in Eppendorf tubes and then kept for incubation at 37 °C for 30 mins in a Microbiology Incubator

Scheme	Plant Extract	Isosaline	Blood	Hyposaline
Test	500µL	-	20µL	500µL
Control	-	500 µL	20µL	500µL

Following incubation, 200µL from each reaction mixture is transferred into a 96 well plate and absorbance of the plate was read at 530 nm.

$$\text{Formula: \%Hemolysis Inhibition} = \frac{\textbf{Absorbance (Positive control - Test)}}{\textbf{Absorbance of Positive Control}} \times 100$$ [13]

Anti-Oxidant Analysis

The method used to assess the anti-oxidant potential was the ability of the extracts to scavenge free radicals in **DPPH (diphenyl -1, 2-picryl hydrazyl)**. [14]

Fresh DPPH solution was prepared in methanol from the powder till its OD showed 1.4.

Various 1:2 serial dilution concentrations of plant extract (0.6, 1.25, 2.5, 5 mg/mL) were prepared in methanol from a 10mg/mL stock solution.

Table 3. Reaction mixtures were prepared as Follows

Scheme	Plant Extract	Methanol	DPPH
Test	100µL	-	100µL
Color Blank	100µL	100µL	-
Control	-	100µL	100µL

The 96 well plate was kept in the dark for incubation for 30 mins. OD was taken at 517 nm.

Formula: %Scavenging activity=

$$\frac{\textbf{[Abs of control]- [Abs of (Test-Blank)]}}{\textbf{Absorbance of Negative Control}} \times 100$$

Preparation of Cosmetic Formulation Using Extract

Oil phase and water phase ingredients were weighed in separate beakers and heated in a water bath over a hot plate at 60°C.

Table 4. Indicating ingredients used for oil phase of cream.

OIL PHASE		
Ingredient	**Amount (gm/100gm)**	**Use**
Stearic Acid	6	Surfactant
Cetostearyl Alcohol	2	Emulsifier
Glycerol Monostearate	1.5	Lubricant
Jojoba Oil	4	Oil for emulsion
Coconut Oil	2	Oil for emulsion
Propyl Paraben	0.25	Preservative

Table 5. Indicating ingredients used for water phase of cream.

WATER PHASE		
Ingredient	**Amount (gm/100gm)**	**Use**
Triethanolamine	1.5	Emulsifier & pH stabilizer
Methyl Paraben	0.25	Preservative
EDTA	0.25	Chelator
Distilled water	70.5	Solvent
Glycerin	5	Humectant (Skin like)

Following this the appropriate amount of plant extract was added to the water phase (depending on whether it is water soluble or not). The temperatures were maintained and the oil phase was slowly poured into the water phase beaker while a standing mixer was used to emulsify the two phases. The mixing continued for around an hour till the desired consistency was reached. The pH of the cream was measured and adjusted to 6 using Citric acid solution.

The cream was then transferred into a container and stored for further use.

RESULTS

The weight of crude extract of fenugreek leaves that was obtained was **1.42 gm** which gave a yield of **9.467%.** And the weight of crude extract of fenugreek seeds was **6.91 gm** that gave a yield of **17.275%**

Preliminary Phytochemical Analysis Results

Table 6. Showing results of the phytochemical detection Tsts, (+ sign) shows the strength of presence of compound

To Detect	**Observation**	Leaves	Seeds
1. Flavonoids	10 mg/mL extract + NaOH gives yellow color. Further dil. HCl gives decoloration	++	+++
2. Alkaloids	10 mg/mL extract + drops of freshly prepared Mayer's reagent, (HgCl & KI) gives yellow-white ppt	+++	++
3. Phenolics	2 mL, 5% FeCl in 1mL 10mg extract gives dark bluish-purple color	++	+++
4. Steroid	1mL 10mg extract + 5mL Chloroform puriss. Conc. H2SO4 added drop by drop along wall of test tube to give a red colour ppt at the interface of chloroform layer	++	++++
5. Terpenoids	2 mL of Trichloroacetic acid in 1mL extract gives deep red color	+++	+++
6. Saponins	Crude extract dissolved in distilled water and shaken vigorously creates froth	++	++

