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Original Article

Characterization, Preclinical Efficacy and Toxicity Evaluations of Flavonoids Glycosides based Standardized Fenugreek Seed Extract (FEFLG)

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ABSTRACT

Introduction: Fenugreek seeds, a natural food chain raw material, is known to have many flavonoid glycosides. **Objective:** Characterization, preclinical efficacy, and safety evaluation of flavonoid glycoside-based standardized fenugreek seed extract (FEFLG). **Methods:** FEFLG was characterized for a group of flavonoid glycoside marker compounds by HPLC. The CD38+ enzyme inhibition efficacy was assessed *in vitro*. In addition, acute oral toxicity (AOT) and subchronic, 90-day repeated-dose oral toxicity (*in vivo*), mutagenicity (AMES test, *in vitro*) and chromosome aberration test (*in vitro*) of FEFLG were evaluated. **Results:** The FEFLG was found to have 49.85% of total flavonoid glycosides content in FEFLG (25.15% of Group 1: vitexin, isovitexin and vitexin 2-o- rhamnoside and 24.70% of Group 2 (vicenin derivatives, schaftoside, iso-schaftoside, orientin and iso-orientin). FEFLG showed CD38+ enzyme inhibition *in vitro* (IC50= 0.96 µg/ml) equivalent to the positive control, apigenin. FEFLG did not show any toxicity at an acute oral dose of more than 2000 mg/kg (median lethal dose, LD50) with a limit dose of 5000 mg/kg. The 90-day repeated-dose oral administration of FEFLG did not induce significant toxicological changes till the maximum dose of 1000 mg/kg in male and female rats, indicating no observed adverse effect level, NOAEL ≥ 1000 mg/kg. FEFLG did not show mutagenicity (up to a concentration of 5000 µg/plate) or structural chromosomal aberrations (up to 5000 µg /ml). **Conclusion:** The CD38+ enzyme inhibitor efficacy *in vitro*, oral safety *in vivo* and absence of mutagenicity or genotoxicity of FEFLG indicated its potential for anti-aging applications.

Key words: Fenugreek seeds, Flavonoid glycosides, CD38+ enzyme inhibition, Acute toxicity, Subchronic toxicity, Mutagenicity, Chromosomal aberration.

INTRODUCTION

Because of increased life expectancy, people worldwide are now experiencing growth in both the size and the proportion of older persons in the population.[1] However, the aging population's health and quality of life is a major concern today.[2-4] Furthermore, the aging process plays a critical role in the etiopathogenesis of several diseases.[5]

A large body of research on antiaging supplementation exists and growing.[6-8] The natural antioxidants and antiinflammatory compounds that provide the benefits of mitochondria protection and energy homeostasis maintenance were suggested to defend against the aging process and promote healthy longevity.[9] The role of nutritional supplements in preventing age-related complications has also been recognized.[10] Moreover, there is an increased global interest in using natural products as dietary supplement ingredients for improved quality of life or their purported health benefits.[11,12]

Aging is a complex phenomenon characterized by progressive loss of cellular function and systemic deterioration of multiple tissues, leading to impaired mitochondrial function and increased vulnerability to death, often accompanied by many pathological diseases.[13,14] The crucial role of free radicals, oxidative stress, and chronic immune inflammation are implicated in the aging process.[13,15] Moreover, mitochondrially targeted agents to mitigate oxidative stress are recommended for multiple forms of skin aging problems.[16]

Recently, a decline of NAD+ (nicotinamide adenine dinucleotide)[17-19] during the aging process by a key degrading enzyme, CD38 (NADase)[20] is reported as important causes for the development of age-associated conditions. During aging, enhanced CD38-dependent NADase activity is responsible for accumulating the pro-inflammatory macrophages in metabolic tissues and reducing tissue NAD+ levels.[17,21-24] Therefore, a specific CD38 inhibitor is an excellent solution to reverse NAD+ decline and ameliorate age-related metabolic dysfunction.[25,26]

The flavonoid glycosides from various plants sources were reported with a set of properties for antiaging and other health applications, namely antiinflammatory[27], anti-complementary[28], antioxidant[29], hepatoprotective[30], anti-diabetic[31], and antibacterial, antifungal[32] properties. The plant-based flavonoids are also reported to maintain energy balance and health of the skin and improve quality of life due to CD38 inhibition activities.[33,34] In addition, apigenin[22] and luteolin[34] obtained from various plant sources are reported for the CD38+ inhibition properties.

Fenugreek (*Trigonella foenum-graecum* L.) seed, a natural food chain raw material, is known to have a significant amount of flavonoid glycosides[35,36] with reports of platelet aggregation inhibition[37] and antioxidant[38] properties. The flavonoid-rich fractions

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of fenugreek seeds have antinociceptive and antiinflammatory effects.[39] Fenugreek seeds' flavonoid glycosides include the apigenin glycosides (vitexin, isovitexin, vicenin derivatives orientin, iso-orientin) and lutenin glycosides (schaftoside and isoschaftoside).[40-42] Apigenin glycosides of fenugreek seeds are responsible for many biological actions, including useful adjuvants for disease management or health products.[43] Many health benefits of vitexin are reported through its antioxidant, antiinflammatory, anticancer, antinociceptive, and neuroprotective effects.[44] Many flavonoid C-glycosides, including apigenin and luteolin glycosides, from fenugreek seeds[40] are reported to inhibit digestive enzymes, activate insulin signaling, and reduce the formation of advanced glycation end products[45,46], and regulate glycolipid metabolism by improving mitochondrial functions in adipocytes *in vitro*.[47] Therefore, fenugreek seed extract with flavonoid glycosides, especially apigenin and luteolin glycosides, has excellent potential for developing antiaging supplementation and wellness products.

While consumers' use of botanical dietary supplements is increasing, the awareness and need for appropriate quality and safety assessment data are ever-rising.[48] Many scientific and regulatory challenges exist in the assurance of safety, quality and efficacy of dietary supplements.[48] The accurate characterization and standardization process helps overcome quality issues such as the complexity and variability of raw materials.[12,49] At the same time, regulatory challenges can be resolved with the availability of information on efficacy and safety assessments.[12] Therefore, the present study was undertaken to prepare and characterize the flavonoids glycosides based on standardized fenugreek seed extract (FEFLG) with efficacy evaluation (as CD38+ inhibition *in vitro*). In addition, the safety assessments of the FEFLG were performed using Organization for Economic Co-operation and Development (OECD) guidelines for acute oral toxicity, 90-day repeated dose oral toxicity, mutagenicity, and genotoxicity studies.

MATERIALS AND METHODS

Preparation and standardization of FEFLG

Fenugreek seeds were collected during the summer from Mandsaur, Madhya Pradesh, India, and authenticated by an expert taxonomist at Agharkar Research Institute, Pune, India. The seeds were air-dried to keep less than 5% moisture content, flaked to a thickness of less than 2 mm, extracted with ethanol-water (70:30 v/v) mixture at 40 °C for 10 h, filtered to 200-mesh cloth and concentrated at 50 °C under vacuum to obtain a semi-solid mass. The resultant mass was dissolved in deionized water and passed through the resin bed comprising a highly cross-linked polymer adsorbent with a high surface area and unique pore size distribution (Dowex Optipore L493) for two h. The resultant elute of the ethanol-soluble bioactive flavonoid glycoside constituents was collected, pooled and concentrated at 50 °C to yield a final composition named as FEFLG.

The standardization was performed for the total flavonoid content of the FEFLG using the aluminum chloride ($AlCl_3$) by colorimetric methods as reported earlier.[50] In brief, 1 mL of FEFLG (0.1 mg/mL methanol), 4 mL distilled water and 1 mL of 5% sodium nitrite solution and mixed well. After 6 min, 1 mL of 10% $AlCl_3$ solution was added, and the mixture was hold for the next 5 min. Thereafter, 2 mL of 1 mol/L sodium hydroxide solution and 2 mL of distilled water was added. The mixture was kept for 20 min, and absorbance was measured at 415 nm. The total flavonoid content was calculated from a calibration curve using standard flavonoid, quercetin, to express the results as mg quercetin equivalent (mg QE) per g.

The characterization of FEFLG was performed for the marker compounds, the flavonoid glycosides using the High-performance liquid chromatography (HPLC) system (Jasco-4000 system, equipped with AS- 4050 auto-sampler and MD-4010 PDA detector). Data acquisition was controlled by JASCO ChromNAV Chromatography Data System software (JASCO GmbH, Grob-Umstadt, Germany). The chromatographic separation for the markers Vicenin 1, Vicenin 2, Vicenin 3, Schaftoside, Isoschaftoside, Orientin, Isoorientin, Vitexin and Isovitexin was accomplished using gradient elution at wavelength 330 nm, temperature: 25 °C as follows: Column: reverse phase C-18 (100 mm × 2.1 mm × 2.6 µm), mobile phase from solution (A) 0.1 % Trifluoroacetic acid and Solution (B) Water : Acetonitrile (50:50) + 0.5 mL Trifluoroacetic acid, flow rate: 0.35 ml/min, runtime: 105 min. The chromatographic separation for marker, Vitexin-2-o-rhamnoside, was accomplished using gradient elution at wavelength 210 nm, temperature: 25 °C as follows: Column: reverse phase C-18 (250 mm X 4.6 mm, 5 µm), mobile phase from solution (A) Water (B) acetonitrile, flow rate: 1 ml/min. 210 nm, a runtime: 30 min. The results were expressed as a percentage of total and flavonoid glycosides and the % content of a group of selected flavonoid glycosides (1) vitexin+isovitexin+vitexin 2-o-rhamnoside (2) vicenin1+vicenin 2+vicenin 3+schaftoside+iso-schaftoside+orientin+iso-orientin using HPLC fingerprinting of FEFLG and corresponding reference standards.

Animals

The toxicological assessments was conducted at good laboratory practices (GLP) compliant test facility (Intox Pvt Ltd, Pune). Wistar rats of age 7-8 weeks old and body weights of 199.14 ± 20% g (males) and 154.84 ± 20% g (females) were obtained from Taconic Biosciences, Inc., USA, through its representative Vivo Bio Tech Ltd., Telangana, India and used for acute and 90-day (sub chronic) toxicity studies. Only female Wistar rats were used for the acute toxicity study as per OECD guideline 423.[51] All the females were nulliparous and non-pregnant. Before the treatment, the rats were kept for 7-12 days for acclimatization. Each animal was assigned a unique identification number on the individual cage tag. Rats were on 'Altromin' brand pelleted rat feed (manufactured by M/s Altromin Spezialfutter GmbH & Co. KG, Germany, and supplied by ATNT Laboratories, Mumbai) and provided with pure potable water through 'Aquaguard' water filter in sterilized bottles with stainless steel sipper tubes *ad labium*. The experimental animal room was supplied with fresh and filtered air, with 10 to 15 air changes per h. The room air was conditioned with ambient temperature (19 to 25 °C), relative humidity (30 % to 70%), and with 12 h light and 12 h dark illumination cycle.

All studies complied with the OECD guidelines for the testing of chemicals[52]. The institutional animal ethics committee of Intox Private Limited, Pune approved all study protocols in compliance with the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

CD38 enzyme inhibitor screening assay

A fluorescence-based etheno-NAD+ assay was employed for the CD38 enzyme inhibitor assay (hydrolase activity) as per the reported procedure.[53,54] The concentrations of test compounds were based on reported half-maximal inhibitory concentration (IC_{50} = 10.3 ± 2.4 µM per litters) of apigenin against CD38 enzyme inhibition data.[55] With the molecular weight of 270.0528 g/mol, the IC_{50} of 10 µM (= 2.7 µg/ml) of apigenin was used as middle concentration in the present study, with ten times lower (i.e. 0.27 µg/ml) and higher (27 µg/ml) than the IC_{50} value (2.7 µg/ml). The corresponding rounded concentrations range (30, 3.0, 0.3 µg/ml) of FEFLG were evaluated in the present study.

The CD38 enzyme inhibition activity was measured using 0.1 unit of recombinant human CD38 (R&D Systems, Minneapolis, USA) in 0.25 mol/L sucrose and 40 mmol/L Tris-HCl (pH 7.4) with utilizing 1, N6-Ethenonicotinamide Adenine Dinucleotide (ε-NAD) as substrate and apigenin as a positive control (inhibitor). The 96-well

plate was immediately put in the plate reader, and the fluorescence (Ex/Em = 320/420) read once per minute up to 35 min. The results were calculated using relative fluorescence units (RFU) as a percentage of CD38 inhibition using the formula: % inhibition = 1-(RFU of the sample/RFU of the negative control))*100. The data was analyzed, and calculations of IC_{50} and the area under the curve (AUC) were made using GraphPad Prism (version 9.3) software. Statistical significance at $p< 0.05$ was considered significant.

