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Formulation, Stability Studies Of Fenugreek Ointment And Its Evaluation For Wound Healing Activity

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ABSTRACT:

Fenugreek (*Trigonella foenum-graecum*) is a legume. It is known for its medicinal qualities such as anti-inflammatory, anti-carcinogenic, anti-oxidant and immunological activities. The fenugreek seed alcohol extraction was used to carry out invitro antioxidant and invivo wound healing studies. The prepared fenugreek ointment formulation was used for anti-oxidant studies to screen its scavenging activity. Three groups of rats were treated with control, fenugreek ointment and povidone iodine ointment to evaluate wound healing activity. The obtained results conclude that, the fenugreek extract ointment treated rats exhibited better results than standard drug in both invitro and invivo studies.

INTRODUCTION:

A wound is a type of injury which happens relatively quickly in which skin is torn, cut, punctured (an open wound), or where blunt force trauma causes a contusion (a closed wound). In pathology, it specifically refers to sharp injury which damages the epidermis of the skin.[1] Wounds that are not healing should be investigated to find the causes and treat them by enhancing wound healing activity[2]. Wound healing means healing of skin, which occurs in four stages, namely; hemostasis, inflammation, proliferation and remodeling or regeneration. (a)The hemostasis phase is initiated when an injury occurs to the skin or tissue. The platelets adhere to the sub-endothelial surface within seconds of the rupture of a blood vessel's epithelial wall. After that, the first fibrin strands begin to adhere in about sixty seconds. As the fibrin mesh begins, the blood is transformed from liquid to gel through pro-coagulants and the release of prothrombin. The formation of a thrombus or clot keeps the platelets and blood cells trapped in the wound area. (b)In the inflammatory phase, the wound starts to close, cellular debris and bacteria are removed and cellular migration is encouraged. In the inflammatory phase, the wound starts to close.[3] (c)The proliferative phase is the phase where granulation tissue forms. Additionally, neovascularization and re-epithelialization occur. Once excess collagen degrades and wound contraction begins to peak, the maturation phase (remodeling) begins. (d) The Remodeling phase starts around week three and can take 12 to 24 months, depending on the patient's age, comorbid medical conditions, and other risk factors. It achieves maximum strength as it matures[4].

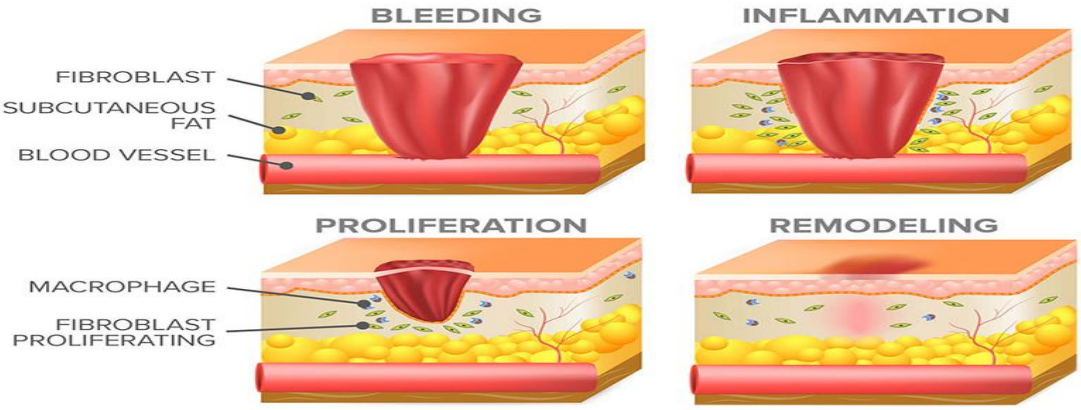

Fig.1 showing different stages of wound healing process

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MATERIALS AND METHODS:

Both invitro anti-oxidant studies and invivo wound healing studies are carried out. Invitro anti-oxidant studies were carried out by using anti-oxidaant screening methods(Catalase assay, Superoxide dismutase (SOD) assay, Lipid peroxidase assay (Lipid peroxidation)). Invivo wound healing studies were done by using incision and excision wound models. Fenugreek seeds were subjected to an alcohol extraction method to get fenugreek extract, which is used in the preparation of ointment.

