

Treatment of Gastric Ulcers with Fenugreek Seed Extract; *In Vitro*, *In Vivo* and *In Silico* Approaches

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Figier, *et al.*: Fenugreek Seed Extract for Gastric Ulcers

The objective of the present study was to evaluate the antiulcer or gastroprotective potential of fenugreek seed aqueous extract using *in vitro* and *in vivo* models and to elucidate the possible mechanism of fenugreek seeds by *in silico* analysis on H⁺/K⁺ ATPase receptor, a crucial target for mediating the gastroprotective effect. Human gastric carcinoma epithelial cell line was used as an *in vitro* model to study the gastro protective effect of fenugreek aqueous extract over a concentration range of 0.1-10 µg/ml by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay against ethanol (15%). *In vivo* gastroprotective effect of the extract was assessed in adult Sprague Dawley rats of either sex at three doses *viz.*, 200, 600 and 1000 mg/kg against ethanol (90%). Pantoprazole sodium (50 mg/kg) and misoprostol sodium (20 µg/kg) were used as positive control. The flavonoids and saponins reported in the extract were assessed for possible interactions at H⁺/K⁺ ATPase receptor site by *in silico* analysis. In the human gastric carcinoma epithelial cells, Fenugreek protected against the damage induced by ethanol at 5 µg/ml; whereas a protection of 67% at the dose of 1000 mg/kg was observed in the animal studies. The flavonoid derivatives namely vitexin-7-O-glucoside, vicenin-2, orientin and luteolin showed good interactions on H⁺/K⁺ ATPase while the saponins lacked good interaction in *in silico* analysis. Fenugreek seed extract showed gastroprotection in both: *in vitro* and *in vivo* studies. The possible mechanism of action for the extract was elucidated by *in silico* studies.

Key words: Fenugreek, AGS cell line, ulcer index, flavonoids, saponins, H⁺/K⁺ ATPase

Fenugreek (*Trigonella foenum-graecum*, Family: Fabaceae) is a commonly used condiment and spice in Indian household. The plant seeds are reported to be of significant medicinal values with its high flavonoid, phenol, saponin and amino acid content^[1]. The antiulcer activity of aqueous and gel fraction has been demonstrated in several animal models^[2-4]. Though these studies have stated several mechanisms behind its gastroprotective effect, most have relied on the use of *in vivo* method.

There have been some studies, which reported use of *in vitro* models like the human gastric carcinoma epithelial cell line (AGS), which is a well-established model and has characteristics of normal gastric epithelial cells^[5]. However, such a model was never used for evaluating the gastroprotective effect in case of herbal extracts. Development of an *in vitro* model for evaluating gastroprotection in herbals can

help reduce the use of large number of experimental animals and the arduous procedure of obtaining ethics committee approvals for the same. In order to take an initiative in this regard it is necessary to have results in *in vitro* model; results of which are comparable to those obtained in *in vivo*.

Elucidation of mechanisms of herbs with substantial experimental evidence involves the isolation and testing of each component for the specific target proposed, which is a time consuming process. Use of *in silico* approaches like docking is useful in characterization of the possible interaction and thereby elucidating the

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binding mode between the herbal constituents with the proposed site of action (enzyme or the receptor)^[6,7]. Unfortunately, a combined approach involving *in vitro*, *in vivo* and *in silico* has been rarely used to study the therapeutic action in case of herbals.

Taking these limitations into consideration, we conducted the present study to evaluate and compare the cytoprotective effect of aqueous extract of fenugreek plant seeds in both *in vitro* and *in vivo* models so as to determine if similar results are obtained using both the models. Also, through this study we aimed to elucidate and support the possible mechanism of action for fenugreek seed extract, by assessing the interactions of *in silico* analysis for the major reported constituents on H⁺/K⁺ ATPase a receptor crucial in mediating gastroprotective effect.

