Pharmacogn. Res.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogres.com | www.phcog.net

ORIGINAL ARTICLE

Preclinical Toxicological Evaluation of IDM01: The Botanical Composition of 4-Hydroxyisoleucine- and Trigonelline-based Standardized Fenugreek Seed Extract

Pallavi O. Deshpande, Vishwaraman Mohan, Prasad Arvind Thakurdesai

Department of Scientific Affairs, Indus Biotech Private Limited, Pune, Maharashtra, India

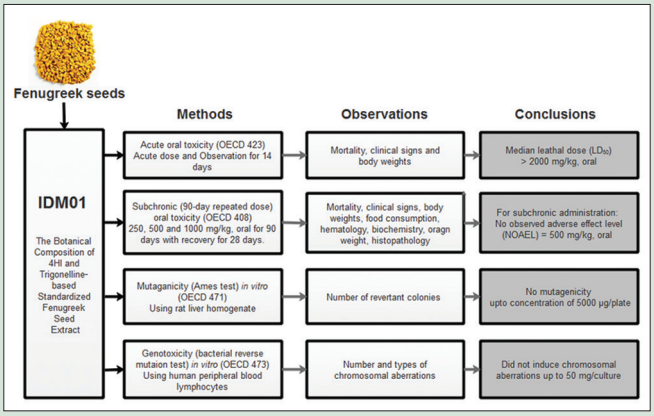
ABSTRACT

Objective: To evaluate acute oral toxicity (AOT), subchronic (90-day repeated dose) toxicity, mutagenicity, and genotoxicity potential of IDM01, the botanical composition of 4-hydroxyisoleucine- and trigonelline-based standardized fenugreek (*Trigonella foenum-graecum* L) seed extract in laboratory rats. **Materials and Methods:** The AOT and subchronic (90-day repeated dose) toxicity were evaluated using Sprague-Dawley rats as per the Organisation for Economic Co-operation and Development (OECD) guidelines No. 423 and No. 408, respectively. During the subchronic study, the effects on body weight, food and water consumption, organ weights with hematology, clinical biochemistry, and histology were studied. The mutagenicity and genotoxicity of IDM01 were evaluated by reverse mutation assay (Ames test, OECD guideline No. 471) and chromosome aberration test (OECD guideline No. 473), respectively. **Results:** The IDM01 did not show mortality or treatment-related adverse signs during acute (limit dose of 2000 mg/kg) and subchronic (90-day repeated dose of 250, 500, and 1000 mg/kg with 28 days of recovery period) administration. The IDM01 showed oral median lethal dose (LD50) >2000 mg/kg during AOT study. The no-observed adverse effect level (NOAEL) of IDM01 was 500 mg/kg. IDM01 did not show mutagenicity up to a concentration of 5000 µg/plate during Ames test and did not induce structural chromosomal aberrations up to 50 mg/culture. **Conclusions:** IDM01 was found safe during preclinical acute and subchronic (90-day repeated dose) toxicity in rats without mutagenicity or genotoxicity.

Key words: Acute oral toxicity, fenugreek seed extract, genotoxicity, mutagenicity, subchronic toxicity

SUMMARY

- Acute oral toxicity, subchronic (90-day) oral toxicity, mutagenicity and genotoxicity of IDM01 (4-hydroxyisoleucine- and trigonelline-based standardized fenugreek seed extract) was evaluated.
- The median lethal dose, LD50, of IDM01 was more than 2000 mg/kg of body weight in rats.
- No observed adverse effect level (NOAEL) of IDM01 was 500 mg/kg of body weight in rats.
- IDM01 was found safe during acute and subchronic oral toxicity studies in rats without mutagenicity or genotoxicity potetial.



Abbreviations Used: 2-AA: 2-aminoanthracene, 2-AF: 2-aminofluorene; 4 NQNO: 4-nitroquinolene-N-oxide, 4HI: 4-hydroxyisoleucine, ANOVA: Analysis of variance, AOT: Acute oral toxicity, DM: Diabetes mellitus, IDM01: The Botanical composition of 4-hydroxyisoleucine- and trigonelline-based standardized fenugreek seed extract, LD50: Median lethal dose, MMS: Methyl methanesulfonate, NAD: No abnormality detected, OECD: Organisation for Economic Co-operation and Development, SD: Standard deviation, UV: Ultraviolet, VC: Vehicle control.