ALCOHOL EXTRACTION METHOD:

30g of fenugreek powder was mixed in 300 ml of ethanol (75%) by shaking in an incubator shaker for 4 h at 30.50°c and 4.5 pH. The extract was centrifuged in a refrigerated centrifuge at 4°c and 5.687 g for 15 min and supernatant was collected. The residue was re-extracted and then further centrifuged. Both the collected supernatants were combined and evaporated in a rotary evaporator under reduced pressure followed by freeze drying under vacuum. Ethanolic extract of fenugreek powder (10g) was dissolved in 40 ml of water and was partitioned with ethyl ether (100 mlx3), ethyl acetate (100 mlx3) and butanol (100 mlx3) successively. All four fractions were evaporated separately under reduced pressure at 45°c for ethyl acetate and ethyl ether fraction at 65°c for butanol and aqueous fraction as per the method of Liu et al. (2012) and freeze dried under vacuum. Ethyl acetate extract powder was light golden yellow color while butanolic extract powder was of light reddish yellow in color. The yields of ethyl ether, ethyl acetate, butanol and aqueous fractions were 0.25 g, 4.83 g, 3.45 g and 2.50 g, respectively. It was further purified by fractionation on a Sephadex G-25 (25-100 µm, Sigma Aldrich) column (450 x 25 mm) with water/ethanol (100:0-0:100) as the eluent and obtained different fractions. All fractions were evaluated for wound healing activity of fenugreek.[5]

INVITRO ANTIOXIDANT STUDIES:

(A) CATALASE ASSAY: Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). Chemically, it is a hemoprotein structurally similar to hemoglobin.

Reaction: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
Procedure: Prepare Catalase Reaction for each sample, positive control and sample HC wells by mixing 1.5 µL fresh 1 mM H2O2 solution with 10.5 µL Catalase Assay Buffer. Prepare a master mix to ensure consistency and add 12 µL diluted H2O2 solution into each sample, positive control and sample HC wells. Incubate reaction at 25ºC for 30 minutes. Then add 10 µL stop solution to each sample and positive control wells. Do Stop Solution to standard dilution or to Sample HC wells. Now Prepare 50 µL of Developer Mix for each reaction and mix enough reagents for the number of assays to be performed. Prepare a master mix of the Developer mix to ensure consistency and add 50 µL of Developer Mix into each standard, sample, sample HC and positive control wells. Then Mix and incubate at 25ºC for 10 min protected from light and measure output immediately at Ex/Em = 535/587 nm on a microplate reader. Catalase activity in the test samples is calculated by using formula.[6]

Catalase activity = (ΔAbs × Total assay volume) /(Δt x ε x l x Enzyme sample volume).
Where,
Δt is the time of incubation (min),
ΔAbs is the change in absorbance,
ε is the extinction coefficient of substrates in units of M-1 cm-1), and
l is the cuvette diameter (1cm).

(B) SUPEROXIDE DISMUTASE (SOD) ASSAY: Superoxide dismutase (SOD) is an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide (O^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Hydrogen peroxide is also damaging and is degraded by other enzymes such as catalase. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen.
Reaction: $2\ HO_2 \rightarrow O_2 + H_2O$
Procedure: Add 200 µL of WST working solution to each well. To the above solution add 20 µL of Dilution Buffer

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to Blank 2 and Blank 3 and also add 20 µL of Enzyme Working Solution to each sample well and Blank1. Then Mix and incubate at 37°C for 20 minutes. Now measure the output (OD450 nm) on a microplate reader. Finally calculate the SOD activity (inhibition rate %) using the following equation.[7]

Formula:

SOD Activity = ({[A blank 1 - A blank 3]-[A sample - A blank 2]}/ [A blank - B blank 3]) x 100

WST Solution: Dilute the 1 mL WST solution with 19 mL of SOD Assay Buffer, To perform the desired number of assays.