The combined approach of *in vitro* and *in vivo* evaluation supported with *in silico* analysis in characterization of the biological activity will thus aid us in proposing the mechanism of fenugreek, which can further help in identifying safer and cost effective alternatives for treating gastric ulcers.

MATERIALS AND METHODS

Soxhlet apparatus (J-Sil, India), Ham's F-12 medium, fetal bovine serum (Invitrogen, USA), tissue culture flasks (BD Falcon, USA), misoprostol sodium as misoprost tablets (Cipla Ltd, India), ethanol (CDH Ltd, India), petroleum ether and sodium bicarbonate (S. D. Fine-Chem Ltd, India), pantoprazole sodium (Blue Cross Labs Pvt. Ltd, Mumbai).

Preparation of the extract:

The seeds of *T. foenum-graecum* were procured from Namdeo Umaji Agritech Pvt. Ltd. Mumbai and were deposited in a herbarium for authentication (sample specimen no: bhf 140611a). Finely ground seed powder was extracted using Soxhlet extraction apparatus by defatting with petroleum ether initially and then by extracting with distilled water. The extract obtained was tested chemically for the presence of various phytoconstituents^[8].

Phytochemical screening of the extract was done to ascertain presence of flavonoids along with other phytoconstituents like saponins and amino acids. Diosgenin, a saponin was used as the marker compound for the extract. Since diosgenin exists in conjugation with aglycone moiety, hydrolysis of the extract with 2 N HCl was performed and partitioned using chloroform. Detection and quantification of

diosgenin was then done by HPTLC densitometry using Camag HPTLC system III, WinCATs software and precoated silica Gel 60 F₂₅₄ (Merck) TLC plates as the stationary phase. The sample applications were done using a Hamilton syringe (100 µl), Linomat 5 applicator with sample band length of 8 mm and migration distance of 180 mm. The mobile phase used was petroleum ether: isopropanol (9:1) with a detection wavelength of 430 nm. The bands were detected using anisaldehyde sulphuric acid as the derivatizing agent. The R_f of diosgenin spot was found to be 0.5.

In vitro studies on fenugreek aqueous extract:

AGS cell line was procured from National Centre for Cell Sciences (NCCS), Pune. Cells were incubated in Ham's F-12 medium containing 100 mg/l of penicillin, 150 mg/l of azithromycin, 1.2 g/l of sodium bicarbonate and 10% foetal bovine serum. The cell monolayer was subcultured in a 25 cm² flask at 1:5 ratios every 72 h by treatment with 0.1% trypsin and 0.03% ethylenediaminetetraacetic acid (EDTA). Cells were grown in an incubator with 5% CO₂ at 37°. Cell passage was maintained in a range of 25-40 for experimentation.

The aqueous extract of fenugreek was evaluated over a concentration range of 0.1 to 400 µg/ml against 15% ethanol as an ulcerogen. The concentration of ethanol to induce damage was obtained after standardization (fig. 1A). The concentrations used were extrapolated from the reported human dose of fenugreek (Nature's Way® Fenugreek seed capsules). The effect on cell viability was assessed by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, fig. 1). The cells were seeded (density of 40×10³ cells/ml) in 96 well plate followed by treatment with different concentrations of the extract. The plate was incubated for 24 h with 5% CO₂ at 37°. Following incubation, MTT was added for 4 h followed by the addition of HCl:isopropanol (1:24); in order to lyse the cells and solubilize the formazan crystals. The absorbance of the resulting solution was read using an enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 570 nm. The amount of colour produced was directly proportional to the number of viable cells. Concentrations of the extracts which did not affect the cell viability were further selected for evaluating the cytoprotective activity.

