Fenugreek Leaf Extract and Its Gel Formulation Show Activity Against *Malassezia furfur*

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ABSTRACT

Malassezia *spp. are commensal yeasts that can cause cutaneous ailments such as dandruff and seborrheic dermatitis. We sought to develop a cost-effective, herbal formulation for the treatment of cutaneous ailments related to* Malassezia *spp. Aqueous and ethanolic extracts of fenugreek (*Trigonellafoenum-graecum *L.) leaves exhibited activity against a clinical isolate and commercial strain of* Malassezia furfur*. The extracts were also found to be active against other pathogenic fungi such as* Aspergillus niger *and* Candida albicans*. Qualitative and quantitative phytochemical evaluation of aqueous extract showed a predominant presence of flavonoids apart from alkaloids, saponins, carbohydrates, phenols, and proteins. Gel formulation of 30% aqueous fenugreek leaf extract was developed and optimized using sodium alginate as a gelling agent. The formulation showed good physicochemical characteristics and retained activity against* M. furfur *during 3-month accelerated stability studies. Furthermore, the developed herbal gel formulation did not show any irritation or sensitization in New Zealand rabbits after topical application, proving its cutaneous safety. Thus, topical gel formulation containing fenugreek leaf aqueous extract could be a safe and effective herbal treatment for various cutaneous fungal infections, including dandruff.*

Keywords: dandruff, *Trigonella*, dermatitis, herbal formulation, *Candida*, *Aspergillus*

INTRODUCTION

Malassezia spp. are commensal lipophilic yeasts that are commonly found in the sebum secretion enriched areas of the skin such as the trunk, back, face, and scalp.[1–4] Studies have shown that *Malassezia* spp. are responsible for a variety of cutaneous as well as systemic infections such as pityriasis versicolor, malassezia folliculitis, seborrheic dermatitis, dandruff, and atopic dermatitis.[2–3,5–8] Furthermore, *Malassezia* spp.-induced skin inflammation can worsen the clinical symptoms of skin ailments such as psoriasis.[3] Finally, the ability of *Malassezia* spp. to form biofilms further complicates the scenario as these biofilms can lead to persistent infections and lower the susceptibility to therapeutic agents.[7] Typically, azole antifungals such as ketoconazole in the form of a solution, cream, foam, or shampoo are used for the treatment of *Malassezia* spp.-related cutaneous disorders.[2,5] However, ketoconazole is toxic to the mammalian cells and additionally may cause urticaria as well.[3,5] Frequent use of these products may also lead to the development of resistance. Thus, there is a clear need to develop better strategies to treat *Malassezia* spp.-related infections, which would be less toxic and free of untoward side effects.

Plants have been a great source for a variety of therapeutic agents, including antifungal agents. Plant-derived bioactive components have been explored as a low-cost, safe, and novel alternative to the existing therapeutic agents for a variety of ailments, including cutaneous infections.[3,5] Multiple phytoconstituents present in the herbal extracts together elicit the synergistic effect, which reduces the chances of development of microbial resistance.[9] Previously, the potential of extracts and/or purified phytochemicals from *Ilex paraguariensis*, *Dittrichia viscosa*, *Asparagus racemosus*, *Hypericumper foratum*, *Embelia ribes,* and *Vitis vinifera* has been demonstrated for the treatment of infections caused by *Malassezia* spp.[1,3,5,7–8,10] Fenugreek (*Trigonellafoenum-graecum* L., family—Fabaceae) is a leguminous herb grown across the globe, and more prominently in India and many Asian and North African countries.[11] Traditionally, fenugreek has been used by Ayurvedic medicine practitioners for its medicinal properties. Furthermore, fenugreek has also been used in the home-based hair care remedies in India. Several studies have shown that fenugreek seed extracts possess antibacterial, antifungal, and antidandruff activity,[12,13] whereas variable results for antibacterial and antifungal activity have been reported for the fenugreek leaf extracts.[14–16] To date, no study has been carried out on the activity of fenugreek leaves extract against *Malassezia* spp. Furthermore, it is much easier to prepare and

process extracts of fenugreek leaves compared to mucilaginous fenugreek seeds. Hence, in this investigation, fenugreek leaves, owing to their abundance and ease of extraction, were chosen for the systematic investigation of antifungal potential and subsequent development of gel-based formulation, which would be safe, efficacious, and commercially viable for the topical treatment of cutaneous fungal infections.

MATERIALS

Fresh leaves of fenugreek (*Trigonellafoenum-graecum L.*) were procured from the local market of Pune, India. The plant was taxonomically identified and authenticated by Western region center of Botanical Survey of India (Ministry of environment, forests, and climate change).

The strain *Malassezia furfur* MTCC-1374 was procured from MTCC (Chandigarh, India). *Candida albicans* NICM-3102 and *Aspergillus niger* NICM-545 were procured from National Chemical Laboratories (Pune, India).

Quercetin and citronella oil were obtained from Yucca Enterprises (Mumbai, India). Methocel® K100LV and K4M (hydroxypropyl methyl cellulose [HPMC] 100 and 4,000 cps grades, respectively) were received as gift samples from Colorcon Asia Pvt. Ltd (Mumbai, India). Klucel® GF Pharm (hydroxypropyl cellulose [HPC]) was obtained as a gift sample from FDC Ltd (Mumbai, India). Sodium alginate, Carbopol® 940 (crosslinked polycarboxylic acid), and sodium carboxymethyl cellulose (Na CMC) were purchased from Analab Fine Chem (Pune, India). Sabouraud dextrose agar was purchased from Hi-media Laboratories (Mumbai, India).

METHODS

Collection of Leaves and Preparation of Extracts

The procured leaves of fenugreek were washed, shade dried, and ground to a fine powder. Powder portions, 200 g each, were soaked in 1,100 mL of ethanol and 1,100 mL of purified water containing 10% ethanol for 7 days with intermittent stirring. The extracts were filtered and concentrated using rotary evaporator (Evator, EV-11; Equitron Medica, Mumbai, India) at a temperature not exceeding 70°C. The concentrated extracts were subjected to determination of weight per milliliter. The pH of the aqueous extract was determined by using pH meter (Equiptronics EQ-614, Mumbai, India). The extracts were kept in well-closed containers at 2°C–8°C.