Acute oral toxicity (AOT) study

The study was conducted in compliance with the OECD Guidelines for Testing of Chemicals No. 423, 'Acute Oral Toxicity - Acute toxic class method.[51] The test suspension of FEFLG was prepared freshly formulated before dosing in analytical grade water to obtain the final concentration of 200 mg/ml. The toxicity of the test item was assessed by the 2-step procedure in female rats in a group of 3 per step. In the first step, three rats were treated with 2000 mg/kg with a 10 ml/kg dose volume. Following the treatment, the rats were observed for the incidence of mortality and clinical signs of toxicity daily for 14 days. On day 15, all rats were euthanized by carbon dioxide asphyxiation, subjected to a complete necropsy, and the gross pathological changes were recorded. The absence or presence of compound-related mortality of the rats dosed at the first step determined the actions for the second step, i.e., no further testing is needed or dosing of three additional rats with the same dose or next lower dose level.

Subchronic (90-day repeated dose) toxicity study

The study was conducted in compliance with the OECD guideline for testing of chemicals (No. 408, Section 4: Health Effects) for the conduct of 'Repeated Dose 90-day Oral Toxicity Study in Rodents'.[56] The dose levels were selected based on the absence of any significant toxicity finding during a 14-day dose range-finding study conducted on rats. A dose of 1000 mg/kg body weight was used as the highest dose level, and it did not result in any remarkable toxicity in the treated animals. Based on these findings, three ascending doses (250 mg/kg, 500 mg/kg and 1000 mg/kg) were selected for the main study.

FEFLG was formulated in analytical grade water. A constant dose volume of 5 ml/kg body weight was maintained across different doses in varying concentrations. AR water was used as vehicle control (VC), and rats were treated with the following schedule for 90 consecutive days:

- G1 – VC (AR water, 5 mL/kg, 90 days) - 10 animals/sex
- G2 – FEFLG-250 (FEFLG, 250 mg/kg, 90 days) - 10 animals/sex
- G3 – FEFLG-500 (FEFLG, 500 mg/kg, 90 days) - 10 animals/sex
- G4 – FEFLG-1000 (FEFLG, 1000 mg/kg, 90 days) - 10 animals/sex
- G1R – VC-R (VC Reversal, AR, 5 mL/kg, 119 days) - 5 animals/sex
- G4R – FEFLG-1000-R (FEFLG Reversal, FEFLG - 1000 mg/kg for 90 days with no treatment from day 91 to day 119) - 5 animals/sex

Daily examination of mortality and clinical signs was done in all the rats. Detailed clinical examination was performed before initiation of treatment, and weekly thereafter, till the study termination. All the rats were subjected to ophthalmoscopic examination before initiation of treatment, and it was repeated in the control and high dose groups at the termination of the treatment. Assessment of sensory reactivity, grip strength and motor activity was conducted in the 13th week of treatment. Weekly recording of body weight and food consumption was done. Blood and urine samples were collected for hematology, blood chemistry (including thyroid function tests), and urinalysis assessments, respectively, at the treatment's termination and the recovery period's end. Vaginal smears were collected from all the female rats at the end of treatment and recovery period for estrus cycle examination. All the rats were terminally sacrificed and subjected to a detailed necropsy with a recording of weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries, heart, pituitary gland, thyroid gland (fixed) and complex of the prostate, seminal vesicles with coagulating glands was done. All the rats from control and high dose groups were examined for histopathological evaluation. Tissues for histopathological evaluation included brain, spinal cord, eyes, pituitary, thyroid, parathyroid, spleen, thymus, adrenals, pancreas, trachea, lungs, heart, liver, kidneys, aorta, esophagus, stomach, duodenum, jejunum, terminal ileum (with Peyer's patch), colon, rectum, urinary bladder, prostate + seminal vesicles with coagulating glands as a whole complex, epididymides, testes, ovaries, utérus with cervix, vagina, skin, sciatic nerve, femur bone, bone marrow (smear), mammary gland (males and females), mesenteric lymph node, axillary lymph node and salivary glands.

Mutagenicity (AMES test)

Salmonella typhimurium reverse mutation assay (AMES test) of FEFLG was carried out in compliance with the OECD Guidelines for Testing of Chemicals (No. 471, Section 4: Health Effects) on the conduct of 'Bacterial Reverse Mutation Test'.[57] FEFLG was evaluated by Ames test to determine its ability to induce reverse mutation at selected histidine loci in five tester strains of *Salmonella typhimurium* viz. TA1535, TA97a, TA98, TA100 and TA102 in the presence and absence of a metabolic activation system (S9). Concurrent VC and strain-specific positive control groups, both with and without metabolic activation, were considered during the assay as specified by OECD guidelines. The assay was conducted in duplicate as Experiment no. 1 and Experiment no. 2.

Based on the preliminary tests of solubility, precipitation and cytotoxicity, the doses for FEFLG were selected as 5000 µg, 1500 µg, 500 µg, 150 µg and 50 µg/plate. Five tester strains were exposed to all the selected doses of FEFLG in triplicate cultures in the absence and presence of a metabolic activation system. Dimethyl sulfoxide (DMSO) was used as vehicle control. The exposed bacteria were plated onto minimal glucose VB agar medium supplemented with L-histidine and D-biotin. The plates were incubated at 37 °C for about 66 and 48 h for Experiment no. 1 and Experiment no.2. After the incubation period, the plates were checked for sterility. The plates were observed for a uniform lawn of auxotrophs (his-) and the colonies for histidine revertant as the prototrophs (his+). Histidine revertant colonies per plate were counted, and the mean number of colonies at each test point was calculated.

Genotoxicity (Chromosomal aberrations test)

The genotoxicity of FEFLG was performed in compliance with the Organization for Economic Cooperation and Development (OECD) guidelines for testing chemicals, section 4, no. 473, for the 'In vitro Mammalian Chromosome Aberration Test.[58] The potential of FEFLG for causing structural chromosome aberrations was assessed in human peripheral blood lymphocytes, which are one of the most widely used cell-type for short-term cultures. Three separate experiments were conducted, two in the absence and one in the presence of a supplementary metabolic activation system (S9). The incidences of chromosome aberrations in the treatment groups were compared with that in the VC groups. The entire study was carried out in duplicate cultures at each concentration.

Rat liver S9 fraction was prepared by phenobarbitone with β-naphthoflavone as inducing agents. S9 fraction was stored at -80°C ± 10 °C. To prepare the S9 mix, a fresh vial was thawed and used. Three exposure levels and concurrent VC and positive control groups were employed. Following the preliminary solubility, precipitation and cytotoxicity studies, cultures of human peripheral blood lymphocytes

were exposed to FEFLG formulated in analytical grade water (vehicle) at concentrations of 5000 µg/ml, 2500 µg/ml, and 1250 µg/ml in the absence of S9 (Experiment 1 and 2) for an exposure period of 3 hours and 24 hours respectively and in the presence of S9 (Experiment 3) for an exposure period of 3 h. All cell cultures were treated with colchicine (0.5 µg/ml) about 2 h before harvesting lymphocytes. Each culture was harvested and processed separately. Chromosomal preparations were made and stained with 5% Giemsa (v/v) for 7 min. Three hundred well-spread metaphases were evaluated microscopically for structural aberrations per test concentration.

Statistical analysis

The data of AOT was analyzed according to the globally harmonized system (GHS) for the classification of chemicals. For the subchronic toxicity study, data for each parameter of hematology, and blood chemistry) were analyzed by one-way ANOVA followed by Dunnett's test (FEFLG-treated v/s VC) or independent-sample t-test (FEFLG-1000-R v/s VC-R) for statistical significance using validated SPSS Version 23.0. For analysis of the AMES test, the mean number of histidine revertant colonies for all the treatment groups was compared with the respective VC group with the assessment of mutagenic evaluation criteria as mentioned in OECD guideline no 471. For the chromosomal aberration test, total numbers of metaphases and metaphases with aberrations were recorded and pooled for both cultures. The aberrations per metaphase and the number of aberrated metaphases were calculated. The data from each treatment level and positive control substances were compared with the VC using a Chi-square test (2×2 contingency tests).

RESULTS

Characterization and standardization of FEFLG

The total flavonoid content of FEFLG was recorded as 94.58% (i.e., 945.8 mg quercetin equivalent (mg QE) per g (colorimetric method). The HPLC analysis (Figure 1 and Figure 2) revealed the assay of marker compounds, total flavonoid glycosides content of FEFLG = 49.85%. In HPLC analysis, the percentage contents of selected flavonoids from group 1 (vitexin+ isovitexin+vitexin 2-O-rhamnoside) was 25.15% (Figure 1) and Group-2 (vicenin 1+vicenin 2+vicenin 3+schaftoside+ isoschaftoside+orientin+iso-orientin) was 24.7% (Figure 2).

CD38 enzyme inhibition assay

The data of concentration (µg/ml) v/s % inhibition of CD38 enzyme data was used to calculate IC_{50} and the AUC for comparisons. FEFLG (30 µg/ml) and apigenin (27 µg/ml) showed 32.51 % and 29.17 % of CD38 inhibition, respectively, at 35 min, at which maximum inhibition was observed. The IC_{50} value for FEFLG and apigenin was 0.9622 µg/ml and 0.4672 µg/ml, respectively (non-linear fit, normalized responses). The AUC values were calculated for each time point (5, 15, 25 and 35 min). The differences in AUC values between the treatment groups, FEFLG and apigenin, were not statistically significant (Two-way Analysis of variance (ANOVA).

Acute oral toxicity (AOT) study

Acute administration of FEFLG did not result in any mortality and abnormal clinical signs on the day of dosing and throughout the 14-day post-treatment evaluation period. No adverse effects on body weight were reported during the 14-day observation period post-dosing. Following administration of FEFLG, no alterations were observed in gross pathology in any of the rats at the end of the study. This evaluation shows that the single oral dose resulted in a median-lethal dose (LD_{50}) greater than 2000 mg/kg and indicates a low order of acute toxicity. FEFLG is classified in GHS Category 5 or unclassified for the obligatory labeling requirement for oral toxicity as per the 'globally harmonized system (GHS) for classifying chemicals. Category 5 corresponds to an LD_{50} value lying between 2000 < Acute Toxicity Estimate ≤ 5000 mg/kg body weight, while the corresponding LD_{50} cut-off value for FEFLG was observed to be 5000 mg/kg body weight.

Subchronic (90-day repeated dose) toxicity study

Sub-chronic administration of FEFLG did not cause death in any tested doses up to 1000 mg/kg and control group rats during dosing and the recovery period. No treatment-related clinical abnormality was observed during daily general and weekly detailed clinical examinations. Slight corneal opacity (left eye) was observed from day 85 of the study till termination (day 91) in one female rat from the FEFLG-500 group. This single observation was considered incidental and unrelated to the treatment. There were no treatment-related ocular abnormalities observed during terminal ophthalmologic examinations. No remarkable and treatment-related incidence of neurological abnormalities was observed when neurological examinations (functional observations) were conducted in the thirteenth week of the study. The other observations in the home cage, open area and manipulative responses of FEFLG treated groups did not show a significant difference as compared to the respective VC group. In addition, no findings indicative of the neurotoxic potential of the FEFLG were encountered during these examinations. The data on body weight gain observed in the weekly schedule is presented in Table 1. Isolated cases with a significant increase in body weight gain of males of the FEFLG-500 group (day 77-84) and FEFLG-1000 group (day 49-56 and 84-90) were found without dose-or sex-dependencies or correlation and considered incidental.

At the end of the treatment period and recovery period, the group means values of hematological (Table 2 and 3) and blood chemistry, including thyroid function tests (Table 4 and 5) parameters of FEFLG treated groups were not significant as compared to VC group except for the statistically significant difference in few parameters. However, all the values were within the historical control range of Intox Pvt. Limited, Pune. Moreover, no dose-or sex- dependant relationships were found for these changes, so these changes were considered incidental and not treatment-related.