Correspondence:
Dr. Prasad Arvind Thakurdesai,
Department of Scientific Affairs, Indus Biotech Private Limited, 1, Rahul Residency, Off Salunke Vihar Road, Kondhwa, Pune - 411 048, Maharashtra, India.
E-mail: prasad@indusbiotech.com

DOI: 10.4103/0974-8490.204649

Access this article online
Website: www.phcogres.com
Quick Response Code:

INTRODUCTION

Recently, there have been growing interests in the application of natural products and botanical compositions as medicinal agents for chronic diseases such as diabetes mellitus (DM)[1,2] and rheumatoid arthritis.[3] The wide range of natural plant-based products is marketed with claims to lower blood glucose levels or prevent and treat complications and comorbidities of DM.[4] One of the most promising products among them is fenugreek (*Trigonella foenum-graecum* L. family: *Fabaceae*) seeds.

Because of a broad spectrum of therapeutic benefits, the fenugreek seeds have received considerable attention as plant-derived food/nutritional supplement.[5,6] Fenugreek seeds are documented in many traditional systems of medicine of Egypt, Asia, and northern Africa for the management of many chronic conditions DM.[7] Most prominent medicinally active phytoconstituents in fenugreek seeds

Cite this article as: Deshpande PO, Mohan V, Thakurdesai PA. Preclinical toxicological evaluation of IDM01: The botanical composition of 4-Hydroxyisoleucine- and trigonelline-based standardized fenugreek seed extract. Phcog Res 2017;9:138-50.

are 4-hydroxyisoleucine (4HI),[8,9] trigonelline,[10] and galactomannans. Fenugreek seeds have previously been shown to have hypoglycemic and hypocholesterolemic efficacy in type 1 and type 2 DM patients,[11,12] and alloxan[13,14] and streptozotocin[15] induced diabetic animals. The modern scientific literature reported beneficial effects of fenugreek seeds powder and extract on glucose and fat metabolism in animal models[16] and patients with DM.[17-19] In the past, the antihyperglycemic activity of IDM01, a composition of 4HI- and trigonelline-based standardized fenugreek seed extract, was reported in animal models of DM.[20-22] Recently, IDM01 showed promising therapeutic efficacy in C57BL/6 mice against high-fat-diet-induced insulin resistance[23] and non-alcoholic steatohepatitis.[24]

With the increasing use of standardized extracts[25] and botanical compositions[26] as plant-derived food supplements, safety evaluation has become crucial for their safe human consumption.[27-31] However, such safety information of IDM01 using scientifically validated and internationally accepted guidelines is not been reported. Therefore, the present study was undertaken with an objective to evaluate the acute oral toxicity (AOT), 90-day repeated dose (subchronic) toxicity, mutagenicity, and genotoxicity of IDM01 using well-accepted guidelines issued by the Organisation for Economic Co-operation and Development (OECD).

MATERIALS AND METHODS

Animals

The Sprague-Dawley rats (125–150 g) of both sexes and 6–8 weeks old were obtained from the Indian Institute of Toxicology, Pune, Maharashtra, India, and used for the studies. The rats kept in polycarbonate cages (a group of 3 per cage) with bedding of padded husk for 7 days before dosing for acclimatization, cages were marked, and individual marking was made on fur for identification. Rats were on pelleted feed (Nav Maharashtra Chakan Oil Mills Ltd., Pune, Maharashtra, India) and provided with filtered potable water in glass bottles *ad labium*. The rats were maintained at ambient temperature (20°C ± 3°C), relative humidity (30%–70%), 10–15 air changes/h, with 12 h and each of dark and light cycle. The females were nulliparous and nonpregnant. The protocol was approved by the Institutional Animal Ethics Committee of Indian Institute of Toxicology, Pune, Maharashtra, India (Registration No. 15/1999/CPCSEA). All the animal experimentations were performed as per the Committee for the Purpose of Control and Supervision of Experiments on Animals and comply with Schedule Y in Drugs and Cosmetics Act (2nd amendment) Rules, 2005, Ministry of Health and Family Welfare, Government of India.