SOD Enzyme Solution: Centrifuge the Enzyme solution for 5 seconds and mix by pipetting, it is essential as the enzyme has 2 layers and must be mixed well before dilution. Dilute 15 µL with 2.5 mL of Dilution Buffer. This is the enzyme working solution and can be stored at +4°C for up to 3 weeks.

(C) LIPID PEROXIDATION ASSAY: Lipid peroxidation is the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in the cell damage. During peroxide formation form fatty acid containing methylene-interrupted double bonds, that is, those found in the naturally occurring polyunsaturated fatty acids, Lipid peroxidation is a chain reaction providing a continuous supply of free radicals that initiate future peroxidation and thus has potentially devastating effects.

Reaction:

1) Initiation: $RH + OH^{*} \rightarrow R^{*} + H_2O$
   $R^{*} + O_2 \rightarrow ROO^{*}$
2) Propagation: $ROO^{*} + RH \rightarrow ROOH + R^{*}$
   $ROOH \rightarrow ROO^{*} + H^{+}$
3) Termination: $ROO^{*} + ROO^{*} \rightarrow RO\text{-}OR + O_2$
   $R^{*} + R^{*} \rightarrow R\text{-}R$

Procedure: Prepare MDA (malondialdehyde) standard dilution for your desired detection method: colorimetric [4 – 20 nmol/well] or fluorometric [0.4 – 2 nmol/well]. Then prepare samples in optimal dilutions so that they fit standard curve readings. To the above, add 600 µL of TBA(Thiobarbituric acid) solution to 200 µL standard and 200 µL test samples and also incubate TBA-standard/TBA-sample mixture at 95°C for 60 minutes. Then Cool to room temperature in an ice bath for 10 minutes and pipette out 200 µL from each 800 µL TBA-standard and TBA-sample reaction mixture into a 96 well microplate. Later measure the plate immediately at OD532 nm for colorimetric assay or Ex/Em =532/553 nm for fluorometric assay.[8] Concentration of MDA in the test samples is calculated by using following formula:

Formula:

Malondialdehyde(µmol/l) = (Absorbance of sample / E0 x L) x D

Where, E**0** = Extinction coefficient $1.56x10^5$ $M^{-1}cm^{-1}$

L = Light path(cm)

D = Dilution factor = $6.7x10^6$

INVIVO WOUND HEALING STUDIES:

(A) INCISION WOUND MODEL: Two parallel six cm paravertebral incisions were made through the full thickness of the skin, 1 cm lateral to the midline of the vertebral column after giving anesthesia. Wounds were closed with interrupted sutures, 1 cm apart with surgical suture. The sutures were removed on the 7th post wounding day. Wound breaking strength (WBS) was measured on the 10th post wounding day in anaesthetized rats. A line was drawn on either side of the incision line 3 mm away from the wound. Two Allis forceps were firmly applied on to the line facing each other. One of the forceps was fixed, while the other was connected to a freely suspended lightweight polypropylene graduated container through a string run over to a pulley. Standard weights were put slowly and steadily into the container. A gradual increase in weight was transmitted to the wound site pulling apart the wound edges. As and when the wound just opened up, the weight was stopped and noted.[9,10]

(B) EXCISION WOUND MODEL: Rats were anesthetized with ketamine (30 mg/kg, IP) and an area of about ≈500 mm2 was marked on the back of the rat by a standard ring. The thickness of the marked skin was then cut carefully. Wounds were traced on 1 mm2 graph paper on the day of wounding and subsequently at a gap