The vaginal smear on day 91 and day 119 confirmed the normal estrous cycle in FELFG-treated and respective VC groups (data not shown). The data on urinalysis (qualitative or semi-quantitative urine parameters, namely color, appearance, graded quantities of analytes such as protein, glucose, ketones, nitrite, bilirubin and occult blood) did not show a significant change between FEFLG and VC groups suggesting no abnormality due to FEFLG treatment at the end of treatment and recovery period (data not shown). The quantitative urinalysis parameters such as specific gravity, urine volume, pH and urobilinogen in the urine of FEFLG-treated rats (both sexes) did not show significant change as compared to the VC group.

The mean values of relative organ weights of male and female rats treated with FEFLG did not show a significant difference as compared to respective VC groups at all dose levels at the termination of the treatment and the recovery period (Table 6 and Table 7) except for statistically significant ($p<0.05$), increase in the pituitary gland (FEFLG-250, FEFLG-500 and FEFLG-1000 in males), uterus weight (FEFLG-500 in females), prostate + seminal vesicles with coagulating glands as a whole complex weight (FEFLG-1000-R) with a decrease in brain and epididymis weight (FEFLG-1000-R in males). No gender or dose-related correlations were found in these changes. Therefore, the changes were considered incidental and not related to treatments. Furthermore, all the values were within the normal historical control range of Intox Private Limited, Pune.

FEFLG at and up to the highest tested dose (1000 mg/kg) did not result in any remarkable and treatment-related gross pathological alterations in any of the tissues of treated rats, as evident at the detailed necropsy examination out at the end of the study and recovery period.

No significant and treatment-related histopathological changes were observed in rats treated with FEFLG at the high dose of 1000 mg/kg body weight and control rats (Table 8). The incidental and spontaneous lesions observed were perivascular lymphocytic aggregation and foam cells in the lungs, with an isolated incidence of mild suppurative inflammatory change in the lung of one male rat from the FEFLG-1000 group. Similarly, a single incidence of minimal granulomatous inflammation of the lungs was observed in the male rat from the FEFLG-1000 group. This change was characterized by a significant number of aggregated, large multinucleated giant cells. Other lesions observed comprised Mineralization in kidneys, minimal sub-mucosal lymphoid hyperplasia in the colon, and minimal cortical vacuolation in adrenals. Female rats' incidental and spontaneous lesions comprised perivascular lymphocytic aggregation and foam cells in lungs; cortical vacuolation and accessory cortical tissue in adrenals; isolated incidence of ectopic thymus ultimobranchial cyst in thyroid and eosinophil cell infiltration in the uterus and minimal sub-mucosal lymphoid hyperplasia in the colon in females. The frequency of all the above-

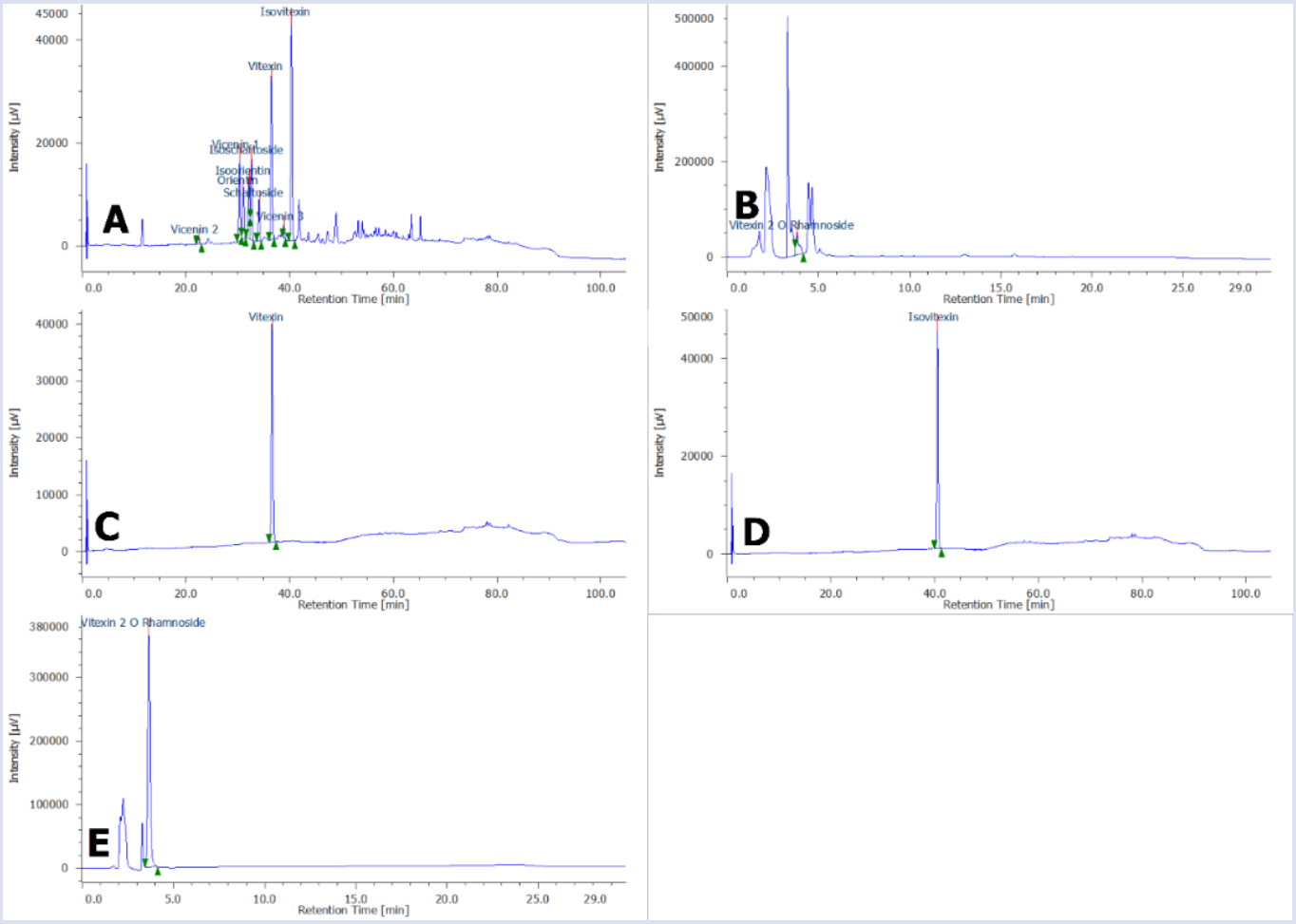


Figure 1: HPLC fingerprint of FEFLG at (A) 330 nm and (B) 210 nm with reference standards of Group 1 glycosides (C) Vitexin (D) Isovitexin (E) Vitexin 2-o-rhamnoside.

Table 1: Effect of FEFLG on body weights (g) of rats for 90 days repeated dose toxicity study.

Days	G1	G2	G3	G4	G1R	G4R
	VC	FEFLG-250	FEFLG-500	FEFLG-1000	VC-R	FEFLG-1000-R
Male						
0	200.20 ± 17.14	197.30 ± 16.86	198.90 ± 16.30	199.47 ± 15.62	-	-
90	372.60 ± 28.83	368.60 ± 28.61	373.90 ± 34.17	393.33 ± 47.73	-	-
118	-	-	-	-	386.40 ± 29.89	426.00 ± 49.64
Female						
0	155.60 ± 8.48	155.50 ± 10.77	152.20 ± 12.09	155.40 ± 12.17	-	-
90	246.87 ± 16.27	247.40 ± 17.51	246.90 ± 15.57	250.27 ± 12.53	-	-
118	-	-	-	-	249.20 ± 22.53	260.40 ± 8.88

Data was represented as Mean ± Standard Deviation (SD). Comparisons with respective days as follows: G2, G3 and G4 V/s. G1; G4R V/s. G1R, n=15 for G1, G2, G3 and G4 whereas n=5 for G1R and G4R

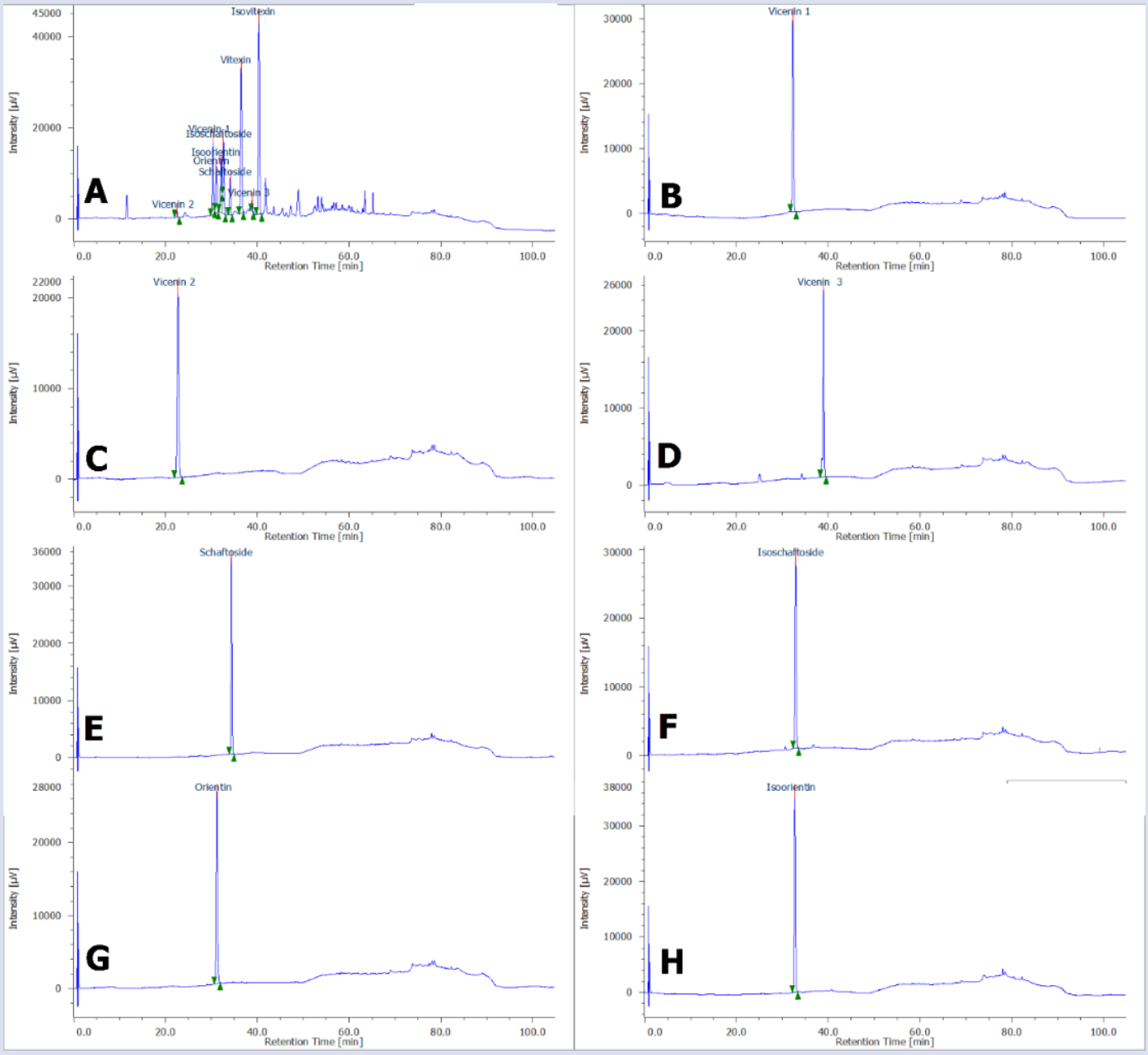


Figure 2: HPLC fingerprint of (A) of FEFLG and reference standards of Group 2 glycosides (B) Vicenin1 (C) Vicenin 2 (D) Vicenin 3 (E) Schaftoside (F) Isoschaftoside (G) Orientin and (H) Iso-orientin.

mentioned microscopic changes was minimal and equal in both treated and VC groups and so not considered toxicological significance.

Mutagenicity Study (AMES test)

The frequencies of histidine revertant colonies of *Salmonella typhimurium* tester strain (TA1535, TA97a, TA98, TA100 and TA102) were observed, both in the presence and absence of a metabolic activation system (Table 9 and Table 10). None of the experiments reveal any dose-dependent increase of colonies compared to the respective VC groups, according to the criteria of evaluation of findings of this Ames Test. Therefore, FEFLG is considered non-mutagenic in *Salmonella typhimurium* tester strains. Plate counts for the spontaneous histidine revertant colonies in the VC groups were within the frequency ranges expected from the laboratory historical control data. Concurrent positive controls demonstrated sensitivity of the assay in the presence and absence of metabolic activation (Table 9 and Table 10).