Evaluation of Phytochemical Constituents

Qualitative analysis. Aqueous and ethanolic extracts of fenugreek leaves were subjected to preliminary qualitative analysis of major phytochemical constituents such as alkaloids, flavonoids, saponins, steroidal saponins, carbohydrates, reducing sugars, phenols, amino acids, and proteins using standard methods as follows[17]:

Alkaloids. Extract (1 mL) was mixed slowly with an equal volume of concentrated hydrochloric acid. Then a few drops of Mayer's reagent were added to the mixture. Formation of green color or white precipitate indicated the presence of alkaloids.

Flavonoids. To 1 mL of extract, 0.5 mL of 10% lead acetate solution was added. The appearance of greenish yellow color indicated the presence of flavonoids.

Saponins. Extract (2 mL) was mixed with an equal volume of purified water in a graduated measuring cylinder for 15 min. Formation of 1 cm layer of foam indicated the presence of saponins.

Steroidal saponins. Extract (1 mL) was mixed with an equal volume of chloroform. Few drops of concentrated sulfuric acid were added gradually from the sides of the test tube to this mixture. The appearance of a brown ring at the interface suggested the presence of steroidal saponins.

Carbohydrates. Extract (1 mL) was mixed with Molisch reagent; concentrated hydrochloric acid was added through the side of the test tube. The appearance of the violet-colored ring at the junction of two liquids indicated the presence of carbohydrates.

Reducing sugars. Mixture of extract and Benedict's reagent was boiled on a water bath. The appearance of green color suggested the presence of reducing sugars.

Phenols. Extract (1 mL) was mixed with 2 mL of distilled water followed by addition of a few drops of 10% $FeCl_3$ solution. The formation of blue or green color indicated the presence of phenols.

Amino acids. Extract (1 mL) was boiled with 1 mL of 0.2% solution of Ninhydrin reagent. The appearance of dark violet color indicated the presence of amino acids.

Protein. Extract was treated with a few drops of 1% sodium hydroxide solution and few drops of copper sulfate solution were added. Color change to violet confirmed the presence of protein.

Quantitative analysis. The aqueous and ethanolic extracts of fenugreek leaves were subjected to analysis of total flavonoid

content. An aqueous solution of extract (10 mg/100 mL) was prepared and 1 mL of this solution was transferred to 10 mL volumetric flask containing 4 mL of distilled water. To the solution, 0.3 mL of 5% sodium nitrite was added. After 5 min, 0.3 mL of 10% w/v aluminum chloride solution was added. At the sixth minute, 2 mL of 1 M sodium hydroxide solution was added, and the final volume was adjusted to 10 mL with distilled water. The absorbance of the solution was measured spectrophotometrically (Shimadzu UV-1700, Japan) at 510 nm against prepared blank reagent. Total flavonoid content of the extract was determined in triplicate by using the standard plot of quercetin, constructed over the concentration range of 10–100 μg/mL by following the similar procedure described above.[18]

Determination of Antidandruff Activity of Extracts

Isolation of dandruff causative yeast. Dandruff flakes were isolated from the hair of a person suffering clinically from dandruff and streaked on sterile Sabouraud dextrose agar (SDA) slant. The slant was incubated at 32°C for 24 h, following which it was refrigerated until further use.[19]

Identification of yeast. The clinical isolate maintained on SDA slant was subjected to following tests

Catalase reaction. A drop of hydrogen peroxide was added to the isolated culture of yeast and observed for bubble formation.[20]

Growth at 32°C. The clinical isolates of yeast cells were streaked on the SDA plate; 0.2 mL of sterile sesame oil was spread over the SDA and incubated at 32°C for 48 h. The plates were observed for the growth of the yeast.

Microscopy. The SDA plates streaked with isolated yeast culture were incubated at 32°C for 1 week. The plates were observed for the growth of the yeast. The morphology of the yeast cells was then studied under the microscope at 40× magnification by staining with methylene blue.[21]

In vitro *antimicrobial assay.* The activity of both aqueous and ethanolic extracts was checked against the isolated Malassezia culture by agar well diffusion method. Previously liquefied sterile SDA, 20 mL, was poured aseptically into each sterile Petri plate having an internal diameter of 10 cm. Care was taken for the formation of uniform thickness of the medium in the plate. For standardization of inoculums, the turbidity of the yeast suspension was adjusted to 0.5 McFarland standards (containing $\approx 1.5 \times 10^8$ colony-forming units [CFU]/mL).[22] After complete solidification of SDA, the plates were inoculated with 0.1 mL of microbial suspension and set aside for 10 min. Wells were then made in the SDA plates aseptically and 0.1, 0.2, and 0.3 mL of aqueous and ethanolic extracts were added carefully to the wells. Ketoconazole (0.1 mL of 2% w/v solution in dimethyl sulfoxide) was used as a positive control. Plates were kept for prediffusion for 30 min followed by incubation for 24 h at 32°C. The zones of inhibition (ZOIs) were measured using zone reader.[23]

Suspension of the commercial culture of *M. furfur*, after adjusting the turbidity to 0.5 McFarland standard, was observed for the susceptibility to the aqueous and ethanolic extracts of fenugreek in exactly the same manner as described above.

Activity of the Leaf Extracts against Pathogenic Fungi

C. albicans and *A. Niger* were the yeasts chosen for the study. Sterilized SDA plates were inoculated with 0.1 mL of each yeast suspension (containing $\approx 1.5 \times 10^8$ CFU/mL). Antimicrobial activity of 0.1, 0.2, and 0.3 mL of aqueous and ethanolic extracts was determined by agar well diffusion method. Ketoconazole (0.1 mL of 2% w/v solution in dimethyl sulfoxide) was used as a positive control. After the incubation period of 24 h at 32°C, respective ZOIs were measured.

Statistical Analysis

The ZOIs observed for various volumes of the extracts were compared statistically using one-way analysis of variance (ANOVA) at $p \leq 0.05$, followed by Tukey's test using GraphPad Prism software (Version 5.1). The antimicrobial activity of 0.3 mL each of ethanolic and aqueous extract was compared with that of the positive control using one-way ANOVA at $p \leq 0.05$ followed by Tukey's test.

Formulation Development of Aqueous Extract

Selection of gelling agent. By using appropriate methods, gels were prepared using different gelling agents such as sodium alginate (1.5%–3% w/w), carbopol 940 (0.3% and 0.5% w/w), Na CMC (2.5%–3% w/w), two different grades of HPMC *viz.* Methocel K100LV (1%–3% w/w) and Methocel K4M (0.5%–1% w/w), and HPC—Klucel GF Pharm (2%–3% w/w). Resultant gels were examined visually for appearance and consistency.