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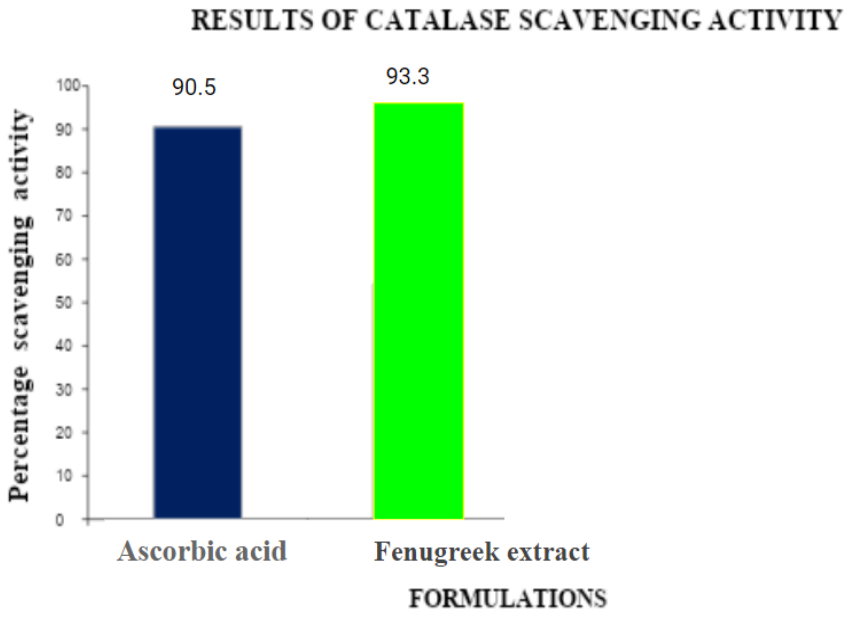
period of 4 days till 12th day, then on the alternate days until healing was complete. Changes in wound area were measured regularly and the rate of wound contraction calculated as given in the formula below. Significance in wound healing of the test groups is derived by comparing healed wound area on respective days with healed wound area of the control group. The period of epithelization, that is, day of fall of eschar and the scar area, was also noted down.[11,12]

% wound contraction = [Healed area /Total wound area] × 100,
(Healed area = original wound area − present wound area)

RESULTS:

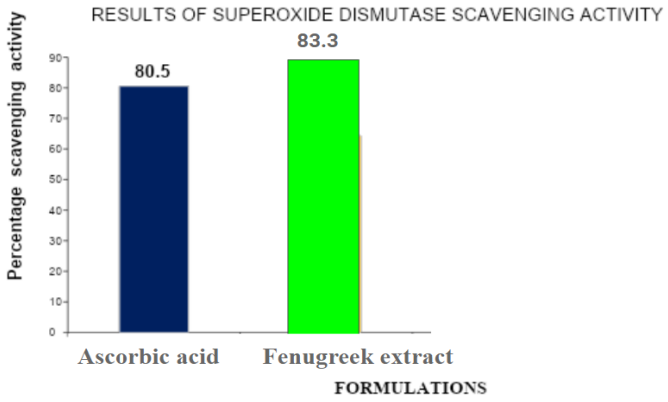
(1) INVITRO ANTIOXIDANT STUDIES:

A) CATALASE ASSAY: Fenugreek extract was compared with standard ascorbic acid.



Graph No.1 Comparing the catalase scavenging activities of selected standard drug and fenugreek extract ointment formulation.

B) SUPEROXIDE DISMUTASE (SOD) ASSAY:



Graph No.2 Comparing the superoxide dismutase scavenging activities of selected standard drug and

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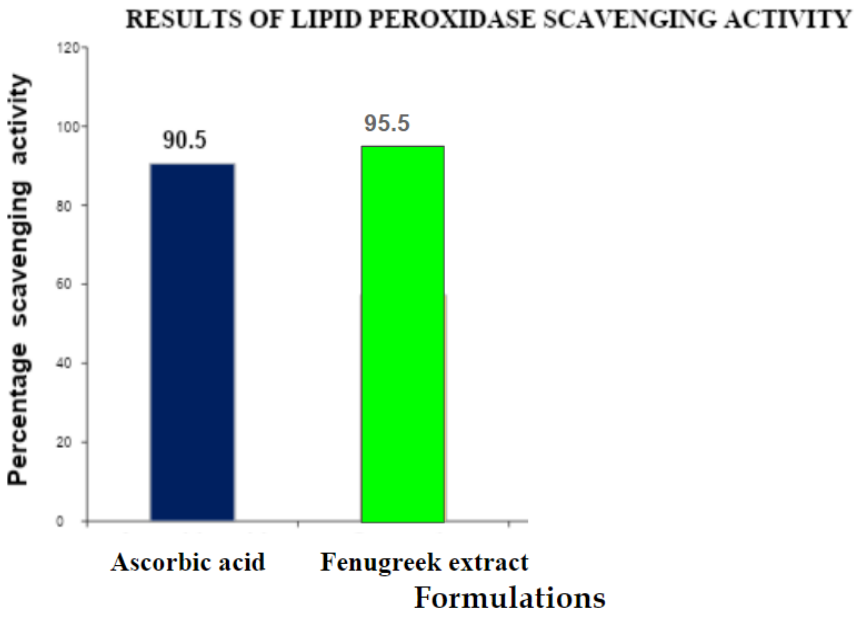
fenugreek extract ointment formulation.