The test compound

The test compound IDM01 was prepared by high-performance liquid chromatography (HPLC) and the sample was provided by Indus Biotech Private Limited, Pune, Maharashtra, India. IDM01 was quantified by reported HPLC methods for assay of marker 4HI[20] and trigonelline.[32] The details of HPLC quantification assay are as follows: HPLC model JASCO LC 2000 with UV-2075; column: Reverse phase C-18 column, L1 (250 mm × 4.6 mm) as defined in USP30/NF25 with 5 µm particle size; detector: Ultraviolet (UV)/visible, injection volume: 20 µL; method time: 30 min; flow rate: 1.5 mL/min; detector: UV at 347 nm. The mobile phase used for 4HI detection was made up of Part A (0.05 mL of trifluoroacetic acid in 100 mL of water) and Part B (40 mL of acetonitrile) mixed at a ratio of 60:40. The mobile phase used for trigonelline detection was trifluoroacetic acid (60) with water: acetonitrile mixture (40). IDM01 was found to have total amino acids with 4HI (28.77%) and trigonelline (34.8%).

The fresh solution of IDM01 was prepared daily using demineralized water (10 mg dissolved in 100 ml) and administered in the volume of 10 mg/kg in rats for acute and subchronic toxicity studies.

Acute oral toxicity study

The acute toxicity study of IDM01 was performed in rats according to the OECD guideline 423.[33] The dose was formulated with distilled water as the vehicle. The dose volume was kept at 10 ml/kg and was administered orally. Rats were observed for 14 days for any sign of morbidity or mortality.

- G1 – Vehicle control (VC) group – Distilled water – 0 mg/kg – five rats per sex
- G2 – Treated group – IDM01 dissolved in distilled water – 2000 mg/kg – five rats per sex.

Subchronic (90-day repeated dose) toxicity study

The subchronic toxicity study was performed in accordance with the OECD guideline for the testing of Chemicals No. 408.[34] One-hundred and sixty rats, i.e., 80 male and 80 female healthy animals, were randomly divided into four groups of 15 animals per sex for dosing up to 90 days and 10 animals per sex as reversal groups for control and high dose. IDM01 dissolved in distilled water was administered to animals at the dose levels 0 mg/kg, 250 mg/kg, 500 mg/kg, and 1000 mg/kg based on AOT and based on preclinical studies. The highest dose of 1000 mg/kg is the recommended limit test dose for 90 days in rats. Oral (gavage) was given once daily for 90 consecutive days. The dose volume was 10 ml/kg body weight. The VC group was administered with the only vehicle.

- G1 – VC (Distilled water, 10 mL/kg, 90 days) - 15 animals/sex
- G2 – IDM01-250 (IDM01, 250 mg/kg, 90 days) - 15 animals/sex
- G3 – IDM01-500 (IDM01, 500 mg/kg, 90 days) - 15 animals/sex
- G4 – IDM01-1000 (IDM01, 1000 mg/kg, 90 days) - 15 animals/sex
- G1R – VC reversal (VC-R) group (Distilled water, 10 mL/kg, 119 days) - 10 animals/sex
- G4R – IDM01-1000-R – IDM01 Reversal group (IDM01, 1000 mg/kg for 90 days) - 90 days treatment of IDM01 followed by vehicle (Distilled water, 10 mL/kg, from day 90 to day 119) - 10 animals/sex.

All animals were observed daily for mortality. The ophthalmoscopic evaluation was carried out. The weight of each rat was recorded at weekly intervals throughout the study period. The consumption of the feed was also observed.

Urine and blood samples were collected for clinical evaluations (urinalysis, hematology, and serum chemistry) from rats during the last week of scheduled day of euthanasia (day 90 for G1 to G4 and day 119 for G1R and G4R) before the scheduled necropsy. For hematological investigations such as hemoglobin, red blood corpuscles, reticulocytes, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), total leukocyte count, and percentage cells in differential leukocyte count, parameters such as neutrophils (N), lymphocytes (L), eosinophils (E), monocytes (M), basophils (B), and prothrombin time were determined. Similarly, for serum biochemistry parameters, blood urea nitrogen, alanine aminotransferase (ALT), aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, creatine phosphokinase, lactate dehydrogenase, blood sugar (Sug), calcium (Ca), phosphorus (P), bilirubin (Bil), albumin (Alb), creatinine (Creat), sodium (Na), potassium (K), chlorine (Cl), cholesterol (Chol), and triglycerides (Trig) were determined. The rats were euthanized on day 91 (G1 to G4) and