Genotoxicity (Chromosomal aberrations test)

Three experiments were conducted either in the absence or presence of a metabolic activation system, following 3 h or 24 h of exposure. There was no significant ($p > 0.05$) concentration-related increase in the % incidence of treated cells with structural chromosome aberrations (excluding gaps) at any of the concentration levels of FEFLG as compared to the incidence in the respective VC group (During experiments (1) chromosome analysis of cultured lymphocytes (2) 24-h treatment, Without metabolic activation (3) 3 h treatment, With metabolic activation). Concurrent positive controls, methyl methane sulphonate (30 µg/mL) and cyclophosphamide monohydrate (60 µg/mL), when tested respectively in the absence and presence of metabolic activation, induced a many-fold increase in the incidence of structural chromosome aberrations over that in the concurrent VC groups, which was statistically significant ($p < 0.05$). Under the experimental conditions described, FEFLG did not induce any dose-dependent increase in the

Table 2: Effect of FEFLG on hematological parameters for 90 days repeated dose toxicity study (Male rats).

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	FEFLG-250	FEFLG-500	FEFLG-1000	VC-R	FEFLG-1000-R
Hb (g %)	15.06 ± 0.49	15.00 ± 0.71	15.23 ± 0.59	15.20 ± 0.58	15.64 ± 0.39	15.32 ± 0.84
PCV (%)	46.97 ± 1.55	46.17 ± 2.01	46.69 ± 2.30	47.06 ± 1.56	47.28 ± 0.97	46.52 ± 2.44
RBC (x106 /µL)	8.67 ± 0.37	8.58 ± 0.48	8.69 ± 0.45	8.77 ± 0.37	8.71 ± 0.14	8.61 ± 0.28
Reticulocytes (%)	2.97 ± 0.90	2.47 ± 0.51	2.60 ± 0.34	2.50 ± 0.50	2.68 ± 0.79	2.05 ± 0.75
MCV ($mm^{3)}$	54.21 ± 1.45	53.83 ± 1.48	53.76 ± 1.17	53.68 ± 1.24	54.26 ± 1.16	53.96 ± 1.11
MCH (pg)	17.38 ± 0.56	17.51 ± 0.59	17.55 ± 0.48	17.33 ± 0.49	17.96 ± 0.42	17.80 ± 0.54
MCHC (%)	32.07 ± 0.50	32.50 ± 0.37	32.63 ± 0.58*	32.32 ± 0.44	33.08 ± 0.61	33.00 ± 0.70
Platelets (x 103 /µL)	1056.50 ± 152.89	1079.70 ± 107.72	997.40 ± 89.88	993.30 ± 135.36	859.60 ± 62.48	905.80 ± 11.43
TLC	10.86 ± 2.36	8.96 ± 1.52	9.88 ± 1.98	10.76 ± 1.97	3.37 ± 0.44	5.55 ± 1.99*
PT (sec)	15.57 ± 1.22	14.89 ± 1.18	15.72 ± 1.04	15.75 ± 0.86	14.30 ± 0.46	13.30 ± 0.85
APTT (sec)	15.44 ± 1.08	15.49 ± 1.23	16.23 ± 0.75	16.52 ± 0.94	15.02 ± 1.10	14.54 ± 1.21
DLC						
N (%)	18.71 ± 3.42	21.80 ± 2.93	20.31 ± 3.00	22.74 ± 4.05*	18.94 ± 2.76	22.20 ± 9.11
L (%)	77.00 ± 3.64	73.07 ± 3.31*	74.81 ± 2.97	72.52 ± 3.74*	77.80 ± 3.12	71.68 ± 9.89
E (%)	1.46 ± 0.43	2.12 ± 0.95*	1.49 ± 0.25	1.42 ± 0.43	1.12 ± 0.18	1.50 ± 0.41
M (%)	1.44 ± 0.41	1.72 ± 0.50	1.93 ± 0.61	2.26 ± 0.81	0.94 ± 0.26	2.68 ± 1.35*
B (%)	0.56 ± 0.08	0.52 ± 0.16	0.58 ± 0.21	0.38 ± 0.13*	0.64 ± 0.44	1.18 ± 0.58

Data was represented as Mean ± Standard Deviation, Comparisons with respective days as follows: G2, G3 and G4 V/s. G1; G4R V/s. G1R,Hb: Hemoglobin; PCV: Packed Cell Volume, RBC: Red Blood Corpuscles, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, TLC: Total leukocyte (White Blood Corpuscles) count, PT.: Prothrombin time, APTT: activated partial thromboplastin time, DLC: differential leucocyte count, N: Neutrophils, L : Lymphocytes, E: Eosinophils, M : Monocytes, B : Basophils. n=15 for G1, G2, G3 and G4 whereas n=10 for G1R and G4R

Table 3: Effect of FEFLG on hematological parameters for 90 days repeated dose toxicity study (Female rats).

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	FEFLG-250	FEFLG-500	FEFLG-1000	VC-R	FEFLG-1000-R
Hb (g %)	14.67 ± 0.63	14.52 ± 0.64	14.60 ± 0.54	14.33 ± 0.67	15.64 ± 0.62	14.98 ± 0.48
PCV (%)	47.15 ± 2.09	45.92 ± 2.08	46.73 ± 1.89	45.00 ± 1.92	46.72 ± 1.38	45.20 ± 1.46
RBC (x106 /µL)	8.01 ± 0.43	7.92 ± 0.41	8.11 ± 0.36	7.83 ± 0.38	8.31 ± 0.15	8.23 ±0.24
Reticulocytes (%)	2.59 ± 0.49	2.22 ± 0.56	2.68 ± 0.78	2.28 ± 0.66	2.30 ± 0.45	1.98 ± 0.43
MCV ($mm^{3)}$	58.90 ± 1.55	58.02 ± 1.91	57.63 ± 1.54	57.56 ± 1.18	56.22 ± 1.17	54.90 ± 0.64
MCH (pg)	18.32 ± 0.42	18.32 ± 0.53	18.05 ± 0.60	18.32 ± 0.31	18.78 ± 0.51	18.16 ± 0.32
MCHC (%)	31.11 ± 0.55	31.60 ± 0.28	31.29 ± 0.56	31.85 ± 0.43*	33.42 ± 0.47	33.06 ± 0.27
Platelets (x 103 /µL)	977.00 ± 100.26	1032.30 ± 123.40	1039.70 ± 96.71	1053.40 ± 137.06	1016.00 ± 49.43	1075.80 ± 76.54
TLC	7.31 ± 1.50	8.23 ± 1.56	9.24 ± 1.83*	9.73 ± 1.26*	6.78 ± 0.50	6.38 ± 1.11
PT (sec)	15.52 ± 1.47	15.86 ± 1.07	15.99 ± 1.12	16.02 ± 1.17	14.76 ± 1.05	14.72 ± 1.08
APTT (sec)	15.70 ± 1.28	15.02 ± 1.59	14.31 ± 1.10	15.27 ± 1.14	17.84 ± 1.01	16.06 ± 0.65*
DLC						
N (%)	19.79 ± 2.94	17.83 ± 4.13	22.08 ± 5.04	18.33 ± 5.24	19.54 ± 3.69	20.20 ± 2.86
L (%)	75.11 ± 3.20	77.27 ± 4.47	72.89 ± 5.81	76.53 ± 6.36	75.36 ± 2.59	74.32 ± 3.25
E (%)	1.76 ± 0.42	1.63 ± 0.33	1.54 ± 0.61	1.63 ± 0.52	1.44 ± 0.33	1.78 ± 0.50
M (%)	1.97 ± 0.64	2.24 ± 0.57	2.52 ± 1.08	2.27 ± 1.29	1.42 ± 0.34	1.90 ± 0.64
B (%)	0.47 ± 0.22	0.36 ± 0.10	0.28 ± 0.09*	0.36 ± 0.10	1.56 ± 1.06	1.10 ± 0.47

Data was represented as Mean ± Standard Deviation, Comparisons with respective days as follows: G2, G3 and G4 V/s. G1; G4R V/s. G1R,Hb: Hemoglobin; PCV: Packed Cell Volume, RBC: Red Blood Corpuscles, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, TLC: Total leukocyte (White Blood Corpuscles) count, PT.: Prothrombin time, APTT: activated partial thromboplastin time, DLC: differential leucocyte count, N: Neutrophils, L : Lymphocytes, E: Eosinophils, M : Monocytes, B : Basophils. n=15 for G1, G2, G3 and G4 whereas n=10 for G1R and G4R

Table 4: Effect of FEFLG on blood chemistry on for 90 days repeated dose toxicity study (male rats).

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	FEFLG-250	FEFLG-500	FEFLG-1000	VC-R	FEFLG-1000-R
Liver function test						
ALT(IU/L)	44.70 ± 10.78	44.20 ± 9.51	47.30 ± 15.93	41.40 ± 15.90	46.20 ± 16.15	36.00 ± 8.94
AST (IU/L)	76.50 ± 12.05	84.70 ± 8.49	88.90 ± 33.16	80.30 ± 11.47	69.60 ± 14.21	70.00 ± 9.56
ALP (IU/L)	82.00 ± 9.92	80.40 ± 11.34	81.60 ± 13.48	88.30 ± 19.51	75.40 ± 20.40	69.40 ± 14.28
Bilirubin (mg/dL)	0.05 ± 0.05	0.05 ± 0.05	0.09 ± 0.06	0.10 ± 0.05	0.08 ± 0.04	0.12 ± 0.04
Total Protein (g/dL)	6.59 ± 0.50	6.49 ± 0.31	6.69 ± 0.33	7.13 ± 0.41*	7.18 ± 0.16	7.04 ± 0.24
Albumin (g/dL)	0.99 ± 0.13	0.91 ± 0.09	0.98 ± 0.14	1.07 ± 0.13	1.24 ± 0.11	1.14 ± 0.15
Globulin (g/dL)	5.60 ± 0.44	5.58 ± 0.29	5.71 ± 0.27	6.06 ± 0.41*	5.94 ± 0.20	5.90 ± 0.33
Kidney function test						
CR (mg/dL)	0.70 ± 0.06	0.69 ± 0.08	0.69 ± 0.12	0.58 ± 0.06	0.61 ± 0.05	0.55 ± 0.06*
BUN (mg/dL)	21.20 ± 1.48	21.30 ± 2.06	21.60 ± 2.32	20.20 ± 2.35	22.40 ± 2.30	21.40 ± 2.07
Urea (mg/dL)	45.50 ± 3.17	45.80 ± 4.47	46.50 ± 5.08	43.20 ± 5.00	48.00 ± 5.00	46.00 ± 4.47
Serum electrolytes						
Ca (mg/dL)	8.82 ± 0.23	8.83 ± 0.11	8.78 ± 0.10	8.98 ± 0.08*	10.24 ± 0.11	10.32 ± 0.21
P (mg/dL)	7.22 ± 1.06	6.56 ± 0.32	7.32 ± 0.61	7.32 ± 0.19	5.30 ± 0.55	6.38 ± 0.89
Na (mmol/l)	146.30 ± 2.45	149.50 ± 2.76*	149.20 ± 2.74	146.80 ± 3.05	144.60 ± 1.95	144.20 ± 1.92
K (mmol/l)	5.12 ± 0.62	4.67 ± 0.37	4.75 ± 0.39	4.90 ± 0.41	5.16 ± 0.29	5.44 ± 0.68
Metabolic function test						
FPG (mg/dL)	62.90 ± 9.69	61.00 ± 5.44	65.50 ± 13.66	67.40 ± 7.80	90.00 ± 6.78	105.60 ± 29.28
Cholesterol (mg/dL)	61.70 ± 7.35	59.10 ± 7.46	59.60 ± 5.40	65.00 ± 7.41	67.80 ± 7.19	55.00 ± 3.24
Triglyceride (mg/dL)	41.00 ± 19.30	44.10 ± 30.37	51.90 ± 26.43	50.20 ± 20.84	56.60 ± 9.99	66.80 ± 20.09
LDL (mg/dL)	11.50 ± 3.10	11.00 ± 2.71	10.40 ± 2.63	10.00 ± 2.75	15.20 ± 8.79	9.20 ± 1.10
HDL (mg/dL)	49.10 ± 6.10	50.00 ± 6.73	51.50 ± 4.65	55.80 ± 6.02*	55.60 ± 5.13	45.40 ± 3.21*
Total bile acid (µmol/L)	24.79 ± 10.33	31.31 ± 19.64	26.01 ± 8.74	25.98 ± 17.61	39.63 ± 31.91	16.14 ± 10.44
Thyroid hormonal test						
Thyroxine (T4)	280.00 ± 32.39	256.00 ± 27.52	268.20 ± 36.17	257.50 ± 30.66	340.60 ± 43.10	341.00 ± 69.69
Triiodothyronine (T3)	6.88 ± 0.43	6.39 ± 0.65	6.16 ± 0.64*	6.83 ± 0.82	6.54 ± 0.28	6.33 ± 0.75
Thyroid stimulating hormone (TSH)	10.57 ± 2.54	10.06 ± 2.01	10.89 ± 2.52	12.89 ± 1.31	12.14 ± 0.72	11.50 ± 2.18

Data was represented as Mean ± Standard Deviation, Comparisons with respective days as follows: G2, G3 and G4 V/s. G1; G4R V/s. G1R, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, CR: Creatinine, BUN: Blood Urea Nitrogen, Ca: Calcium, P: Phosphorus, Na: Sodium, K: Potassium, FPG: Fasting plasma glucose, LDL: low-density lipoprotein, HDL: high-density lipoprotein. n=15 for G1, G2, G3 and G4 whereas n=10 for G1R and G4R

Table 5: Effect of FFLG on blood chemistry on during 90 days repeated dose toxicity study (female rats).