Preformulation studies. Aqueous leaf extract was added in 10% concentration to each of the prepared gel vehicles mentioned above. The resultant mixtures were observed for appearance and consistency.

Formulation trials. Gel formulations were prepared using the formulae depicted in *Table 1*. The weighed quantities of methyl and propyl paraben were dissolved in boiling water. To this solution, glycerin was added and mixed properly. Well-

Table 1. Development Trials for Gel Formulation of Aqueous Leaf Extract of Fenugreek

Ingredients	Quantity (% w/w)					
	F1	F2	F3	F4	F5	F6
Fenugreek extract	20	20	20	30	30	30
Sodium alginate	2	2.5	3	2	2.5	3
Glycerin	8	8	8	8	8	8
Propyl paraben	0.1	0.1	0.1	0.1	0.1	0.1
Methyl paraben	0.2	0.2	0.2	0.2	0.2	0.2
Citronella oil	0.2	0.2	0.2	0.2	0.2	0.2
Purified water	qs 100	qs 100	qs 100	qs 100	qs 100	qs 100

qs, quantity sufficient.

soaked aqueous slurry of sodium alginate was added to the above-prepared solution with moderate stirring. Weighed quantity of extract was added gradually and mixed thoroughly followed by addition of citronella oil. Finally, the weight of the formulation was made up to 100% with purified water.

Evaluation of Gel Formulations

Organoleptic properties. The formulations were examined for their overall appearance, consistency, homogeneity, and odor.

pH. The pH of the formulations was checked in triplicate using pH meter (Equiptronics EQ-614, Mumbai, India).

Viscosity. The measurement of viscosity of gel formulations was carried out at 25°C using viscometer (DV-E; Brookfield). Spindle number 63 was rotated at 50 rotations per minute (rpm) and the corresponding viscosity (in cps) was noted. The viscosity determination was done in triplicate on three consecutive days.

Spreadability. The weighed quantity of gel (about 0.5 g) was sandwiched between two glass slides. The weight of 100 g was placed on the slides for 10 min, after which the diameter of the spread smear of the gel was measured at different points. Spreadability of the gel was calculated by using the formula

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

Where S—spreadability, M—weight placed on the slide, L—diameter of the circle in cm, and T—time in seconds. The spreadability was determined in triplicate for each trial formulation.

Assay. The weighed quantity of gel (0.1 g) was dissolved in 30 mL of distilled water, sonicated in a bath sonicator for 10 min, and the resulting solution was filtered through Whatman filter paper. The filtrate, 1 mL, was transferred to 10 mL volumetric flask containing 4 mL of distilled water. To the solution, 0.3 mL of 5% sodium nitrite was added. After 5 min, 0.3 mL of 10% w/v aluminum chloride solution was added. At the sixth minute, 2 mL of 1 M sodium hydroxide solution was added and the final volume was adjusted to 10 mL with distilled water. The absorbance of the solution was measured spectrophotometrically (Shimadzu UV-1700, Japan) at 510 nm against prepared blank reagent and the flavonoid content was determined from the standard plot of quercetin. The assay was done in triplicate for each formulation.

In vitro *release study.* Formulation F2 and F5 were subjected to *in vitro* release study using Franz Diffusion cells. Cellophane membrane (molecular weight cutoff: 8,000 Da) was placed in between the donor and receptor compartments of each cell. Receptor compartment contained distilled water maintained at 32°C ± 0.5°C, which was stirred continuously at 600 rpm using a magnetic stirrer. Donor compartment consisting of about 1 g of gel spread uniformly on cellophane membrane was covered with aluminum foil to avoid water loss from the formulation. Aliquots of 2 mL each were withdrawn from the receptor compartment at 1-, 2-, 3-, 4-, 5-, and 6-h intervals and subjected to determination of total flavonoid content, which was determined as described earlier in the section of assay of the gel formulations. The quantity of the receptor medium was kept constant throughout the experiment by replacing an equal quantity of distilled water at every interval. The study was carried out in triplicate.

Determination of in vitro *antidandruff activity.* The antidandruff activity of the formulations F2 and F5 was studied on the pure culture of *M. furfur* by well diffusion method, wherein 0.1, 0.2, and, 0.3 g of each gel formulation were added to the well and the SDA plates were incubated at 32°C for 24 h. The ZOIs exhibited by varying amounts of each formulation were compared by one-way ANOVA at $p \leq 0.05$, followed by Tukey's test, whereas comparison between the ZOI shown by 0.3 g each of formulation F2 and F5 was made using Student's two-tailed *t*-test at $p \leq 0.05$.

The antidandruff activity of formulations was also checked by oil overlay method where 0.2 mL of previously sterilized sesame oil was spread over the solidified SDA plates inoculated with a culture of *M. furfur* and holding 0.3 g quantity each of formulation F2 and F5 in the wells.[24] ZOIs were noted after the incubation at 32°C for 24 h. The comparison of the ZOIs obtained with and without oil overlay was done for each formulation (F2 and F5) using Student's two-tailed *t*-test at $p \leq 0.05$.

Accelerated Stability Studies

The gel formulation was prepared in larger bulk as per the formula F5, filled in aluminum collapsible tubes, and stored at accelerated conditions of 40°C ± 2°C and 75% ± 5% relative humidity. Samples were withdrawn at 0-, 1-, 2-, and 3-month intervals and subjected to the evaluation parameters like organoleptic examination, pH, viscosity, spreadability, assay, *in-vitro* release study, and antidandruff activity. The results of the antidandruff activity at all the time points were compared using one-way ANOVA at $p \leq 0.05$.

Skin Irritation Test

After obtaining the approval of the Institutional Animal Ethics Committee (ICP/IAEC/2017-18/01), New Zealand male albino rabbits ($n = 5$) were procured from LACSMI Biofarms Pvt Ltd (Pune, India). The animals were acclimatized for a period of 7 days. Gel formulation F5 (about 1 g) was applied to the 2.5 × 2.5 cm marked area of left-hand dorsal surface of the shaved intact skin of all the rabbits. A placebo formulation prepared with the same formula as F5, but without the extract, was applied in a similar manner to the right-hand dorsal surface of the back of each rabbit. The sites were occluded with gauze piece and were washed thoroughly after 4 h of application. Skin reaction at the sites of application in the form of erythema or edema was assessed and scored (as per the scheme given in *Table 2*) at 1 h, 24 h, 48 h, 72 h, 84 h (7 days), and 168 h (14 days) after application of the formulations. The scores for test and control sites were calculated using the following formula:

Skin irritation score = $\sum$ Erythema and edema scores at 1, 24, 48, 72, 84, and 168 h/number of rabbits

The scores of both the test and control sites were compared statistically using Student's two-tailed *t*-test at $p \leq 0.05$.[25] The animal experimentation complied with the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forest and Climate Change, Government of India.