To determine the maximum effect of ethanol as ulcerogen on AGS cells, the cells were incubated in serum free medium containing 0-15% v/v ethanol for

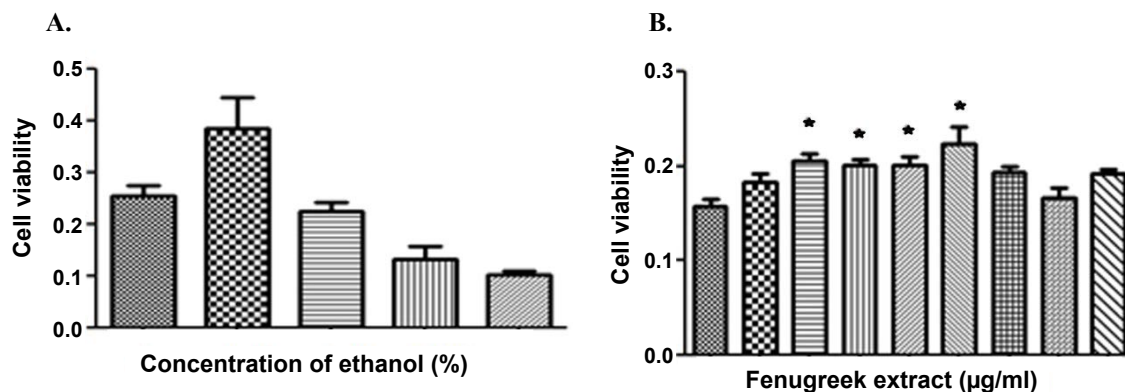


Fig. 1: Cell viability assay

(A) Standardization of ethanol concentration for cell viability, ☒ control, ☒ 5%, ☒ 10%, ☒ 12.50%, ☒ 15%. (B) Effect of extract concentration on cell viability against ethanol-induced damage, ☒ ethanol control, ☒ 0.1 µg/ml, ☒ 0.5 µg/ml, ☒ 1 µg/ml, ☒ 2.5 µg/ml, ☒ 5 µg/ml, ☒ 7.5 µg/ml, ☒ 10 µg/ml, ☒ standard. (Data represented as mean±SEM for n=6. *Indicates significance with respect to ethanol treated positive control at P<0.05, using one way ANOVA with Turkey multiple comparison test)

30 min. Following incubation, viability of the cells was assayed using MTT assay. The concentration inducing maximum damage was further used to study cytoprotective effect of the extracts.

AGS cells were seeded in 96 well plates with cell density of 4×10^3 cells/well followed by treatment with various concentrations of the extracts over a range 0.1-10 µg/ml for 24 h. The cytoprotective role of the extracts against ethanol was assayed by exposing the treated AGS cells to 15% ethanol for 30 min. After exposure the cell viability was measured using MTT assay. AGS cells treated only with ethanol served as control to assess the cytoprotective effect. Based on the similarity for the mechanism of action, misoprostol sodium a prostaglandin analogue was used as a reference standard for evaluating cytoprotective activity of fenugreek phytoconstituents against ethanol damage.

In vivo studies for evaluation of gastroprotection in ethanol-induced ulcer model:

Sprague Dawley rats (nos-84) in weight range 150-250 g were used for study. The protocol for *in vivo* studies was approved by the animal ethics committee and was prepared as per committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines (MVC/IAEC/10/2012).

Animals fasted for over 24 h were administered with the extract suspension in doses of 200, 600 and 1000 mg/kg. An hour later, ethanol (90%) was administered orally. After 1 h the animals were euthanized by injecting xylazine ketamine. The euthanized animals were dissected and stomachs were opened along greater curvature. The gastric

juice was collected and the stomach was washed with saline and examined for the presence of ulcers. The ulcer index, curative ratio, pH, and titratable acidity were measured for the gastro protective assessment parameters^[2,4,9]. Two reference standards having different gastroprotection mechanism: pantoprazole sodium, a proton pump inhibitor (causes reduction of gastric acidity) and misoprostol sodium, a prostaglandin analogue (offers mucosal protection) were used for gastroprotective comparison. The histopathology of sample was performed by embedding tissue sample of each of the study groups in paraffin and cross sections of 4 µm were taken using a microtome. The sections were stained using eosin and haematoxylin and observed under the electron microscope of resolution of 100x.