day 119 (G1R and G4R) and underwent gross pathological examination for signs of toxicity via necropsy. All organs, mucosa, body cavities, etc., were examined for gross pathological changes. Major organs and major endocrine glands (pituitary, adrenal, thymus, thyroid, sex, etc.,) were weighed. The absolute values were recorded and relative values (i.e., percentage of the body weight) were calculated. Tissue samples from selected organs (heart, kidney, liver, lung, spleen, stomach, pancreas and skeletal muscle) from the VC (G1 and G1R) and 1000 mg/kg (G4 and G4R) were preserved, fixed, and stained for histopathological evaluation via light microscopy. The remaining tissues/organs were preserved in 10% neutral buffered formalin from control and different dose groups.

Mutagenicity (Ames test)

Mutagenic activity of IDM01 was performed in full compliance with the OECD guidelines for mutagenicity testing Test No: 471[35,36] and USFDA (40 CFR 79.68). The bacteria used were histidine auxotrophic strains of *Salmonella typhimurium* (TA97a, TA 98, TA 100, TA 1535 and TA 102). All the strains were stored in liquid nitrogen (−160°C), in cryocans. Strains were maintained on master plates and checked periodically for viable counts and genotype characteristics. For the assay, cultures were grown for 16 h in nutrient broth at 37°C. The cell density of cultures, 1×10^9 cells per ml, was assessed by cell count.

The preparation for mutagenicity experiment

IDM01 was dissolved in sterile water for injection and was tested in plate incorporation assay at a concentration of 5000.00, 1666.67, 555.55, 185.18, and 61.72 μg/plate. The concentrations selected were based on dose range finding study that used concentration range of 5000–1 μg/plate; there was no cytotoxicity observed up to the concentration of 5000 μg/plate. The number of groups formed was 7 and were kept for incubation for 48–72 h at 37°C. Minimal glucose agar medium (Vogel-Bonner minimal medium E), soft agar (overlay agar containing 0.5 mM histidine and biotin, Minimal glucose agar with biotin, Minimal glucose agar with histidine and biotin), minimal glucose agar (histidine, biotin, and ampicillin), nutrient agar, and nutrient broth mediums were prepared. The bacterial strains were cultured in nutrient broth. Vogel-Bonner minimal medium E was chosen as the selective medium. The top agar contained 0.6% agar, 0.5% NaCl, and 0.05 mM histidine-biotin.

Preparation of liver homogenate and S-9 mix fraction

Sprague-Dawley male rats (6–8 weeks old, 175–200 g), bred at the Indian Institute of Toxicology (Pune, Maharashtra, India), were used. They were used for the preparation of liver homogenate S-9 mix fraction. Mixed function oxidase systems in the rat liver were stimulated following an intraperitoneal injection of sodium phenobarbital (diluted in corn oil) at a dosage of 80 mg/kg/day for 5 consecutive days. On the 6th day of induction, following an overnight fasting, the rats were killed and livers aseptically removed.[37]

The preparation of liver homogenate was carried out with sterile glassware and solutions at 4°C. Excised samples of liver were transferred to a beaker containing 0.15 M KCl. After weighing and washing, livers were transferred to a beaker containing 0.15 M KCl (3 ml KCl: 1 g liver) minced with sterile scissors and homogenized. The homogenate was centrifuged for 10 min at 9000 r/min and the supernatant divided into small aliquots. These were stored at − 160°C in cryocan and tested with mutagen 2-aminofluorene (2-AF) before use.