Table 2. Evaluation of Skin Irritation Scores

Reaction	Score
(A) Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness to eschar formation)	4
(B) Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well raised)	2
Moderate edema (raised approx.1mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Total possible score for primary irritation	8

RESULTS

Preparation of Extracts

The aqueous and ethanolic extracts of fenugreek leaves prepared by cold maceration method had characteristic fenugreek odor. The weight per milliliter of aqueous and ethanolic extract was found to be 1.33 and 1.31 g/mL, respectively. The pH of the aqueous extract was found to be 4.15.

Evaluation of Phytochemical Constituents

Phytochemical evaluation of the aqueous and ethanolic extracts showed the presence of saponins, steroidal saponins, flavonoids, phenols, and proteins in both the extracts. In addition to these, the aqueous extract showed the presence of carbohydrates and alkaloids, unlike the ethanolic extract.

The total flavonoid content of the aqueous extract was 22.85 ± 2.47 μg/mg and that of ethanolic extract was 19.12 ± 1.19 μg/mg.

Determination of Antidandruff Activity of Extracts

Isolation and identification of dandruff causative yeast. Dandruff-causing microbe was isolated from the hair of the person suffering from dandruff. Catalase test and microscopic examination confirmed that the isolated yeast belonged to dandruff-causing *Malassezia* spp. The culture of the clinical isolate of *Malassezia* was maintained by regular subculturing on the SDA slants. Commercial culture of *M. furfur* was also procured to compare the activity of both the extracts.

In vitro *antidandruff activity of the extract.* In the case of isolated strain, as well as the commercial strain of *M. furfur,* ZOI shown by the aqueous extract was significantly higher compared with the ethanolic extract. As the quantity of extract increased from 0.1 up to 0.3 mL, ZOI increased significantly. The clinical isolate of *Malassezia* showed greater susceptibility to the extract compared to MTCC 1374 culture, which could be attributed to the difference between the two strains (*Fig. 1*). Ketoconazole, a synthetic antifungal with

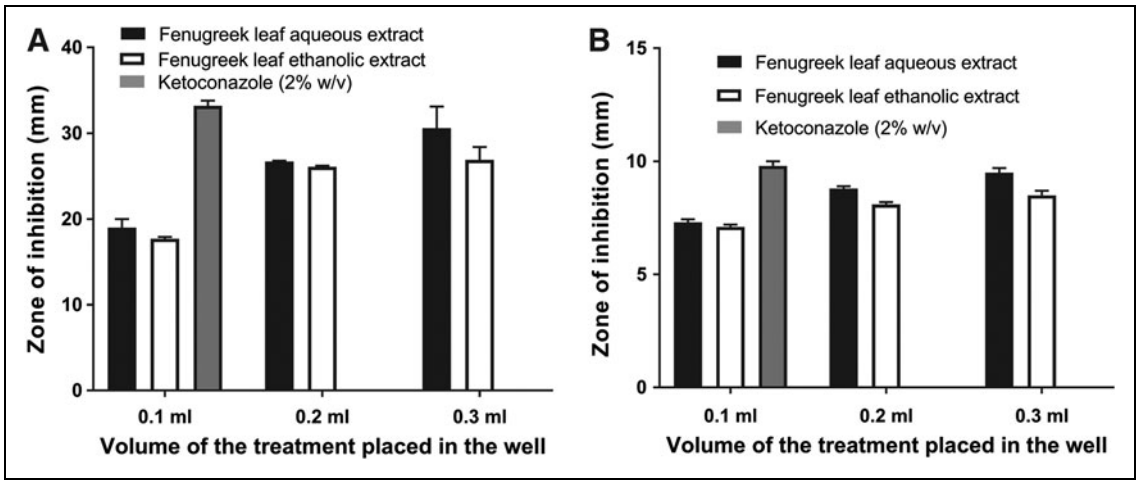

Fig. 1. Fenugreek leaf aqueous and ethanolic extract show activity against *Malassezia furfur*. Fenugreek leaf aqueous extract (specific gravity: 1.33 g/mL) and ethanolic extract (specific gravity: 1.31 g/mL) and ketoconazole solution in DMSO (2% w/v) showed activity against **(A)** clinical isolate of *M. furfur* and **(B)** commercially procured *M. furfur* (MTCC-1374). Clinical isolate of *M. furfur* was more sensitive to fenugreek leaf extracts and positive control (ketoconazole) compared to the commercial strain of *M. furfur*. (Data expressed as mean ± SD; $n = 3$). DMSO, dimethyl sulfoxide; SD, standard deviation.

known activity against *Malassezia* spp., was used as a positive control. The concentration of ketoconazole solution (2% w/v) used for the testing was same as that of topical ketoconazole formulations used in the clinical practice. The ZOI shown by the 0.3 mL of aqueous extract was statistically comparable to that shown by ketoconazole, whereas 0.3 mL of the ethanolic extract showed significantly lower ZOI. Hence, aqueous extract was selected for the gel formulation development.

Activity of the Leaf Extracts Against Pathogenic Fungi

Findings of considerable activity against dandruff-causing yeasts prompted us to explore the activity of the extracts against the pathogenic fungi like *A. niger* and *C. albicans*. The aqueous extract showed activity against both *A. niger* and *C. albicans*, whereas ethanolic extract showed activity only against *A. niger* with little or no activity against *C. albicans* (*Fig. 2*). Various phytoconstituents present in the herbal extracts are together responsible for showing certain pharmacological activity. Presence of alkaloids along with the rest of the phytoconstituents in aqueous extract could be responsible for its activity against *Candida*, unlike the ethanolic extract in which alkaloids were absent. The aqueous extract thus showed overall better antifungal activity compared to the ethanolic extract (*Supplementary Fig. S1*).

Formulation Development of Aqueous Extract

To develop a topical gel formulation for the extract, gel vehicles were prepared using various gelling agents. Upon addition of 10% extract to Carbopol 940 gel base, the gel lost its consistency owing to the shift in the pH to about 4, the viscosity of which could not be regained after adjusting the pH to 6. Gel vehicles prepared with cellulose-based gelling agents like Na CMC, HPMC, and HPC, upon addition of extract, showed a drop in the viscosity and significant thinning after standing for a few hours to few days. Such incompatibility was not observed between the extract and the sodium alginate gel base upon storing the mixture at room temperature for 1 month.