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	FEFLG-250	FEFLG-500	FEFLG-1000	VC-R	FEFLG-1000-R
Liver function test						
ALT(IU/L)	31.80 ± 7.58	52.30 ± 32.57	37.20 ± 15.02	39.10 ± 12.77	35.00 ± 9.46	28.40 ± 3.51
AST (IU/L)	98.30 ± 19.97	93.10 ± 16.02	83.30 ± 14.07	98.90 ± 19.06	81.20 ± 13.95	75.00 ± 7.78
ALP (IU/L)	42.50 ± 6.93	43.40 ± 10.71	39.40 ± 5.06	33.90 ± 3.81*	33.80 ± 6.26	25.80 ± 6.98
Bilirubin (mg/dL)	0.11 ± 0.06	0.09 ± 0.03	0.10 ± 0.07	0.06 ± 0.05	0.08 ± 0.04	0.10 ± 0.00
Total Protein (g/dL)	7.21 ± 0.28	7.03 ± 0.33	7.13 ± 0.37	7.15 ± 0.50	7.28 ± 0.41	7.54 ± 0.49
Albumin (g/dL)	1.24 ± 0.18	1.26 ± 0.16	1.27 ± 0.18	1.30 ± 0.23	1.38 ± 0.28	1.52 ± 0.21
Globulin (g/dL)	5.97 ± 0.20	5.77 ± 0.22	5.86 ± 0.30	5.85 ± 0.48	5.90 ± 0.27	6.02 ± 0.41
Kidney function test						
CR (mg/dL)	0.92 ± 0.12	0.84 ± 0.13	0.86 ± 0.12	0.83 ± 0.11	0.66 ± 0.06	0.64 ± 0.07
BUN (mg/dL)	23.70 ± 3.16	22.00 ± 2.63	21.90 ± 2.73	23.30 ± 3.65	23.60 ± 4.78	24.20 ± 2.49
Urea (mg/dL)	50.90 ± 6.81	47.40 ± 5.72	47.00 ± 6.04	50.00 ± 7.92	50.80 ± 10.40	52.20 ± 5.40
Serum electrolytes						
Ca (mg/dL)	9.04 ± 0.14	8.96 ± 0.08	8.92 ± 0.10	9.13 ± 0.50	10.00 ± 0.34	10.18 ± 0.36
P (mg/dL)	7.94 ± 0.98	7.53 ± 0.61	7.15 ± 0.56*	7.35 ± 0.44	4.10 ± 0.65	4.48 ± 0.53
Na (mmol/l)	144.50 ± 1.18	145.10 ± 1.97	146.10 ± 1.10	145.40 ± 2.17	145.40 ± 0.89	144.40 ± 2.07
K (mmol/l)	4.62 ± 0.37	4.38 ± 0.27	4.14 ± 0.44*	4.42 ± 0.21	4.60 ± 0.47	5.02 ± 0.33
Metabolic function test						
FPG (mg/dL)	51.10 ± 5.88	55.40 ± 8.28	55.30 ± 5.33	56.10 ± 8.12	84.00 ± 9.51	80.60 ± 2.97
Cholesterol (mg/dL)	54.90 ± 12.18	50.30 ± 9.23	45.70 ± 8.81	64.30 ± 10.17	52.60 ± 14.40	55.40 ± 12.66

Triglyceride (mg/dL)	40.90 ± 15.14	40.90 ± 15.59	39.70 ± 14.38	49.30 ± 6.70	55.80 ± 12.85	53.20 ± 10.76
LDL (mg/dL)	6.00 ± 1.76	6.20 ± 1.32	5.20 ± 0.92	6.90 ± 1.37	4.40 ± 1.14	4.80 ± 1.30
HDL (mg/dL)	52.20 ± 15.05	46.40 ± 8.88	41.20 ± 8.60	55.90 ± 9.11	41.40 ± 9.84	43.00 ± 11.98
Total bile acid (µmol/L)	62.59 ± 51.19	27.47 ± 17.61*	33.98 ± 9.66	17.23 ± 7.85*	29.89 ± 25.70	37.13 ± 38.15
Thyroid hormonal test						
Thyroxine (T4)	272.40 ± 22.81	258.00 ± 44.78	244.60 ± 17.80	260.90 ± 20.32	304.80 ± 25.84	411.80 ± 112.76
Triiodothyronine (T3)	7.28 ± 0.75	6.73 ± 0.73	7.54 ± 0.85	8.38 ± 1.22*	6.45 ± 0.67	6.97 ± 0.56
Thyroid stimulating hormone (TSH)	10.72 ± 0.89	10.00 ± 1.39	9.17 ± 0.74*	10.19 ± 0.62	12.56 ± 2.22	12.24 ± 1.56

Data was represented as Mean ± Standard Deviation, Comparisons with respective days as follows: G2, G3 and G4 V/s. G1; G4R V/s. G1R, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, CR: Creatinine, BUN: Blood Urea Nitrogen, Ca: Calcium, P: Phosphorus, Na: Sodium, K: Potassium, FPG: Fasting plasma glucose, LDL: low-density lipoprotein, HDL: high-density lipoprotein. n=15 for G1, G2, G3 and G4 whereas n=10 for G1R and G4R

Table 6: Effect of FEFLG on relative organ weights (g) of during 90 days repeated dose toxicity study (Male rats).

Organ	G1	G2	G3	G4	G1R	G4R
	VC	FEFLG-250	FEFLG-500	FEFLG-1000	VC-R	FEFLG-1000-R
Brain	0.57 ± 0.05	0.59 ± 0.04	0.57 ± 0.05	0.56 ± 0.07	0.57 ± 0.03	0.52 ± 0.04*
Liver	2.62 ± 0.13	2.45 ± 0.11	2.50 ± 0.18	2.70 ± 0.31	2.62 ± 0.40	2.67 ± 0.16
Kidneys	0.63 ± 0.04	0.62 ± 0.04	0.62 ± 0.03	0.68 ± 0.08	0.55 ± 0.04	0.57 ± 0.05
Adrenals	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Testes	0.99 ± 0.06	0.97 ± 0.07	1.00 ± 0.08	0.99 ± 0.07	0.91 ± 0.08	0.87 ± 0.07
Heart	0.28 ± 0.03	0.28 ± 0.03	0.30 ± 0.03	0.29 ± 0.03	0.29 ± 0.04	0.31 ± 0.22
Spleen	0.22 ± 0.05	0.17 ± 0.02	0.20 ± 0.05	0.22 ± 0.07	0.19 ± 0.02	0.19 ± 0.01
Thymus	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
Epididymis	0.35 ± 0.03	0.39 ± 0.04	0.40 ± 0.04	0.40 ± 0.06	0.41 ± 0.01	0.37 ± 0.03*
Pituitary	0.00 ± 0.00	0.01 ± 0.00*	0.01 ± 0.00*	0.01 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00
Thyroid	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Prostate**	0.70 ± 0.15	0.82 ± 0.14	0.77 ± 0.15	0.84 ± 0.18	0.65 ± 0.09	0.78 ± 0.08*

Data was represented as Mean ± Standard Deviation. Comparisons with respective days as follows: G2, G3 and G4 V/s. G1; G4R V/s. G1R, n=15 for G1, G2, G3 and G4 whereas n=10 for G1R and G4R .** Prostate + Seminal vesicles with coagulating glands as a whole complex

Table 7: Effect of FFFLG on relative organ weights of during 90 days repeated dose toxicity study (Female rats).

Organ	G1	G2	G3	G4	G1R	G4R
	VC	FEFLG-250	FEFLG-500	FEFLG-1000	VC-R	FEFLG-1000-R
Brain	0.82 ± 0.04	0.85 ± 0.06	0.83 ± 0.05	0.82 ± 0.06	0.83 ± 0.10	0.80 ± 0.05
Liver	2.62 ± 0.23	2.69 ± 0.28	2.74 ± 0.29	2.72 ± 0.27	2.43 ± 0.36	2.54 ± 0.08
Kidneys	0.63 ± 0.06	0.60 ± 0.03	0.64 ± 0.05	0.65 ± 0.05	0.58 ± 0.04	0.59 ± 0.03
Adrenals	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Ovaries	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.02
Heart	0.30 ± 0.03	0.31 ± 0.03	0.33 ± 0.03	0.32 ± 0.02	0.31 ± 0.03	0.30 ± 0.01
Spleen	0.25 ± 0.04	0.23 ± 0.02	0.23 ± 0.04	0.23 ± 0.03	0.23 ± 0.04	0.22 ± 0.04
Thymus	0.14 ± 0.02	0.14 ± 0.03	0.14 ± 0.02	0.14 ± 0.03	0.16 ± 0.03	0.20 ± 0.03
Uterus	0.35 ± 0.10	0.37 ± 0.11	0.50 ± 0.21*	0.31 ± 0.10	0.27 ± 0.06	0.26 ± 0.03
Pituitary	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Thyroid-fixed	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00

Data was represented as Mean ± Standard Deviation. Comparisons with respective days as follows: G2, G3 and G4 V/s. G1; G4R V/s. G1R, n=15 for G1, G2, G3 and G4 whereas n=10 for G1R and G4R

Table 8: Summary of histopathology findings.

Sex	Male		Female	
Group	VC	FEFLG-1000	VC	FEFLG-1000
Number of Animals examined:	10	10	15	15
Adrenals				
Corticular vacuolation, minimal	4	1	NAD	1
Accessory cortical tissue	NAD	NAD	1	NAD
Aorta	NAD	NAD	NAD	NAD
Brain	NAD	NAD	NAD	NAD
Caecum	NAD	NAD	NAD	NAD
Colon				
Sub-mucosal lymphoid hyperplsia, minimal	2	4	2	2
Duodenum	NAD	NAD	NAD	NAD
Epididymis	NAD	NAD		
Eyes	NAD	NAD	NAD	NAD
Heart	NAD	NAD	NAD	NAD
Ileum	NAD	NAD	NAD	NAD
Jejunum	NAD	NAD	NAD	NAD
Kidneys				
Mineralization	2	3	NAD	NAD
Liver	NAD	NAD	NAD	NAD
Lungs				
Perivascular lymphocytic aggregation, minimal	7	4	6	2
Foam cells, minimal	6	3	1	NAD
Suppurative inflammation, mild	NAD	1	NAD	NAD
Granulomatous inflammation, minimal	NAD	1	NAD	NAD
Lymph Node	NAD	NAD	NAD	NAD
Ovaries	-	-	NAD	NAD
Skin	NAD	NAD	NAD	NAD
Oesophagus	NAD	NAD	NAD	NAD
Pancreas	NAD	NAD	NAD	NAD
Pituitary	NAD	NAD	NAD	NAD
Prostate	NAD	NAD	-	-
Rectum	NAD	NAD	NAD	NAD
Sciatic nerve	NAD	NAD	NAD	NAD
Seminal vesicles	NAD	NAD	-	-
Skeletal muscle	NAD	NAD	NAD	NAD
Spleen	NAD	NAD	NAD	NAD
Spinal cord	NAD	NAD	NAD	NAD
Sternum with bone marrow	NAD	NAD	NAD	NAD
Stomach	NAD	NAD	NAD	NAD
Testes	NAD	NAD	-	-
Trachea	NAD	NAD	NAD	NAD
Thymus	NAD	NAD	NAD	NAD
Thyroid				
Ectopic thymus and ultimobranchial cyst	NAD	NAD	NAD	1
Urinary Bladder	NAD	NAD	NAD	NAD
Uterus				
Eosinophilic cell infiltration, minimal	-	-	NAD	2

NAD = No Abnormality Detected

Table 9: Summary data of histidine revertants in *Salmonella typhimurium*, Reverse Mutation Assay (AMES Test) - Experiment No.: 1.