The results obtained for *in vitro* and *in vivo* were expressed as ±SEM. The data was evaluated by using one way ANOVA (Tukey multiple comparison test) for *in vitro* study and paired t-test for *in vivo* studies. Value of P<0.05 was considered to be statistically significant.

In silico computational studies:

The docking studies of the fenugreek constituents with the binding site of H⁺/K⁺ ATPase receptor was performed using Surflex-1.3 module in Sybyl-X 2.0 Suite^[10]. Since experimentally determined structure of H⁺/K⁺ ATPase pump was not available, it was built using the principles of comparative protein modelling in Modeller 9v9^[9,11-13]. The template sequences of structures, sodium-potassium pump (Na⁺/K⁺-ATPase) and gastric H⁺/K⁺-ATPase (PDB code 3A3Y and 2XZB, respectively) were obtained from the protein data bank^[10] and aligned using Clustal X2^[14]. The

sequence similarity of 62% was observed between the query and the template sequences, which is considered as acceptable to proceed with the homology modelling. The best model was chosen based on De-optimized potential energy (DOPE score)^[11] and profile-3D^[15] scores and Ramachandran's plot of the models. The best model thus selected was prepared using the protein preparation wizard workflow in the Schrödinger Suite 2010.

The 3D model of H⁺/K⁺-ATPase pump was minimized using molecular dynamics simulations (MDS). Prior to MDS the model was embedded in dipalmitoyl phosphatidylcholine (DPPC) membranes based on the co-ordinates provided by orientation of proteins in membrane database (OPM) and then soaked in TIP3P water explicit model^[16]. The salt concentration of the system was adjusted to 0.15 M of NaCl by adding appropriate Na⁺ and Cl⁻ ions. This generated the system necessary to carry out the MDS. The system was then relaxed using NPT relax protocol for transmembrane proteins in Desmond. Subsequently the system was simulated for 5 ns, with the temperature of the system maintained at 323 K by coupling to an external temperature bath based on the Berendsen algorithm. Initial velocities were generated randomly from a Maxwell distribution at 323 K in accordance with the masses assigned to the atoms. The trajectories and corresponding energies were sampled every 5 ps.

The ligands of the fenugreek constituents selected for docking were mainly flavonoids and saponins i.e. vitexin-7-O-glucoside, vicenin-2, vicenin-2, orientin, luteolin, naringenin, quercetin, diosgenin, yamogenin, yuccagenin, tigogenin and smilagenin. The active site of amino acids reported in the literature for H⁺/K⁺ ATPase^[17,18] is with respect to ligands SCH28080 and oubain, respectively. The amino acid residues found important in the active site of H⁺/K⁺ ATPase pump were: Val³⁴⁴, Ala³⁴⁵, Phe³³⁸, Gly³⁴¹, Phe⁸⁰⁵, Thr⁸¹⁹, Asp³³⁴, Tyr⁸⁰⁸, Arg⁹⁰² and Asp⁹⁰⁸. The SurFlex docking protocol was employed to dock the said ligand, which is based on an incremental construction of the ligands in the active site of the protein. A set of diverse poses were obtained based on the ranking by the inbuilt empirical scoring function of SurFlex^[19,20].

RESULTS AND DISCUSSION

Phytochemical assessment indicated presence of flavonoids, saponins and phenols (Table 1). In *in vitro* studies on fenugreek aqueous extract showed statistically significant inhibition of cell death

(fig. 1A) at concentrations of 0.5, 1.0, 2.5 and 5.0 µg/ml. The extract was also found to exhibit better *in vitro* cytoprotective activity than misoprostol sodium (reference standard) against ethanol-induced damage to the cells (fig. 1B). A decrease in the activity of the extract was observed beyond the concentration of 5.0 µg/ml.