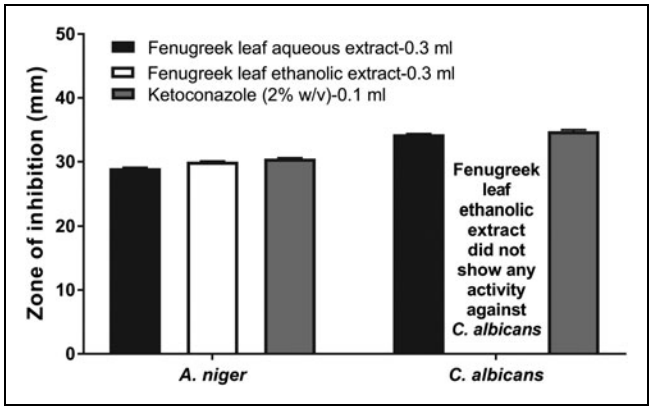

Fig. 2. Fenugreek leaf aqueous and ethanolic extract show activity against other pathogenic fungi. Fenugreek leaf aqueous extract (0.3 mL) showed activity against *Aspergillus niger* as well as *Candida albicans*, whereas ethanolic extract (0.3 mL) was only active against *A. niger*. Around 0.1 mL of 2% w/v ketoconazole solution in DMSO was used as a positive control. (Data expressed as mean ± SD; $n = 3$).

Gel formulations were developed with varying concentrations of sodium alginate as a gelling agent, methyl and propyl paraben as antibacterial preservatives, glycerin as a humectant, and citronella oil as a fragrance to mask the characteristic odor of the fenugreek leaf extract. Formulations comprised two different concentrations of extract *viz.* 20% (formulations F1, F2, and F3) and 30% (F4, F5, and F6). This was to evaluate the impact of the quantity of extract on viscosity and antidandruff activity of the formulation.

Evaluation of Gel Formulations

The results of the evaluation of gel formulations are shown in *Table 3*.

Formulations F2 containing 20% extract and F5 containing 30% extract showed acceptable consistency, spreadability, and viscosity, and hence, these formulations were further evaluated for the *in vitro* release and antidandruff activity.

Both the formulations released the flavonoids completely over a period of 6 h (*Fig. 3*). The higher release rate was observed from formulation F5 at initial time points owing to the presence of a higher quantity of extract compared to that in formulation F2, thus creating a greater concentration gradient across the donor and receptor compartment. However, toward the end of the 6-h study period, both the formulations showed almost complete release.

Formulation F5 showed significantly better *in vitro* antidandruff activity compared to formulation F2, owing to a higher quantity of the extract present in it. The marginally higher viscosity of formulation F5 did not affect the release of the actives from the formulations. There was no significant difference between the ZOIs observed in the SDA plates with oil overlay and the ones without oil overlay (*Supplementary Fig. S2*). Overlay of oil on the growth medium simulates the condition of oily scalp, which is observed commonly in those suffering from dandruff. Presence of oil favors the growth of dandruff-causing fungi.[26] However, similar antimicrobial activity in the presence and absence of oil postulates equal efficiency of the formulation in inhibiting the fungal growth in case of dry as well as oily scalp. Formulation F5 with higher ZOI than F2 was considered the optimum formulation.

Table 3. Evaluation of Fenugreek Aqueous Leaf Extract Containing Gel Formulations

Sr. no.	Formulation	pH	Viscosity (cps)	Spreadability (g·cm/s)	Assay %
1	F1	6.6 ± 0.15	590 ± 12	2.53 ± 0.03	101.07 ± 0.95
2	F2	6.62 ± 0.18	742 ± 15	2.18 ± 0.07	98.48 ± 2.00
3	F3	6.59 ± 0.2	753 ± 04	2.06 ± 0.1	99.38 ± 7.76
4	F4	6.12 ± 0.11	614 ± 10	2.41 ± 0.04	94.40 ± 1.25
5	F5	6.21 ± 0.12	754 ± 10	2.11 ± 0.05	98.77 ± 1.25
6	F6	6.23 ± 0.04	810 ± 11	1.88 ± 0.9	98.40 ± 2.25

The values reported are average ± standard deviation of $n = 3$.

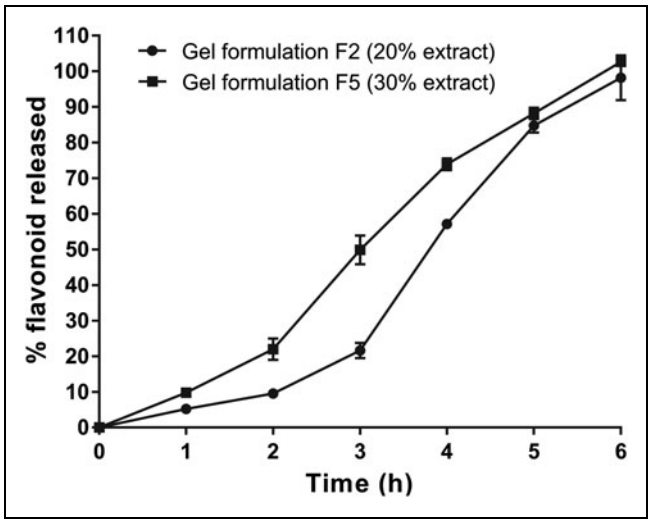

Fig. 3. *In vitro* release studies of fenugreek aqueous leaf extract-containing gel formulations from cellophane membrane using Franz-diffusion cells. The gel formulation (F5) containing higher amount of extract showed quicker release of flavonoids and by the end of 6 h, both the gel formulations (F2 and F5) completely released flavonoids in the receptor medium. (Data expressed as mean ± SD; $n = 3$).

Skin Irritation Test

Application of formulation F5 to the skin of New Zealand albino rabbits did not lead to any erythema or edema compared to the placebo formulation. The scores of erythema and edema calculated over the observation period of 14 days for the test as well as control were zero (*Table 4*). This confirmed the innocuous nature of the formulation and its safety in cutaneous application.

Accelerated Stability Testing

Stability samples of formulation F5 drawn at 1-, 2-, and 3-month intervals showed comparable organoleptic properties, consistency, and spreadability characteristics with no significant change in the viscosity, pH, and assay values. *In vitro* release profiles of the stability samples were compared with that of the initial sample (*Fig. 4*). The formulation very well retained the antifungal activity throughout the study period as confirmed by similar ZOIs obtained for all the stability samples against *M. furfur* in the absence as well as the presence of oil overlay.