Preliminary Hemolysis Results

O.D. of Positive Control (Blood + distilled water) = 2.995
O.D. of Negative Control (Blood + Isosaline) = 0.097
O.D. of Color Blank (Isosaline + distilled water) = 0.062

Table 7. Showing the results of hemolysis assay of fenugreek leaves

Concentration (mg/mL)	Test O.D.	%Hemolysis
1	0.162	3.339
2	0.246	6.143
3	0.407	11.519
4	0.675	20.467

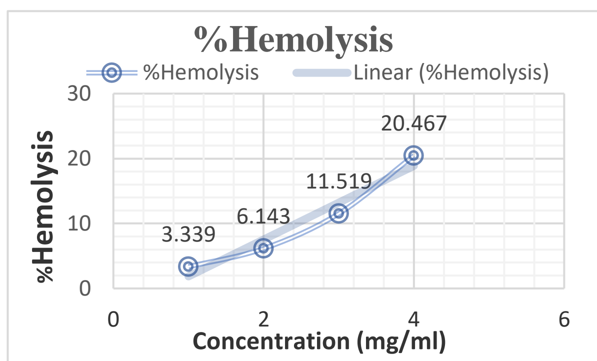

Figure 1. Showing the results of hemolysis assay of fenugreek leaves

Table 8. Showing the results of hemolysis assay in fenugreek seeds

Concentration (mg/mL)	Test O.D.	%Hemolysis
1	0.146	2.804
2	0.221	5.309
3	0.313	8.381
4	0.397	11.185

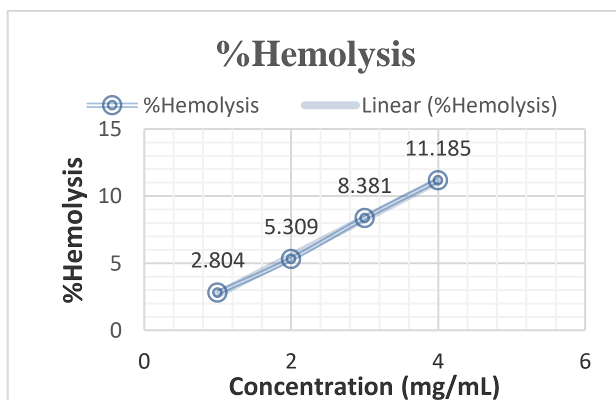

Figure 2. Showing the results of hemolysis assay of fenugreek seeds

Hyposaline Induced Hemolysis Inhibition Results

O.D. of Positive Control (Blood+ Hyposaline 0.15%+ Isosaline) =0.751

Table 9. Showing the results of hyposaline induced hemolysis inhibition assay of fenugreek leaves

Concentration (mg/mL)	Test O.D.	%Hemolysis Inhibition
0.1	0.241	67.9094
0.3	0.095	87.350
0.6	0.101	86.551
1.25	0.115	84.687
2.5	0.087	88.415

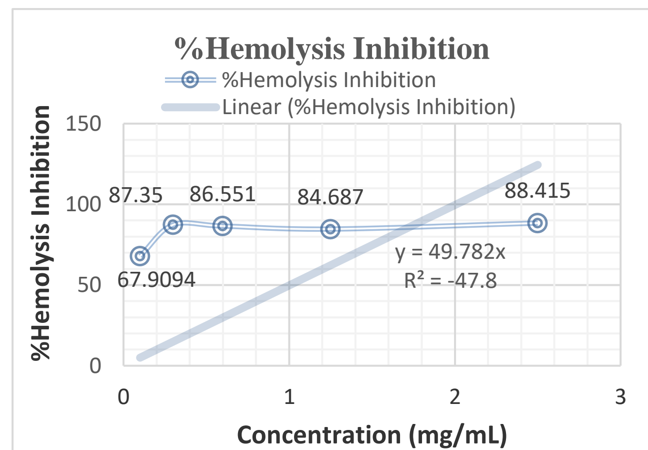

Figure 3. Showing the results of hyposaline induced hemolysis inhibition assay of fenugreek leaves