In *in vivo* model, a positive correlation existed between the doses of extract and protective response i.e. an increase in protection with increase in dose was observed. The most effective concentration of 1000 mg/kg of extract was found to prevent ulcer lesions completely (fig. 2). However, an increase in titrable acidity was seen at high dose. Similarly the pH increased towards the acidic range at the protective dose of 1000 mg/kg. Curative ratio indicated 67% protection with respect to positive control group. The extract exhibited better gastric acidity reduction and mucosal protection effect in comparison to reference standards-pantoprazole sodium and misoprostol sodium against ethanol as an ulcerogen (Table 2).

In *in silico* model, flavonoid constituents like vitexin-7-O-glucoside, vicenin-1, luteolin and orientin were found to show good binding, with H⁺/K⁺ ATPase

TABLE 1: PHYTOCHEMICAL PARAMETERS

Phytochemical parameter	Value
Water soluble extractive value	36.80%
Total flavonoid content	10.54 mg/g of extract
Total phenolic content determined as mg equivalent of gallic acid	65.82 mg/g of extract
Percent content of total saponins in hydrolysed extract	20.56%
Diosgenin content in total saponins	3.80%

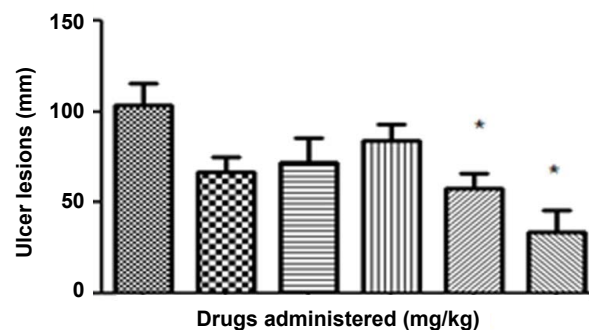


Fig. 2: Effect of fenugreek extract against ethanol-induced damage in rats

⊠ Positive control, ⊞ standard I (pantoprazole), ⊞ standard II (misoprostol), ⊞ low dose (200 mg/kg), ⊞ medium dose (600 mg/kg), ⊞ high dose (1000 mg/kg). High dose was found to be most effective in reducing lesions (Data represents ±SEM for n=6. *Indicates significance with respect to positive control group at P<0.05, using paired t test for all treatment groups)

as compared to other compounds (Table 3). Diosgenin, which was used as a biomarker was found to show moderate binding. The key amino acids involved in interaction were mainly Ile³³⁷, Ile³⁴⁰, Asp³³⁴, Asn¹⁴³, Asn¹⁴⁴ and Gly¹³³ (fig. 3). Also the presence of hydroxyl groups on the glycosidic scaffold was found to contribute significantly to the overall polar interactions shown by the compounds.

The present study aims to establish a correlation between *in vivo*, *in vitro* and *in silico* approaches for predicting gastroprotection of fenugreek seed extract. The AGS cell line used *in vitro* closely mimics the gastric epithelial lining *in vivo* and thus it was easier to correlate the response elicited by the extract. A dose-dependent increase in cytoprotection was seen *in vitro* with an increase in the concentration of the fenugreek aqueous extract. The damage induced by ethanol *in vitro* is through generation of toxic free radicals^[21] and presence of phytoconstituents like flavonoids and saponins, which exhibit antioxidant and gastroprotective activity^[22,23] in the extract, could have contributed to this protective response. The possible free radical scavenging effect of the extract observed *in vitro* is in sync with a study conducted by Madhava *et al.*, who found up to 70% free radical scavenging activity in fenugreek extracts^[24].