Table 4. Score of Erythema and Edema After Application of Gel Formulation F5 (Test) and Placebo Formulation (Control) to the Skin of the Back Portion of New Zealand Rabbits

Rabbit no.	Reaction	Score for the skin reaction											
		Control						Test					
		1 h	24 h	48 h	72 h	84 h	168h	1 h	24 h	48 h	72 h	84 h	168h
1	Erythema	0	0	0	0	0	0	0	0	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0
2	Erythema	0	0	0	0	0	0	0	0	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0
3	Erythema	0	0	0	0	0	0	0	0	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0
4	Erythema	0	0	0	0	0	0	0	0	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0
5	Erythema	0	0	0	0	0	0	0	0	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0

DISCUSSION

Malassezia spp.-associated cutaneous disorders are estimated to affect more than 140 million individuals worldwide every year.[3] The current drug therapies against *Malassezia* spp. such as ketoconazole and other azole antifungal agents have issues such as mammalian cell toxicity and the emergence of resistant strains.[2–3,5] As most of the *Malassezia* spp.-related cutaneous disorders require long-term treatment, it is essential to develop low-cost and effective therapies against *Malassezia* spp. Plant extracts or pure phytochemicals containing lotions, solutions, or shampoos have been used for the treatment of cutaneous disorders, including *M. furfur*-related dandruff. This investigation focused on evaluating the activity of fenugreek leaf extract against *M. furfur*.

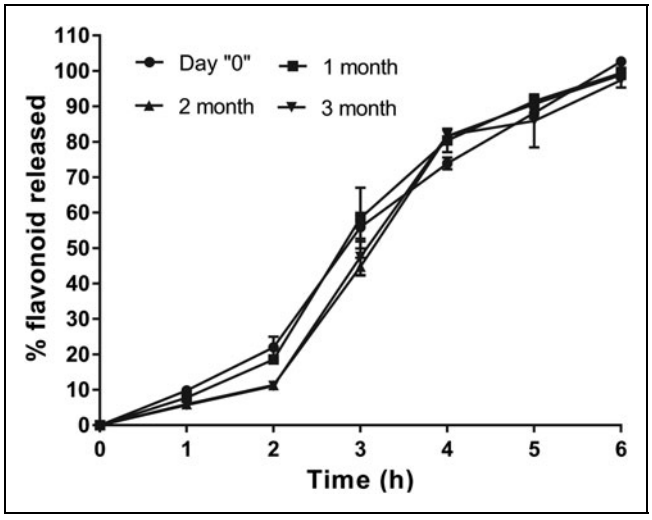

Fig. 4. *In vitro* release profile of flavonoids from fenugreek aqueous leaf extract containing gel formulation (F5) did not show any significant change over the course of 3 months. The formulations were stored at 40°C ± 2°C and 75% ± 5% RH. (Data expressed as mean ± SD; $n = 3$). RH, relative humidity.

Trigonellafoenum-graecum L. (fenugreek) is an edible leguminous plant that is traditionally used for its medicinal properties. Fenugreek is also known for its medicinal values in other Asian, Mediterranean, and African regions. Studies show that fenugreek can be used for the treatment of inflammation, wound, abscesses, and arthritis.[11,27–29] Several studies have shown that fenugreek seed extracts possess antibacterial, antifungal, antidandruff, antidiabetic, antioxidant, cholesterol lowering, and anticancer activity.[12,13] Seed extracts have particularly shown potential in the treatment of diabetes and related neuropathy[30–34] and urolithiasis.[35] Fenugreek leaves, although more abundant in nature compared to the seeds, have been relatively less explored for bioactivity. The reported literature demonstrates the antioxidant, antiplasmodial, wound healing, and antiulcer activity of the fenugreek leaves,[15,36,37] whereas variable results for antibacterial and antifungal activity have been reported for the

fenugreek leaf extracts.[14–16] Thus far, no study has been carried out to explore the activity of fenugreek leaf extract against *Malassezia spp*. We focused on the preparation and evaluation of aqueous and ethanolic extract of fenugreek leaves with the hope of developing herbal formulation suitable for the treatment of cutaneous fungal infections.

The cold maceration process was used for the extraction of leaves. The extraction process was optimized by determining the flavonoid content of the extract on day 4, 7, and 9 of maceration and the study showed significantly higher flavonoid content on the seventh day compared to the fourth day, whereas no significant increment was noted in the content on day 9 (*Supplementary Table S1*). Hence, the cold maceration process was carried out for 7 days to prepare the extracts. The *in vitro* assay showed that aqueous and ethanolic extracts of fenugreek leaves were significantly active against clinical isolate as well as the commercial strain of *M. furfur*. It was noteworthy that the clinical isolate was more sensitive to the fenugreek leaf extract as well as ketoconazole compared to the commercial strain. It should also be noted that aqueous fenugreek extract showed similar anti-*Malassezia* activity when compared to 2% w/v ketoconazole solution. Currently, all the clinically used ketoconazole formulations contain 2% w/v of ketoconazole.[38] Hence, the similarity of anti-*Malassezia* activity of the fenugreek leaf aqueous extract to that of 2% w/v ketoconazole could be correlated to the antidandruff potential of the herbal extract.

Fenugreek leaves contain a considerable amount of flavonoids such as quercetin, vitexin, and kaempferol and their glycosides, phenolic compounds, and ascorbic acid.[39–43] Flavonoids and phenolic compounds are known to possess antimicrobial, anti-inflammatory, and antioxidant property.[5,7–9] Studies have shown that leaf extracts rich in flavonoids are active against *M. furfur*.[5,7–9] Recently, it has been shown that polyphenolic compounds, including flavonoids such as quercetin and kaempferol, show activity against *Malassezia globosa* by selectively inhibiting *Malassezia* β-carbonic anhydrase, a validated antidandruff target.[44] Thus, the anti-*Malassezia* activity of fenugreek leaf extract could be attributed to the presence of flavonoids. As flavonoids such as quercetin and kaempferol are also active against pathogenic fungi and their biofilms,[45,46] it was not surprising to see that fenugreek leaf extract was also found to be active against pathogenic fungi such as *C. albicans* and *A. niger*. The studies showed that the aqueous extract was generally more active than ethanolic extract in most of the cases. Hence, the aqueous extract of fenugreek leaves was chosen for further development.