Treatment	Dose (µg/plate)	S-9	Mean Number of Revertants (Mean ± S.D.)				
			TA1535	TA97a	TA98	TA100	TA102
FEFLG	5000	-	12.33 ± 4.73	99.33 ± 2.31	21.00 ± 7.94	156.67 ± 4.62	260.00 ± 6.93
		+	15.00 ± 3.61	174.00 ± 8.72	43.00 ± 7.55	153.33 ± 30.09	285.33 ± 18.04
	1500	-	13.00 ± 2.00	99.33 ± 5.03	31.67 ± 4.51	200.67 ± 17.01	248.00 ± 32.74
		+	14.67 ± 0.58	171.33 ± 8.08	31.33 ± 1.53	172.00 ± 19.70	254.67 ± 12.22
	500	-	13.00 ± 5.29	106.00 ± 12.49	34.33 ± 4.04	156.67 ± 23.86	246.67 ± 34.49
		+	14.67 ± 3.79	168.67 ± 11.72	35.00 ± 2.65	170.00 ± 21.07	250.67 ± 15.14
	150	-	13.33 ± 0.58	97.33 ± 7.02	36.33 ± 7.57	157.33 ± 22.12	258.67 ± 10.07
		+	13.33 ± 1.15	174.00 ± 10.58	31.00 ± 1.00	156.00 ± 27.50	253.33 ± 6.11
	50	-	17.67 ± 2.52	114.67 ± 15.14	28.00 ± 2.65	184.67 ± 28.45	246.67 ± 10.07
		+	12.67 ± 3.51	170.67 ± 11.02	44.33 ± 5.77	176.00 ± 7.21	257.33 ± 6.11
DMSO (VC)	100 µl	-	11.00 ± 3.61	124.67 ± 13.01	31.67 ± 4.04	181.33 ± 28.94	262.67 ± 11.55
		+	14.00 ± 3.46	173.33 ± 4.16	48.67 ± 5.86	178.00 ± 32.19	258.67 ± 16.17
Sodium azide	2	-	493.33 ± 68.04	-	-	-	-
ICR 191	1	-	-	912.00 ± 113.42	-	-	-
4 NQNO	0.5	-	-	-	562.67 ± 24.44	-	-
3-MMS (µl/plate)	1	-	-	-		1042.67 ± 60.58	1888.00 ± 65.48
2 - AA	10	+	485.33 ± 41.05	-	-	-	-
2 - AF	20	+		874.67 ± 51.43	498.67 ± 39.46	1066.67 ± 56.19	-
DANTHRON	30	+	-	-	-	-	1816.00 ± 108.22

VC – Vehicle control, DMSO- Dimethyl sulfoxide, data was represented as Mean ± Standard deviation. Data was analyzed by unpaired 't' test. - represents plates without metabolic activation (without S9 mix), + represents plates with metabolic activation (with S9 mix), Data of each strain was compared with respective positive control group. ICR 191 - Acridine Mutagen, NQNO - 4-Nitroquinolene-N-Oxide, MMS - 3-Methylmethane Sulphonate, 2-AA - 2-Aminoanthracene, 2-AF - 2-Aminofluorene

Table 10: Summary data of histidine revertants in *Salmonella typhimurium*, Reverse Mutation Assay (AMES Test) - Experiment No.: 2.

Treatment	Dose (µg/plate)	S-9	Mean Number of Revertants (Mean ± S.D.)				
			TA1535	TA97a	TA98	TA100	TA102
FEFLG	5000.00	-	12.33 ± 2.52	103.33 ± 4.16	39.67 ± 8.74	130.00 ± 4.00	292.00 ± 18.33
		+	11.67 ± 2.08	161.33 ± 4.16	40.00 ± 8.89	134.00 ± 4.00	268.00 ± 18.33
	1500.00	-	14.00 ± 2.65	122.00 ± 2.00	36.33 ± 6.11	130.00 ± 3.46	253.33 ± 8.33
		+	16.00 ± 3.61	170.00 ± 7.21	37.00 ± 6.24	130.67 ± 6.11	269.33 ± 10.07
	500.00	-	18.00 ± 1.00	132.67 ± 3.06	40.33 ± 4.04	131.33 ± 8.33	258.67 ± 8.33
		+	17.67 ± 4.93	158.67 ± 3.06	38.67 ± 5.69	132.00 ± 7.21	312.00 ± 12.00
	150.00	-	16.33 ± 6.43	142.67 ± 5.03	41.00 ± 4.58	129.33 ± 9.24	276.00 ± 8.00
		+	12.33 ± 4.93	148.00 ± 4.00	40.00 ± 5.29	133.33 ± 5.03	264.00 ± 10.58
	50.00	-	11.33 ± 3.21	137.33 ± 14.19	38.33 ± 6.51	130.00 ± 5.29	264.00 ± 14.42
		+	15.00 ± 1.00	147.33 ± 7.02	40.00 ± 9.17	133.33 ± 6.11	272.00 ± 18.33
DMSO (VC)	100 µl	-	17.00 ± 4.58	138.67 ± 11.02	41.33 ± 3.21	140.00 ± 3.46	256.00 ± 10.58
		+	17.67 ± 2.52	164.67 ± 3.06	35.33 ± 4.51	139.33 ± 3.06	252.00 ± 8.00
Sodium azide	2.00	-	552.00 ± 24.00	-	-	-	-
ICR 191	1.00	-	-	968.00 ± 118.39	-	-	-
4 NQNO	0.50	-	-	-	642.67 ± 12.22	-	-
3-MMS (µl/plate)	1.00	-	-	-		1008.00 ± 36.66	1685.33 ± 58.97
2 - AA	10.00	+	541.33 ± 20.13	-	-	-	-
2 - AF	20.00	+		864.00 ± 32.00	581.33 ± 20.13	978.67 ± 39.46	-
DANTHRON	30.00	+	-	-	-	-	1621.33 ± 100.66

VC – Vehicle control, DMSO- Dimethyl sulfoxide, the data was represented as Mean ± Standard deviation. Data was analyzed by unpaired 't' test. - represents plates without metabolic activation (without S9 mix), + represents plates with metabolic activation (with S9 mix), Data of each strain was compared with respective positive control group. ICR 191 - Acridine Mutagen, NQNO - 4-Nitroquinolene-N-Oxide, MMS - 3-Methylmethane Sulphonate, 2-AA - 2-Aminoanthracene, 2-AF - 2-Aminofluorene

incidences of cultured mammalian cells with structural chromosome aberrations, either in the absence or presence of a metabolic activation system, and so considered non-clastogenic.

DISCUSSION

Fenugreek is emerging as a vital ingredient source for diverse health and nutrition benefits.[59-61] The present study reported the preparation, standardization, and efficacy of CD38+ inhibition and preclinical safety profile FEFLG, a flavonoid glycosides-based standardized fenugreek seed extract. FEFLG was characterized for a group of flavonoid glycosides by HPLC and evaluated for efficacy as a CD38+ enzyme inhibitor *in vitro*. Furthermore, safety evaluations were performed *in vivo* (AOT and 90-day repeated dose subchronic toxicity) and in vitro (mutagenicity and genotoxicity).

The flavonoid content of FEFLG was found to be 49.85% with a colorimetric method using aluminum chloride ($AlCl_3$), which is the most reliable and commonly used for estimation of flavonoids from natural products.[50,62-64] In addition, a detailed characterization of FEFLG by HPLC revealed the presence of many apigenin (vitexin, vicenin, orientin and their derivatives) and luteolin (schaftoside and isoschaftoside) flavonoid glycosides (Figure 1 and Figure 2).

In the present study, the potential of FEFLG as a CD38+ enzyme inhibitor was evaluated using hydrolyzed NAD+ molecules that produce a fluorescent etheno-ADP-ribose product.[65] The CD38+ enzyme and NAD+ system involve energy metabolism, DNA repair, epigenetic regulation, immune response, and cell senescence.[66] In the present study, FEFLG was found to have dose-dependent inhibition of the CD38 enzyme. Furthermore, The AUC values of FEFLG and peak inhibition time of 35 min were close to the AUC of the positive control, apigenin. Furthermore, the obtained difference in IC_{50} values of FEFLG and apigenin was 48.55% (i.e., 0.9622 µg/ml v/s 0.4672 µg/ml). Considering the total flavonoid glycosides content of FEFLG is about half (43.83%) as that of pure apigenin, the potencies of FEFLG and apigenin are equivalent. In the present study, apigenin showed IC_{50} = 0.4672 µg/ml, which is lower than reported IC_{50} of 2.78 µg/ml (10.3 ± 2.4 mmol/L) for the CD38/NAD+ase activity.[33] This difference can be attributed to procedural wavelength variations during fluorescence measurement or dilution procedures.

The enzyme CD38+ is emerging as the critical target of novel compounds against many conditions via NAD+-dependent pathways[19,55,67], mitochondrial oxidative stress[68] and age-related diseases.[69] Moreover, CD38+ inhibition is a potential option for managing many age-related disorders[70-73] and known to improve endurance and exercise performance.[74] Therefore, the efficacy of FEFLG, as shown in the present study as a CD38+ inhibitor, suggests its potential to prevent age-related NAD+ decline.

The safety assessments of the FEFLG were performed using OECD guidelines[52], which are well-accepted worldwide for safety assessments of any chemical that can be exposed or consumed by human beings and are recommended by most regulatory agencies worldwide AOT study determines the exposure ranges and maximum dose at which animal lethality was observed.[75] Female rats are preferred for acute toxicity studies as females are generally sensitive.[76] Results of acute toxicity allow a substance to be ranked and classified according to the GHS for classifying chemicals that cause acute toxicity.[77,78] FEFLG was found safe with a median lethal cut-off dose of 5,000 mg/kg in female rats and therefore classified as category 5 or unclassified according to the GHS of classification of chemicals.

Subchronic toxicity study involves continuous treatment exposure for 90-days, and the highest safe dose is defined as no observed adverse effect level (NOAEL). NOAEL is selected after comparing the severity of toxic effects between treated and control groups.[75] The doses for subchronic toxicity of FEFLG (250, 500 and 1000 mg/kg) were selected from the results of AOT to assess safe oral dose levels for long-term human exposure. Repeated oral administration for 90 days of FEFLG did not cause any mortality or toxicity signs at and up to the dose level of 1000 mg/kg body weight during the dosing period and post-dosing recovery period. The ophthalmoscopic and functional observation of treated animals also confirmed the normal physiological responses like the VC group's findings. Hematological evaluation provides vital information about animals' physiological and pathological health, especially the administration of toxic compounds.[79] Although few alterations in hematological values were observed compared to the VC group, these changes cannot be considered confirmatory changes in blood profile as no correlated gross pathological and histopathological changes were observed. Moreover, the alterations were found to be within the historical control range. Therefore, these changes were considered incidental and unrelated to treatments.

Hormone cycling in females is crucial in social interaction and impacts behavior due to hormone fluctuation throughout the cycle. The sensitivity of social interaction to the estrus cycle may be correlated with neurodevelopmental disorders and neuropsychiatric disease.[80] During the current study, vaginal smears between FEFLG treated and VC groups were comparable concerning estrous cycle stages, suggesting normal estrous cycle and hormonal status. The relative organ weights indicate sensitivity to predict the toxicity and correlate well with histopathological changes.[81] The changes observed in organ weights in the present study were found to be within the historical control range of the facility at the end of the treatment and recovery period. All the histopathological observations noted in the present study are reported as common occurrences in the rats housed in laboratory conditions.[82] Mineralization characterized by basophilic deposits was observed in the rats from both VC and FEFLG-1000 treated rats. The presence of eosinophils in the endometrium and mineralization is known to be present during the normal estrous cycle.[82] Similarly, spontaneous, age-related changes, such as cortical vacuolization and congenital changes in adrenals of rats or ectopic thymus and ultimobranchial cyst in the thyroid gland, are commonly reported in histology of normal rats.[82]

The human-equivalent dose (HED) can be derived from animal NOAEL by referring to US Food and Drug Administration guidance for industry.[83] Considering the NOAEL of 1,000 mg/kg in male and female rats, the HED of FEFLG can be considered as 9.7 g per day (assuming the average human weight of 60 kg), indicating a reasonable margin of safety.