In *in vivo* model similarly, the gastroprotective activity was observed at higher doses with significant reduction in ulcer lesions. However, at the highest protective dose (1000 mg/kg) an increase in acidic pH and titratable acidity was seen. This possibly indicates that the protective mechanisms of the extract were not based solely on alteration of pH or a decrease in titratable acidity. A close understanding of the mechanism behind ethanol-induced damage based on previous reports shows that the detrimental effect of ethanol on gastric mucus is not due to an increased secretion of gastric acid^[25], but due to an alteration in the permeability of the gastric mucus by interference

with the activity of cyclic AMP. The extract exhibited good mucosal protective effect as there was an increase in total mucosal weight in treated groups as compared to the ethanol group. The aqueous extract being rich in presence of galactomannans could have formed a protective barrier around the gastric epithelial lining, as galactomannans have been reported to possess mucosal protective effect^[26]. Also, a significant reduction in the degree of degeneration, necrosis and inflammation on treatment with the extract *in vivo* indicates a possible role as an antiinflammatory and antisecretory agent apart from mucus protection which is in sync with the observation of previously published reports^[2]. The antisecretory mechanisms suggested for the extract could possibly involve certain key receptors like the H⁺/K⁺ ATPase. Interestingly, key flavonoid constituents reported to be present in the extract like vitexin-7-O-glucoside, vicenin-1, luteolin and orientin have shown remarkable degree of interaction with H⁺/K⁺ ATPase receptor binding site *in silico* (fig. 4).

The extract tested positive for the presence of flavonoids. The *in vitro* gastroprotective activity of fenugreek extract can be attributed to the free radical scavenging effect of the extract on the AGS cell line. The *in vivo* studies in rats suggested that the extract due to the presence of galactomannans formed a protective barrier over the gastric epithelial lining and thus exhibited a good mucosal protective effect. Findings from *in vivo* studies also suggested that the gastroprotective mechanism was not attributed solely due to alteration of gastric pH or acidity. The gastroprotective response of the extract was consistent in both *in vitro* and *in vivo* models. The crude form of the extract is an amalgam of a series of phytoconstituents, each individually possessing a therapeutic value with the possibility of synergism. Thus, to avoid the arduous, time consuming and expensive process of isolation of each component, *in silico* analysis after confirming activity through *in vitro* and *in vivo* models is, in our view, appropriate.

TABLE 2: GASTROPROTECTION OBSERVED IN VARIOUS GROUPS OF RATS IN ETHANOL-INDUCED ULCER MODEL

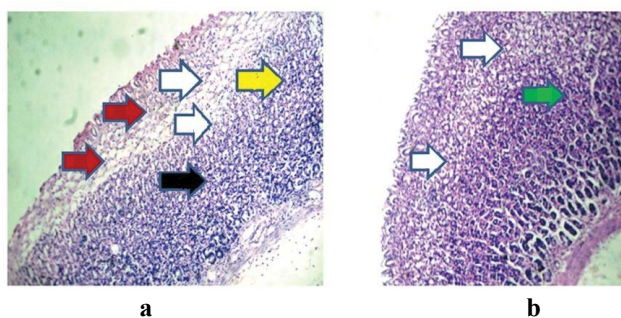
Groups (dose administered in rats per body weight)	Ulcer index (mm)	Curative ratio (%)	Titratable acidity (mEq/l)	pH
Ethanol group (2 ml)	103.40±11.97	-	320	2.5
Std. I, pantoprazole (50 mg/kg)	67.20±8.027	35	24	6.98
Std. II, misoprostol sodium (20 µg/kg)	72.20±13.29	30.17	24	6.98
Low dose of fenugreek extract (200 mg/kg)+ethanol	84.00±9.072	18.70	56	5.03
Medium dose of fenugreek extract (600 mg/kg)+ethanol	58.20±8.224*	43.71	48	6.03
High dose of fenugreek extract (1000 mg/kg)+ethanol	34.00±11.92*	67.11	160	4.45
Normal (2 ml)	NIL	NIL	160	4.6