C) LIPID PEROXIDASE ASSAY (LIPID PEROXIDATION):



Graph No.3 Comparing the lipid peroxidase scavenging activities of selected standard drug and fenugreek extract ointment formulation.

B) INVIVO WOUND HEALING STUDIES:

(A) INCISION WOUND MODEL:

S.NO.	**TREATMENT**	**WBS (10^{th} POST WOUND DAY)**
1	CONTROL	282.2 ± 19.6 g
2	POVIDONE IODINE EXTRACT	373.3 ± 13.8 g
3	FENUGREEK EXTRACT OINTMENT	404.0 ± 20.1 g

Table no.1 showing WBS results of incision wound model

Where,

WBS = Wound breaking strength

(B) EXCISION WOUND MODEL:

S.NO.	**TREATMENT**	**% WOUND CONTRACTION**

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		DAY 4	DAY 12	DAY 14	DAY 20
1	CONTROL	21.3%	68.1%	80.5%	98.2%
2	POVIDONE IODINE EXTRACT	32.3%	85.5%	92.6%	100%
3	FENUGREEK EXTRACT OINTMENT	32.5%	87.8%	93.2%	100%

Table no.2 showing % wound contraction results by excision wound model

S.NO.	TREATMENT	MEAN EPITHELIALIZATION PERIOD	MEAN SCAR AREA
1	CONTROL	12.8 days	99.8 mm^2
2	POVIDONE IODINE EXTRACT	10.4 days	75.4 mm^2
3	FENUGREEK EXTRACT OINTMENT	10 days	73.2 mm^2

Table no.3 showing mean epithelialization period and mean scar area results by excision wound model.

DISCUSSION:

The objective of the study is to evaluate fenugreek extract and its role in wound healing activity and antioxidant activity. The extract of fenugreek was extracted by using suitable alcohol extraction method and equipment in our laboratory. The formulation was evaluated for its physical parameters, Invitro anti-oxidant and Invivo wound healing studies. The formulation appears in semi solid consistency with different colour appearance. The formulation was shown to be having a pH range of 5.5 and pH is adjusted to normal.[13]The formulation was evaluated for its scavenging activity by using antioxidant screening methods such as catalase, superoxide dismutase and lipid peroxidation assays.[14] It was also evaluated for their wound healing activity by using excision and incision wound healing activity methods. By comparing standard drug, ascorbic acid with fenugreek extract formulation, we have concluded that the fenugreek extract formulation has better scavenging activity than ascorbic acid. In incision wound healing model results, fenugreek extract ointment showed better wound breaking strength than the standard drug povidone iodine ointment. In the excision wound healing model, fenugreek extract ointment showed better mean epithelialization period and scar area results than the standard drug povidone iodine ointment. By these better results in the incision and excision wound model, we have concluded that fenugreek extract ointment had better wound healing activity than povidone iodine ointment.[15-18]