Ames assay can detect the mutagenicity of compounds accurately. In the present study, the mutagenicity of FEFLG was assessed to explore the potential genetic hazards using the Ames assay.[84,85] In the present study, FEFLG did not show any signs of significant mutagenicity in either the presence or absence of a metabolic activator up to 5,000 µg/plate concentration. Therefore, FEFLG was found to be non-mutagenic.

A genotoxicity assessment is performed during drug development to evaluate the ability of test compounds to induce DNA damage, such as cross-links, adducts, and cleavage.[86] Genotoxins can further lead to tumor progression[87] and congenital malformations.[88] In the present study, FEFLG did not induce any significant chromosomal aberrations in cultured mammalian cells in either the presence or absence of metabolic activation and is considered non-clastogenic.

Considering the efficacy as a CD38+ inhibitor and the preclinical safety profile as shown in the present study strongly suggests potential of FEFLG as a promising oral supplementation for various antiaging applications. However, suitable clinical studies on FEFLG in relevant populations are required to establish the clinical evidence.

CONCLUSIONS

The present study demonstrated the efficacy as CD38+ enzyme inhibitor *in vitro* and preclinical oral safety of FEFLG for possible anti-aging applications. The acute oral LD_{50} and NOAEL on sub-chronic, 90-day repeated oral dose administration of FEFLG in rats is more than 2000 mg/kg (limit dose 5000 mg/kg) and 1000 mg/kg, respectively, with the absence of mutagenic or clastogenic potential.

ABBREVIATIONS

2-AA: 2-aminoanthracene; 2-AF: 2-aminofluorene; $AlCl_3$: Aluminum chloride; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AMES test: *Salmonella typhimurium* reverse mutation assay; ANOVA: Analysis of variance; AOT: Acute oral toxicity; APTT: Activated partial thromboplastin time; AUC: Area under the curve ; B: Basophils;

BUN: Blood urea nitrogen; Ca: Calcium; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals ; CPM: Cyclophosphamide monohydrate; CR: Creatinine; DLC: Differential leucocyte count; DMSO: Dimethyl sulfoxide; E: Eosinophils; FEFLG: Flavonoids glycosides based on standardized fenugreek seed extract; FPG: Fasting plasma glucose; GHS: Globally harmonized system; Hb: Hemoglobin; HDL: High-density lipoprotein; HED: Human-equivalent dose; HPLC: High-performance liquid chromatography; IC_{50}: Half-maximal inhibitory concentration; ICR 191: Acridine mutagen; K: Potassium; L: Lymphocytes; LD_{50}: Median-lethal dose; LDL: Low-density lipoprotein; M: Monocytes; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; MCV: Mean corpuscular volume,; Mg QE: Mg quercetin equivalent ; N: Neutrophils; Na: Sodium; NAD: Nicotinamide adenine dinucleotide; NAD: No abnormality detected; NOAEL: No observed adverse effect level; NQNO: 4-nitroquinolene-n-oxide; OECD: Organization for Economic Co-operation and Development; P: Phosphorus; PCV: Packed cell volume; PT: Prothrombin time; RBC: Red blood corpuscles; RFU: Relative fluorescence units; TLC: Total leukocyte (White Blood Corpuscles) count; VC: Vehicle control

SUMMARY

Characterization, efficacy, and oral toxicity of flavonoids glycosides-based standardized fenugreek seed extract (FEFLG) was reported.

FEFLG showed efficacy as CD38+ enzyme inhibitor during *in vitro* testing.

The acute oral median-lethal dose (LD_{50}) of oral FEFLG was greater than 2000 mg/rat/day.

No observed adverse effect level (NOAEL) of oral FEFLG was greater than 1000 mg/rat/day.

FEFLG was found as non-mutagenic and non-clastogenic.

REFERENCES

1. Robine J-M. Ageing populations: We are living longer lives, but are we healthier. United Nations, Department of Economics and Social Affairs, Population Division. 2021.
2. Takeda M. Economic consequences of anti-aging. Glycative Stress Res. 2022;9:24-32.
3. Chang AY, Skirbekk VF, Tyrovolas S, Kassebaum NJ, Dieleman JL. Measuring population ageing: an analysis of the Global Burden of Disease Study 2017. Lancet Public Health. 2019;4(3):e159-67.
4. Solé-Auró A, Deeg DJH. New thinking about health expectancy: introduction to the special section. Eur J Ageing. 2021;18(4):439-41.
5. Torregrosa-Muñumer R, Vara E, Fernández-Tresguerres J, Gredilla R. Resveratrol supplementation at old age reverts changes associated with aging in inflammatory, oxidative and apoptotic markers in rat heart. Eur J Nutr. 2021;60(5):2683-93.
6. Fusco D, Colloca G, Lo Monaco MR, Cesari M. Effects of antioxidant supplementation on the aging process. Clin Interv Aging. 2007;2(3):377-87.
7. Banez MJ, Geluz MI, Chandra A. A systemic review on the antioxidant and anti-inflammatory effects of resveratrol, curcumin, and dietary nitric oxide supplementation on human cardiovascular health. Nutr Res. 2020;78:11-26.
8. Simioni C, Zauli G, Martelli AM. Oxidative stress: role of physical exercise and antioxidant nutraceuticals in adulthood and aging. Oncotarget. 2018;9(24):17181-98.
9. Tan BL, Norhaizan ME, Liew WP, Sulaiman Rahman H. Antioxidant and Oxidative Stress: A Mutual Interplay in Age-Related Diseases. Front Pharmacol. 2018;9:1162.
10. Bruins MJ, Van Dael P, Eggersdorfer M. The Role of Nutrients in Reducing the Risk for Noncommunicable Diseases during Aging. Nutrients. 2019;11(1):85.
11. Thakkar S, Anklam E, Xu A. Regulatory landscape of dietary supplements and herbal medicines from a global perspective. Regul Toxicol Pharmacol. 2020;114:104647.
12. Ratajczak M, Kaminska D, Świątły-Błaszkiewicz A, Matysiak J. Quality of Dietary Supplements Containing Plant-Derived Ingredients Reconsidered by Microbiological Approach. Int J Env Res Public Health. 2020;17(18):6837.
13. Cui H, Kong Y, Zhang H. Oxidative Stress, Mitochondrial Dysfunction, and Aging. J Signal Transd. 2012;2012:646354.
14. Amorim JA, Coppotelli G, Rolo AP, Palmeira CM, Ross JM, Sinclair DA. Mitochondrial and metabolic dysfunction in ageing and age-related diseases. Nat Rev Endocrinol. 2022;18(4):1-16.
15. Fuente MDl, Miquel J. An Update of the Oxidation-Inflammation Theory of Aging: The Involvement of the Immune System in Oxi-Inflamm-Aging. Curr Pharm Des. 2009;15(26):3003-26.
16. Brand RM, Wipf P, Durham A, Epperly MW, Greenberger JS, Falo LD. Targeting Mitochondrial Oxidative Stress to Mitigate UV-Induced Skin Damage. Front Pharmacol. 2018;9:920.
17. Covarrubias AJ, Kale A, Perrone R. Senescent cells promote tissue NAD(+) decline during ageing via the activation of CD38(+) macrophages. Nat Metab. 2020;2(11):1265-83.
18. Janssens GE, Grevendonk L, Perez RZ. Healthy aging and muscle function are positively associated with NAD+ abundance in humans. Nat Aging. 2022;2(4):254-63.
19. McReynolds MR, Chellappa K, Baur JA. Age-related NAD+ decline. Exp Gerontol. 2020;134:110888.
20. Schultz MB, Bochaton T, Bonkowski M. NAD+ depletion as a cause of inflammaging. Innovation in Aging. 2018;2:746.
21. Yaku K, Okabe K, Nakagawa T. NAD metabolism: Implications in aging and longevity. Ageing Res Rev. 2018;47:1-17.
22. Chini EN, Chini CCS, Espindola Netto JM, de Oliveira GC, van Schooten W. The Pharmacology of CD38/NADase: An Emerging Target in Cancer and Diseases of Aging. Trends Pharmacol Sci. 2018;39(4):424-36.
23. Wagner S, Manickam R, Brotto M, Tipparaju SM. NAD+ centric mechanisms and molecular determinants of skeletal muscle disease and aging. Mol Cell Biochem. 2022;477(6):1829-48.
24. Elhassan YS, Kluckova K, Fletcher RS. Nicotinamide Riboside Augments the Aged Human Skeletal Muscle NAD+ Metabolome and Induces Transcriptomic and Anti-inflammatory Signatures. Cell Rep. 2019;28(7):1717-1728.e1716.
25. Tarragó MG, Chini CCS, Kanamori KS. A potent and specific CD38 inhibitor ameliorates age-related metabolic dysfunction by reversing tissue nad+ decline. Cell Metab. 2018;27(5):1081-1095.e1010
26. Aman Y, Qiu Y, Tao J, Fang EF. Therapeutic potential of boosting NAD+ in aging and age-related diseases. Trans Med Aging. 2018;2:30-7.
27. Aquila S, Giner RM, Recio MC, Spegazzini ED, Ríos JL. Anti-inflammatory activity of flavonoids from Cayaponia tayuya roots. J Ethnopharmacol. 2009;121(2):333-7.
28. Liu J-Y, Li S-Y, Feng J-Y. Flavone C-glycosides from the flowers of Trollius chinensis and their anti-complementary activity. J Asian Nat Prod Res. 2013;15(4):325-31.
29. Jiang W, Qu H, Yuan D, Yang G, An F, Wang S. Protection of orientin and vitexin from flowers and flower buds of Trollius chinensis on brain injury in aging mice induced by D-galactose. Chinese Trad Herbal Drugs. 2012;43:1376-80.
30. Hoffmann-Bohm K, Lotter H, Seligmann O, Wagner H. Antihepatotoxic C-glycosylflavones from the leaves of Allophyllus edulis var. edulis and gracilis. Planta Med. 1992;58(6):544-8.

31. Peng X, Zheng Z, Cheng K-W. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. Food Chem. 2008;106(2):475-81.

32. Brango-Vanegas J, Costa G, Ortmann C. Glycosylflavonoids from Cecropia pachystachya Trécul are quorum sensing inhibitors. Phytomedicine. 2014;21(5):670-5.

33. Escande C, Nin V, Price NL. Flavonoid apigenin is an inhibitor of the NAD+ ase CD38: implications for cellular NAD+ metabolism, protein acetylation, and treatment of metabolic syndrome. Diabetes. 2013;62(4):1084-93.

34. Kellenberger E, Kuhn I, Schuber F, Muller-Steffner H. Flavonoids as inhibitors of human CD38. Bioorganic & medicinal chemistry letters. Bioorg Med Chem Lett. 2011;21(13):3939-42.

35. Shang M, Cai S, Han J. Studies on flavonoids from Fenugreek (*Trigonella foenumgraecum* L.). Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi. China J Chin Materia Med. 1998;23(10):614-6.

36. Nagulapalli Venkata KC, Swaroop A, Bagchi D, Bishayee A. A small plant with big benefits: Fenugreek (*Trigonella foenum-graecum* Linn.) for disease prevention and health promotion. Mol Nutr Food Res. 2017;61(6):1600950.

37. Pang X, Cong Y, Yu H-S. Spirostanol saponins derivated from the seeds of *Trigonella foenum-graecum* by β-glucosidase hydrolysis and their inhibitory effects on rat platelet aggregation. Planta Med. 2012;78(3):276-85.

38. Kenny O, Smyth T, Hewage C, Brunton N. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (*Momordica charantia*) fruit. Food Chem. 2013;141(4):4295-302.

39. Mandegary A, Pournamdari M, Sharififar F, Pournourmohammadi S, Fardiar R, Shooli S. Alkaloid and flavonoid rich fractions of fenugreek seeds (*Trigonella foenum-graecum* L.) with antinociceptive and anti-inflammatory effects. Food Chem Toxicol. 2012;50(7):2503-7.

40. Rayyan S, Fossen T, Andersen OM. Flavone C-glycosides from seeds of fenugreek, *Trigonella foenum-graecum* L. J Agr Food Chem. 2010;58(12):7211-7.