*Indicates significance with respect to positive control group

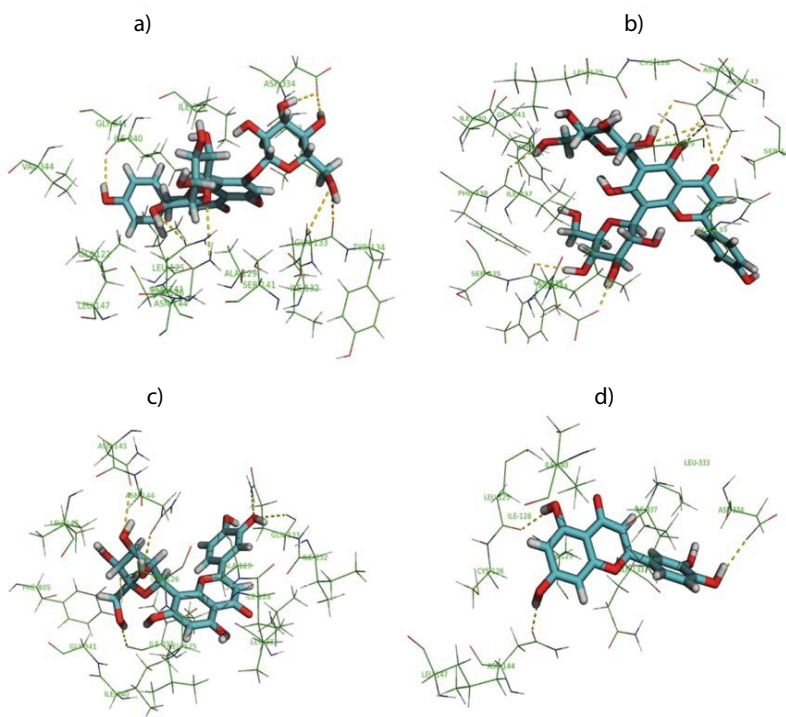
TABLE 3: DOCK SCORES OF FENUGREEK CONSTITUENT ON INTERACTION WITH H⁺/K⁺ ATPase

Compound	Total score	Crash score*	Polar score**
Vitexin-7-O-glucoside	6.17	-2.26	6.02
Vicenin-2	6.1	-1.85	6.99
Isovitexin	5.35	-1.36	2.31
Luteolin	5.19	-0.43	3.63
Orientin	5.1	-1.47	4.05
Sarsasapogenin	4.8	-0.75	0
Isorientin	4.4	-1.85	2.63
Neotigogenin	4.35	-0.57	0
Diosgenin	4.26	-1.47	1.47
Tricin	4.17	-0.46	1.64
Yuccagenin	3.45	-2.44	1.16
Gitogenin	3.45	-1.19	0.002
Quercetin	3.22	-0.39	3.14
Naringenin	2.97	-0.42	2.15

*Crash scores close to 0 are favourable negative numbers indicate penetration; **Polar score indicates the degree of H-bonding for a given molecule; molecules with low polar scores were excluded from further screening

**Fig. 3: Histopathology of tissue**

(a) Ethanol positive control, (b) tissue post treatment with fenugreek extracts (1000 mg/kg). ➡ Haemorrhage, ➡ inflammation, ➡ necrosis, ➡ degeneration, ➡ oedema

**Fig. 4: Interactions of active constituents with H⁺/K⁺ ATPase**

(a) Vitexin 7-O-glucoside, (b) vicenin 2, (c) orientin, (d) luteolin

The flavonoid constituents during *in silico* analysis showed remarkable degree of interaction with H⁺/K⁺ ATPase receptor binding site. Thus, this integrated study using *in vitro*, *in vivo* and *in silico* models demonstrates the promising therapeutic potential of fenugreek seed extract as gastroprotective and indicates scope for isolation of individual components of fenugreek seed extract to discover effective, safe and economical gastroprotective agents.

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Conflict of interest:

There are no conflicts of interest.

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