The study focused on the development of aqueous gel formulation as it can be used for the treatment of a variety of cutaneous fungal disorders. Preformulation studies showed that fenugreek leaf aqueous extract is incompatible with commonly used gelling agents such as carbopol and cellulose derivatives such as CMC, HPMC, or HPC. Studies have shown that phenolic compounds could affect the gelling of cellulose derivatives such as HPMC[47] and the phenolic compounds present in the fenugreek leaf extract could be responsible for the incompatibility. Such an incompatibility was not observed when sodium alginate was used as a gelling agent. The gel formulation F5 containing 30% fenugreek leaf extract retained its activity against *M. furfur*. Furthermore, the formulation did not show any irritation to rabbits' skin, indicating its *in vivo* safety and tolerability. Finally, the optimized gel formulation F5 exhibited good physical and chemical stability and retained the antidandruff activity throughout the 3-month storage period at accelerated conditions.

CONCLUSION

Due to its activity against the *Malassezia* spp., fenugreek leaf aqueous extract can be further developed as herbal remedy for the treatment of dandruff and other *Malassezia* spp.-related cutaneous infections. The developed topical gel containing 30% fenugreek leaf extract retained the activity against *Malassezia* spp. Hence it can be further evaluated as an alternative to the currently available 2% topical ketoconazole formulations.

DISCLOSURE STATEMENT

No competing financial interests exist.

AUTHORS' CONTRIBUTION

V.H. conceptualized the project, M.K. supervised the project, V.J. carried out the laboratory work, and A.A.D. contributed in research discussions, drafting, and editing the article.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1

REFERENCES

1. Onlom C, Khanthawong S, Waranuch N, Ingkaninan K: In vitro anti-Malassezia activity and potential use in anti-dandruff formulation of *Asparagus racemosus*. *Int J Cosmet Sci* 2014;36:74–78.
2. Angiolella L, Carradori S, Maccallini C, Giusiano G, Supuran CT: Targeting Malassezia species for novel synthetic and natural antidandruff agents. *Curr Med Chem* 2017;24:2392–2412.
3. Sivasankar C, Gayathri S, Bhaskar JP, Krishnan V, Pandian SK: Evaluation of selected Indian medicinal plants for antagonistic potential against *Malassezia* spp. and the synergistic effect of embelin in combination with ketoconazole. *Microb Pathog* 2017;110:66–72.
4. Schwartz RA: Superficial fungal infections. *Lance*t 2004;364:1173–1182.
5. Filip R, Davicino R, Anesini C: Antifungal activity of the aqueous extract of *Ilex paraguariensis* against *Malassezia furfur*. *Phytother Res* 2010;24:715–719.
6. Schwartz J, Messenger A, Tosti A, *et al.*: A comprehensive pathophysiology of dandruff and seborrheic dermatitis—towards a more precise definition of scalp health. *Acta Derm Venereol* 2013;93:131–137.
7. Simonetti G, Tocci N, Valletta A, *et al.*: In vitro antifungal activity of extracts obtained from *Hypericumper foratum* adventitious roots cultured in a mist bioreactor against planktonic cells and biofilm of *Malassezia furfur*. *Nat Prod Res* 2016;30:544–550.
8. Simonetti G, D'Auria FD, Mulinacci N, *et al.*: Anti-dermatophyte and Anti-Malassezia activity of extracts rich in polymeric flavan-3-ols obtained from *Vitis vinifera* seeds. *Phytother Res* 2017;31:124–131.
9. Matthew CJ, Aishwarya I, Blonk B, Ian CE: Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? *Pharmacogn Rev* 2017;11: 57–72.
10. Rhimi W, Ben Salem I, Immediato D, Saidi M, Boulila A, Cafarchia C: Chemical composition, antibacterial and antifungal activities of crude *dittrichia viscosa* (l.) greuter leaf extracts. *Molecules* 2017;22:E942.
11. Wani SA, Kumar P: Fenugreek: A review on its nutraceutical properties and utilization in various food products. *J Saudi Soc Agric Sci* 2018;17: 97–106.
12. Zia T, Hasnain SN, Hasan S: Evaluation of the oral hypoglycaemic effect of Trigonellafoenum-graecum L. (methi) in normal mice. *J Ethnopharmacol* 2001; 75:191–195.
13. Husain FM, Ahmad I, Khan MS, Al-Shabib NA: *Trigonellafoenum-graceum* (Seed) extract interferes with quorum sensing regulated traits and biofilm formation in the strains of Pseudomonas aeruginosa and Aeromonas hydrophilia. *Evid Based Complement Alternat Med* 2015;2015:879540.
14. Sharma V, Singh P, Rani A: Antimicrobial activity of *Trigonellafoenum-graecum* L. (fenugreek). *Eur Exp Biol* 2017;7:1–4.
15. Ramya P, Sudisha J, Lakshmi DN, Aradhya SM: Antibacterial and anti-oxidant activities of fenugreek (*Trigonellafoenum-graecum* L.) leaves. *Res J Med Plant* 2011;5:695–705.
16. Haouala R, Hawala S, El-Ayeb A, Khanfir R, Boughanmi N: Aqueous and organic extracts of *Trigonellafoenum-graecum* L. inhibit the mycelia growth of fungi. *J Environ Sci* 2008;20:1453–1457.
17. Gupta M, Thakur S, Sharma A, Gupta S: Qualitative and quantitative analysis of phytochemicals and pharmacological value of some dye yielding medicinal plants. *Orient J Chem* 2013;29:475–481.
18. Al-Matani SK, Al-Wahaibi RN, Hossain MA: Total flavonoid content and antimicrobial activity of crude extract from leaves of Ficussycomorus native to Oman. *Karbala Int J Mod Sci* 2015;1:166–171.
19. Pingili M, Vanga S, Rapala RK: Antifungal activity of plant extracts against dandruff causing organism *Malassezia furfur*. *Int J Bioassays* 2016;5:5047–5049.
20. Kindo A, Sophia S, Kalyani J, Anandan S: Identification of Malassezia species. *Indian J Med Microbiol* 2004;22:179–181.
21. Tarazooie B, Kordbacheh P, Zaini F, *et al.*: Study of the distribution of Malassezia species in patients with pityriasis versicolor and healthy individuals in Tehran, Iran. *BMC Dermatol* 2004;4:5.
22. Sutton S: Determination of inoculum for microbiological testing. *J GXP Compliance* 2011;15**:**49–53.
23. Ali I, Khan FG, Suri KA, Gupta BD, *et al.*: In vitro antifungal activity of hydroxychavicol isolated from Piper beetle L. *Ann Clin Microbiol Antimicrob* 2010;9:7.
24. Agrawal V, Bhagwat A, Sawant C: Sesame oil incorporated medium for isolation and enumeration of lipophilic yeasts. *Int J Pharm Sci Res* 2004;5: 2972–2979.
25. More BH, Sakharwade SN, Tembhurne SV, Sakarkar DM: Evaluation for skin Irritancy testing for developed formulations containing extract of Butea monosperma for its topical application. *Toxicol Appl Pharmacol* 2013;3:10–13.
26. Borda LJ, Wikramanayake TC: Seborrheic dermatitis and dandruff: a comprehensive review. *J Clin Investig Dermatol* 2015;3:10.
27. Aher R, Belge S, Kadam S, Kharade S, Misal A, Yeole P: Therapeutic importance of fenugreek (*Trigonellafoenum-graecum* L.). A review. *J Plant Sci Res* 2016;3:49.
28. Srinivasan K: Fenugreek (Trigonellafoenum-graecum): A review of health beneficial physiological effects. *Food Rev Int* 2006;22:203–224.
29. Yadav UC, Baquer NZ: Pharmacological effects of Trigonellafoenum-graecum L. in health and disease. *Pharm Biol* 2014;52:243–254.
30. Bodhankar SL, Ghule AE, Jadhav SS: Trigonelline ameliorates diabetic hypertensive nephropathy by suppression of oxidative stress in kidney and reduction in renal cell apoptosis and fibrosis in streptozotocin induced neonatal diabetic (nSTZ) rats. *Int Immunopharmacol* 2012;14:740–748.
31. Morani AS, Bodhankar SL, Mohan V, Thakurdeasi PA: Ameliorative effects of standardized extract from *Trigonellafoenum-graecum* L. seeds on painful peripheral neuropathy in rats. *Asian Pac J Trop Med* 2012;5:385–390.
32. Raju J, Gupta D, Rao AR, Yadava PK, Baquer NZ: Trigonella foenum-graecum (Fenugreek) seed powder improves glucose homeostasis in alloxan diabetic rat tissues by reversing the altered glycolytic, gluconeogenic and lipogenic enzymes. *Mol Cell Biochem* 2001;224:45–51.
33. Kumar GS, Shetty AK, Sambaiah K, Salimath PV: Antidiabetic property of fenugreek seed mucilage and spent turmeric in streptozotocin-induced diabetic rats. *Nutr Res* 2005;25:1021–1028.
34. Hannan JM, Ali L, Rokeya B, *et al.*: Soluble dietary fibre fraction of Trigonellafoenum-graecum (fenugreek) seed improves glucose homeostasis in animal models of type 1 and type 2 diabetes by delaying carbohydrate digestion and absorption, and enhancing insulin action. *Br J Nutr* 2007;97: 514–521.
35. Kapse CU, Bodhankar SL, Vishwaraman M, Thakurdeasi PA: Therapeutic effect of standardized fenugreek seed extract on experimental urolithiasis rats. *J Appl Pharm Sci* 2013;3:29–32.
36. Bhatia K, Kaur M, Atif F, *et al.*: Aqueous extract of Trigonellafoenum-graecum L. ameliorates additive urotoxicity of buthionine sulfoximine and cyclophosphamide in mice. *Food Chem Toxicol* 2006;44:1744–1750.
37. Palaniswamy M, Pradeep BV, Sathya R, Angayarkanni J: In vitro anti-plasmodial activity of *Trigonellafoenum-graecum* L. *Evid Based Complement Alternat Med* 2010;7:441–445.
38. Choi FD, Juhasz MLW, Mesinkovska AN: Topical ketoconazole: a systematic review of current dermatological applications and future developments. *J Dermatolog Treat* 2019;22:1–12.
39. Rababah TM, Hettiarachchy NS, Horax R: Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and tert-butylhydroquinone. *J Agric Food Chem* 2004;52:5183–5186.