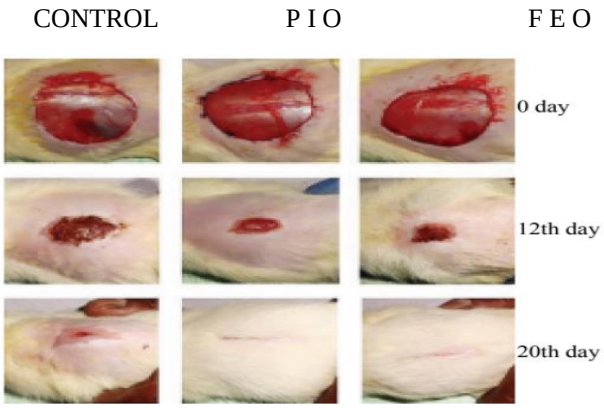

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Fig.2 Photographic representation of contraction rate showing %wound contraction area on different post excision days of control, PIO (Povidone Iodine ointment) and FEO (fenugreek extract ointment)

REFERENCES:

1. O'Mathúna DP (August 2016). O'Mathúna DP (ed.). "Therapeutic touch for healing acute wounds". *The Cochrane Database of Systematic Reviews* (8): CD002766.
2. Velnar T.Bailey T. Smrkolj V. "**The wound healing process: an overview of the cellular and molecular mechanisms".***J Int Med Res.* 2009 Sep–Oct; **37**: 1528-1542.
3. Zarbock A., Polanowska-Grabowska R.K,.Ley K. **Platelet - neutrophil - interactions: linking hemostasis and inflammation.** *Blood Rev.* 2007 Mar; **21**: 99-111.
4. Yang J.,Weinberg R.A., **Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis.** *Dev Cell.* 2008 Jun; **14**: 818-829
5. L.B. Gu, X.N. Liu, H.M. Liu, H.L. Pang, G.Y. Qin, Extraction of fenugreek (Trigonella foenum-graceum L.) seed oil using subcritical butane: characterization and process optimization, Molecules 22 (2017) 2–14. doi:10.3390/molecules22020228.
6. Goth LA. Simple method for determination of serum catalase activity and revision of reference range. Clinica Chimica Acta. 1991 Feb; 196(2-3):143–51.
7. Boveris, A., and Cadenas, E. (1982) in Superoxide Dismutase (Oberley, L. W., Ed.), Vol. 2, pp. 15-29, CRC Press, Boca Raton, FL.
8. Sato Y, Hotta N, Sakamoto Net al.: Lipid peroxide level in plasma of diabetic patients. Biochem Med 25: 373-378, 1981
9. Cetin EO, Yesil-Celiktas O, Cavusoglu T, et al. Incision wound healing activity of pine bark extract containing topical formulations: A study with histopathological and biochemical analyses in albino rats. *Pharmazie*. 2013;68:75–80.
10. Lindblad WJ. Considerations for selecting the correct animal model for dermal wound-healing studies. J Biomater Sci Polym Ed 2008; 19: 1087–96.
11. Singer AJ, McClain SA. Development of a porcine excisional wound model. *Acad Emerg Med.* 2003;10(10):1029–33.
12. Herndon DN, Parks DH. Comparison of serial debridement and autografting and early massive excision with cadaver skin overlay in the treatment of large burns in children. *J Trauma.* 1986;26(2):149–52.
13. Altuntas E, Ozgoz E, Taser F (2005) Some physical properties of Fenugreek (Trigonella foenum-graceum L.) seeds. J Food Eng 71:37–435
14. Dixit, P., Ghaskadbi, S., Mohan, H. and Devasagayam, T. P. A. (2005), Antioxidant properties of germinated fenugreek seeds. Phytotherapy Research, 19: 977–983
15. Akbari M, Rasouli H, Bahdor T (2012) Physiological and pharmaceutical effect of fenugreek: a review. IOSRPHR 2:49–53.
16. Taranalli, A.D. and I.J. Kuppast, 1996. Study of wound healing activity of seeds of Trigonella foeum graceum in rats. Indian J. Pharm. Sci., 58: 117-119.
17. Basch, E., Ulbricht, C., Kuo, G., Szapary, P., & Smith, M. (2003). Therapeutic applications of fenugreek. Alternative Medicinal Review, 8(1), 20–27.
18. Puri, D. (1998). Therapeutic potential of fenugreek. Indian Journal of Physiology and Pharmacology, 42(3), 423–424.