41. Kenny O, Smyth TJ, Hewage CM, Brunton NP. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (Momordica charantia) fruit. Food Chem. 2013;141(4):4295-302.

42. Benayad Z, Gomez-Cordoves C, Es-Safi NE. Characterization of flavonoid glycosides from fenugreek (*Trigonella foenum-graecum*) crude seeds by HPLC-DAD-ESI/MS analysis. Int J Mol Sci. 2014;15(11):20668-85.

43. He M, Min JW, Kong WL, He XH, Li JX, Peng BW. A review on the pharmacological effects of vitexin and isovitexin. Fitoterapia. 2016;115:74-85.

44. Babaei F, Moafizad A, Darvishvand Z, Mirzababaei M, Hosseinzadeh H, Nassiri-Asl M. Review of the effects of vitexin in oxidative stress-related diseases. Food Sci Nutr. 2020;8(6):2569-80.

45. Yao D, Zhang B, Zhu J. Advances on application of fenugreek seeds as functional foods: Pharmacology, clinical application, products, patents and market. Crit Rev Food Sci Nutr. 2020;60(14):2342-52.

46. Xiao J, Capanoglu E, Jassbi AR, Miron A. Advance on the flavonoid C-glycosides and health benefits. Crit Rev Food Sci Nutr. 2016;56(1):S29-S45.

47. Luan G, Wang Y, Wang Z. Flavonoid Glycosides from Fenugreek Seeds Regulate Glycolipid Metabolism by Improving Mitochondrial Function in 3T3-L1 Adipocytes in Vitro. J Agr Food Chem. 2018;66(12):3169-78.

48. Dwyer JT, Coates PM, Smith MJ. Dietary Supplements: Regulatory Challenges and Research Resources. Nutrients. 2018;10(1):41.

49. Shipkowski KA, Betz JM, Birnbaum LS. Naturally complex: Perspectives and challenges associated with Botanical Dietary Supplement Safety assessment. Food Chem Toxicol. 2018;118:963-71.

50. Chang C-C, Yang M-H, Wen H-M, Chern J-C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2002;10.

51. OECD. Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris: OECD Publishing; 2002.

52. OECD. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris: OECD Publishing; 1998.

53. de Oliveira GC, Kanamori KS, Auxiliadora-Martins M, Chini CC, Chini EN. Measuring CD38 Hydrolase and Cyclase Activities: 1, N6-Ethenonicotinamide Adenine Dinucleotide (ε-NAD) and Nicotinamide Guanine Dinucleotide (NGD) Fluorescence-based Methods. Bio-protocol. 2018;8(14):e2938.

54. Schultz MB, Lu Y, Braidy N, Sinclair DA. Assays for NAD+-Dependent Reactions and NAD+ Metabolites. ADP-ribosylation and NAD+ Utilizing Enzymes: Springer; 2018:77-90.

55. Escande C, Nin V, Price NL. Flavonoid apigenin is an inhibitor of the NAD+ ase CD38: implications for cellular NAD+ metabolism, protein acetylation, and treatment of metabolic syndrome. Diabetes. 2013;62(4):1084-93.

56. OECD. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents; 2018.

57. OECD. Test No. 471: Bacterial Reverse Mutation Test. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris, France: OECD Publishing; 1997.

58. OECD. Test No. 473: In vitro Mammalian Chromosome Aberration Test. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. 1st ed. Paris, France: OECD Publishing; 1997.

59. Sarwar S, Hanif MA, Ayub MA, Boakye YD, Agyare C. Chapter 20 - Fenugreek. In: Hanif MA, Nawaz H, Khan MM, Byrne HJ, eds. Medicinal Plants of South Asia: Elsevier. 2020;257-71.

60. Srinivasan K. Chapter 3.15 - Fenugreek (*Trigonella foenum-graecum* L.) Seeds Used as Functional Food Supplements to Derive Diverse Health Benefits. In: Nabavi SM, Silva AS, eds. Nonvitamin and Nonmineral Nutritional Supplements: Academic Press; 2019;217-21.

61. Basu SK, Zandi P, Cetzal-Ix W. Chapter 28 - Fenugreek (*Trigonella foenum-graecum* L.): Distribution, Genetic Diversity, and Potential to Serve as an Industrial Crop for the Global Pharmaceutical, Nutraceutical, and Functional Food Industries. In: Singh RB, Watson RR, Takahashi T, eds. The Role of Functional Food Security in Global Health: Academic Press; 2019;471-97.

62. Baba SA, Malik SA. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of Arisaema jacquemontii Blume. J Taibah Uni Sci. 2015;9(1):449-54.

63. Marinova D, Ribarova F, Atanassova M. Total phenolics and total flavonoids in Bulgarian fruits and vegetables. J Uni Chem Technol Metal. 2005;40(1):255-60.

64. Pourmorad F, Hosseinimehr S, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr J Biotechnol. 2006;5(12):e2100551.

65. Muller HIM, Muller C, Schuber F. NAD+ glycohydrolase, an ecto-enzyme of calf spleen cells. Biochem J. 1983;212(2):459-64.

66. Covarrubias AJ, Perrone R, Grozio A, Verdin E. NAD(+) metabolism and its roles in cellular processes during ageing. Nat Rev Mol Cell Biol. 2021;22(2):119-41.

67. Chini CCS, Zeidler JD, Kashyap S, Warner G, Chini EN. Evolving concepts in NAD+ metabolism. Cell Metab. 2021;33(6):1076-87.

68. Ogura Y, Kitada M, Xu J, Monno I, Koya D. CD38 inhibition by apigenin ameliorates mitochondrial oxidative stress through restoration of the intracellular NAD(+)/NADH ratio and Sirt3 activity in renal tubular cells in diabetic rats. Aging (Albany NY). 2020;12(1):11325-36.

69. Hou Y, Wei Y, Lautrup S. NAD+ supplementation reduces neuroinflammation and cell senescence in a transgenic mouse model of Alzheimer's disease via cGAS-STING. Proc NatL Acad Sci. 2021;118(37):e2011226118.

70. Clement J, Wong M, Poljak A, Sachdev P, Braidy N. The Plasma NAD+ Metabolome Is Dysregulated in "Normal" Aging. Rejuvenation Res. 2018;22(2):121-30.

71. Shi B, Wang W, Korman B. Targeting CD38-dependent NAD+ metabolism to mitigate multiple organ fibrosis. iScience. 2021;24(1):101902.

72. Avvedimento EV, Gabrielli A. Linking NAD metabolism and DNA repair to inflammation in SSc. Nat Rev Rheumatol. 2021;17(7):381-2.

73. Peclat TR, Shi B, Varga J, Chini EN. The NADase enzyme CD38: an emerging pharmacological target for systemic sclerosis, systemic lupus erythematosus and rheumatoid arthritis. Curr Opin Rheumatol. 2020;32(6):488-96.

74. Peclat TR, Thompson KL, Warner GM. CD38 inhibitor 78c increases mice lifespan and healthspan in a model of chronological aging. Aging Cell. 2022;21(4):e13589.

75. Ganapathi SC, Holla R, Ym SS, Mundugaru R. Acute oral toxicity study of ethanolic extract of *Actinoscirpus grossus* (L.F) Goetgh. and DA Simpson. Asian J Pharm Clin Res. 2018;11(1):321-3.

76. Lipnick RL, Cotruvo JA, Hill RN. Comparison of the up-and-down, conventional LD50, and fixed-dose acute toxicity procedures. Food Chem Toxicol. 1995;33(3):223-31.

77. Winder C, Azzi R, Wagner D. The development of the globally harmonized system (GHS) of classification and labelling of hazardous chemicals. J Hazard Mat. 2005;125(1-3):29-44.

78. OECD. Test No. 423: Acute Oral toxicity - Acute Toxic Class Method; 2002.

79. Mukinda JT, Eagles PF. Acute and sub-chronic oral toxicity profiles of the aqueous extract of *Polygala fruticosa* in female mice and rats. J Ethnopharmacol. 2010;128(1):236-40.

80. Chari T, Griswold S, Andrews NA, Fagiolini M. The Stage of the Estrus Cycle Is Critical for Interpretation of Female Mouse Social Interaction Behavior. Front Behav Neurosci. 2020;14:113.

81. Kluwe WM. Renal function tests as indicators of kidney injury in subacute toxicity studies. Toxicol Appl Pharmacol. 1981;57(3):414-24.

82. Cesta M, Malarkey D, Herbert R, Brix A, Sills R. Nonneoplastic lesion atlas. Environ Health Perspect. 2014;122(3):A76-9.

83. Centre for Drug Evaluation and Research. Guidance for industry: estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. In: Department of Health Human Services, ed. Rockville, MD: US FDA; 2005.

84. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res. 1975;31(6):347-64.

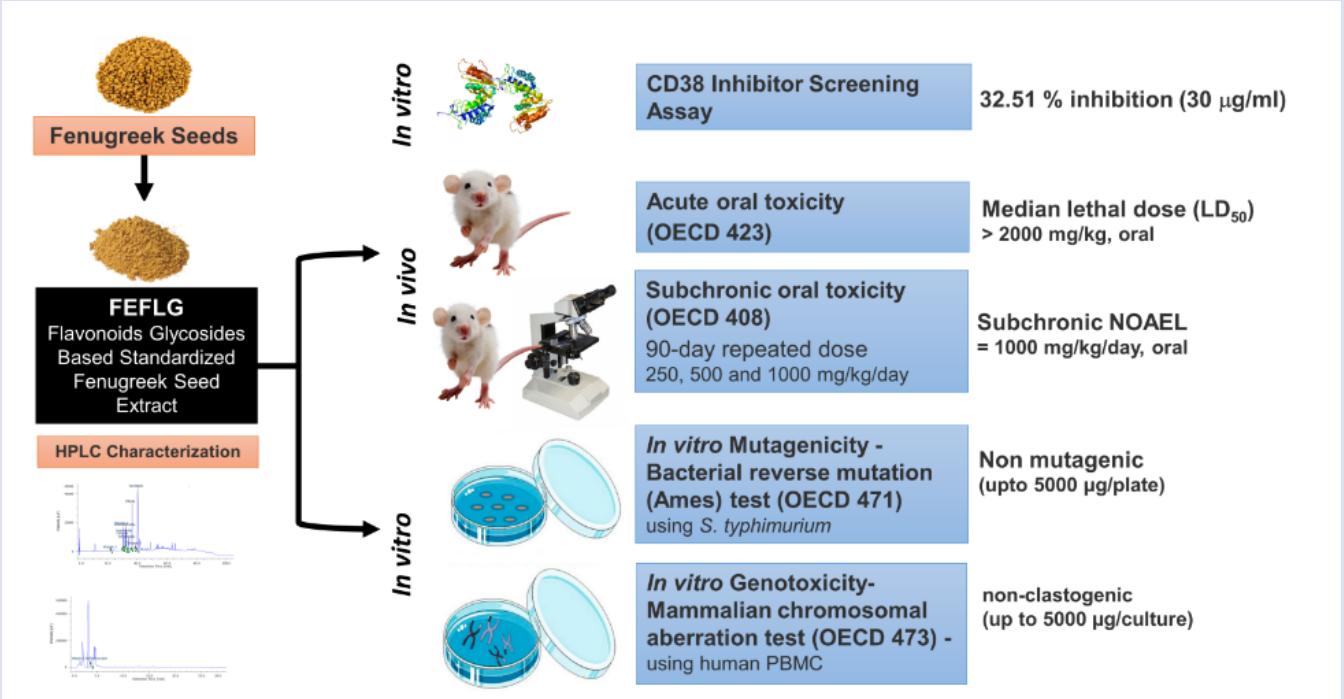
85. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res. 1983;113(3-4):173-215.

86. Williams GM. Mechanisms of chemical carcinogenesis and application to human cancer risk assessment. Toxicology. 2001;166(1-2):3-10.

87. Lee SJ, Yum YN, Kim SC. Distinguishing between genotoxic and non-genotoxic hepatocarcinogens by gene expression profiling and bioinformatic pathway analysis. Sci Rep. 2013;3:1-9.

88. Grazuleviciene R, Kapustinskiene V, Vencloviene J, Buinauskiene J, Nieuwenhuijsen MJ. Risk of congenital anomalies in relation to the uptake of trihalomethane from drinking water during pregnancy. Occup Environ Med. 2013;70(4):274-82.

GRAPHICAL ABSTRACT



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