40. Al-Dabbagh B, Elhaty IA, Al Hrout A, *et al.*: Antioxidant and anticancer activities of *Trigonellafoenum-graecum*, *Cassia acutifolia* and *Rhazya stricta*. *BMC Complement Altern Med 2018*;18:240.
41. Singh P, Vishwakarma SP, Singh RL: Antioxidant, oxidative DNA damage protective and antimicrobial activities of the plant *Trigonellafoenum-graecum*. *J Sci Food Agric* 2014;94:2497–2504.
42. Omezzine F, Bouaziz M, Daami-Remadi M, Simmonds MSJ, Haouala R: Chemical composition and antifungal activity of *Trigonellafoenum-graecum* L. varied with plant ploidy level and developmental stage. *Arab J Chem* 2017;10:S3622–S3631.
43. Bahmani M, Shirzad H, Mirhosseini M, Mesripour A, Rafieian-Kopaei M: A Review on ethnobotanical and therapeutic uses of fenugreek (*Trigonellafoenum-graceum*L). *J Evid Based Complementary Altern Med* 2016;21:53–62.
44. Nocentini A, Bua S, Del Prete S, *et al.*: Natural polyphenols selectively inhibit β-carbonic anhydrase from the dandruff-producing fungus *Malassezia globosa*: activity and modeling studies. *Chem Med Chem* 2018;13:816–823.
45. Rocha MFG, Sales JA, Rocha MG, *et al.*: Antifungal effects of the flavonoids kaempferol and quercetin: a possible alternative for the control of fungal biofilms. *Biofouling* 2019;8:1–9.
46. Gao M, Wang H, Zhu L: Quercetin assists fluconazole to inhibit biofilm formations of fluconazole-resistant *Candida albicans* in in vitro and in vivo antifungal managements of vulvovaginal candidiasis. *Cell Physiol Biochem* 2016;40:727–742.
47. Banks SR, Pygall SR, Bajwa GS, Doughty SW, Timmins P, Melia CD: The influence of substituted phenols on the sol: gel transition of hydroxypropyl methylcellulose (HPMC) aqueous solutions. *Carbohydr Polym* 2014;101:1198–1204.

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Abbreviations Used

ANOVA = analysis of variance
CFU = colony-forming units
HPC = hydroxypropyl cellulose
HPMC = hydroxypropyl methyl cellulose
Na CMC = sodium carboxymethyl cellulose
SDA = Sabouraud dextrose agar
ZOI = zone of inhibition