Table 10. Showing the results of hyposaline induced hemolysis inhibition assay of fenugreek seeds

Concentration (mg/mL)	Test O.D.	%Hemolysis Inhibition
0.1	0.226	69.907
0.3	0.257	65.779
0.6	0.136	81.891
1.25	0.076	89.880
2.5	0.075	90.013

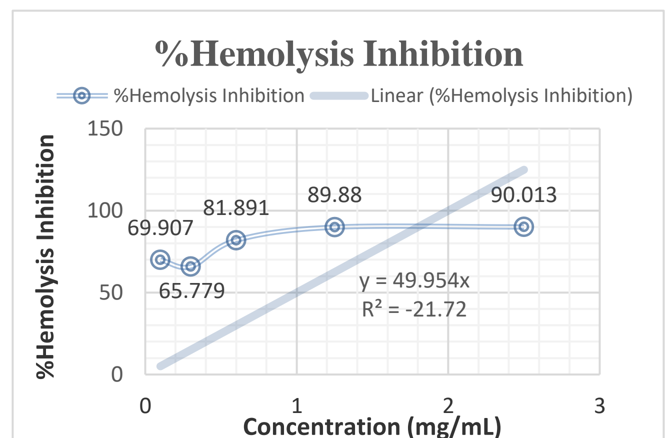

Figure 4. Showing the results of hyposaline induced hemolysis inhibition assay of fenugreek seeds

Analysis of Tests

Finding Potency by using IC50 value- The Half-maximal inhibitory concentration (IC50) is the most widely used and informative measure of a drug's efficacy. It indicates how much drug is needed to inhibit a biological process by half, thus providing a measure of potency of an antagonist drug in pharmacological research.

Calculation by obtaining a linear trendline of the X-Y scatter graph and to get its slope equation, y=mx+c. Following which, calculate x at 50%y.

Calculating the IC50 values for Fenugreek leaves, obtained concentration of extract for cream:

y = 49.782x

Equating y as 50 for 50% inhibitory concentration 50 = 49.782x

Therefore x = 49.782/ 50

= 1.0044 mg/mL

Calculating the IC50 values for Fenugreek seeds, obtained concentration of extract for cream:

Y = 49.954X

Equating y as 50 for 50% inhibitory concentration50 = 49.954x

Therefore X = 49.954/ 50

From the above calculation we can infer that both fenugreek leaves and seed extract are potent inhibitors of membrane lysis of HRBC (human red blood cell). Moreover, seeds showed a better result hence the seed extract was further used to prepare a cosmetic formulation.

Seed Extract in Cream

Calculation of how much crude extract to use in cream:

IC50 value of seeds = 1.0009 mg/mL, this potency is takentimes ten. Therefore 10.009 mg/mL.

Now, for 100 mg cream (100× 10.009)/1= 1000.9 mg or1.0009 gm of extract/100gm of cream.

CONCLUSION

The study contained extraction, phytochemical analysis and secondary analyses on the medicinal plant of Fenugreek to show its anti-inflammatory and immunomodulatory properties. The presence of various compounds such as **flavonoids, alkaloids, phenolics, steroids, terpenoids and saponins** was successfully demonstrated in both fenugreek leaves and seeds.

Moreover, it was shown that fenugreek does not cause much membrane lysis, and its **hemolysis is under 21% for leaves and under 12% for seeds**. The leaves and seeds are also excellent hemolysis inhibitors, showing **%hemolysis inhibition of over 88% and 90%** respectively.

Thus, the seed extract was used to make a cosmetic formulation. In terms of future prospects, there is possibility to carry out in vitro tests, temperature stability tests, skin penetration and absorption tests so that the product meets all the quality guidelines and can be proved as a good contender against other creams in the market in terms of its anti-inflammatory properties.

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