The mutagenicity experiments with metabolic activation

Metabolic activation was performed using a cofactor supplemented postmitochondrial fraction (S9 fraction). The characterization of S-9 preparation was carried out by total protein estimation (3.83 g/100 ml, Lowry method) and sterility of S-9 preparation by examining the plates that had no microbial contamination. The S-9 mix was prepared immediately before its use in the experimental procedure. The microsomal enzyme reaction mixture contained S-9 (1.00 ml/10 ml), 0.4 M MgCl2, 1.65 M KCl salt solution (0.20 ml/10 ml), 1.0 M G-6-P (0.05 ml/10 ml), 0.1 M NADP (0.40/10 ml), 0.2 M Phosphate buffer pH 7.4 (5.00 ml/10 ml), and distilled water (3.35/10 ml).

The strain-specific positive control chemicals used in nonactivation studies are 4-Nitroquinolene-N-Oxide at 0.5 μg/plate for TA 98/TA 97a strains; methyl methanesulfonate at 1 μl for TA 100 and TA 102 strain, and sodium azide at 0.5 μg/plate for TA 1535 strain. The positive control chemicals used in the presence of metabolic activation are 2-AF at 10 μg/plate for TA 98, TA 97a and TA 100 whereas 2-aminoanthracene at 0.5 μg/plate and danthron at 30 μg/plate were used as positive control for TA 1535 and TA 102 strain. VC and phosphate buffer was used as negative control for nonactivated and activated procedure, respectively. Overnight cultures were prepared by transferring a colony from the appropriate master plate to 25 ml of nutrient broth. Inoculated cultures were kept incubated for 16 h at 37°C temperature to achieve a cell density of about 10^9 cells/ml.

The mutagenicity experiments without metabolic activation

For experiment without metabolic activation, a cell suspension of a tester strain (0.1 ml), 0.5 ml of phosphate buffer, and 100 μl of the vehicle or freshly prepared test substance were added to 2 ml of top agar with L-histidine-biotin. These mixtures were overlaid on the hardened surface of 25 ml minimal glucose agar plates. The plates were inverted and incubated at 37°C for 48 h. Each concentration was tested in triplicates. For the experiment with metabolic activation, above-mentioned procedures were repeated except that 0.5 ml of the S-9 mix was added to the top agar instead of 0.5 ml of phosphate buffer.

The plates were checked for sterility and scored for a uniform lawn of auxotrophs (his-) and the colonies of histidine revertants as the prototrophs. Histidine revertant colonies were counted manually. The mean number of histidine revertants for all the treatment groups was compared with the number of revertants in the respective VC group. The mutagenic activity of the test substance was considered for positive in case of increased concentration over the range tested and a reproducible increase at one or more concentrations in a number of revertant colonies per plate in at least one strain with or without metabolic activation system. The test substance was considered to be toxic if there was a decrease in the number of revertants and/or thinning or absence of background lawn.

Genotoxicity (chromosomal aberrations test)

Chromosome aberration assay of IDM01 was performed in full compliance with the OECD guidelines Test No: 473.[38] Heparinized peripheral blood was added to the culture medium, distributed as 10 ml to each vial and incubated at 37°C for 48 h. Cultures were exposed to the 25 mg/culture, 12.5 mg/culture, and 6.25 mg/culture of IDM01 without and with metabolic activation for the duration of 4 h with addition of 0.5 ml of S9 mix to the cultures with metabolic activation and addition of 0.5 ml of sodium phosphate buffer (pH 6.8) to cultures without metabolic activation. Cultures were centrifuged at 1000 rpm, and the medium was replaced with fresh culture medium. The cultures were further incubated for 24 h. Cell cultures were treated with 100 μl of distilled water for the solvent control group and with 20 μl of Benzo(a)pyrene-containing 2 μg, for positive mutagen with the addition of 0.5 ml of the S9 mix for metabolic activation.

In the second experiment, cultures without metabolic activation were exposed to the 25 mg/culture, 12.5 mg/culture, and 6.25 mg/culture of IDM01 continuously for 24 h. Cell cultures were treated with 100 μl

of distilled water for the solvent control group and with 10 µl of ethyl methanesulfonate containing 1200 µg, for positive mutagen without metabolic activation. Four hours after exposure, cell cultures with metabolic activation were washed and fed with fresh medium. Three hours before harvesting cell cultures were treated with colchicine at a final concentration of 2 µg/culture to arrest cells in metaphase stage. Cells were centrifuged at 1000 rpm for 10 min. The cell pellet was suspended in a hypotonic solution of 0.075 M KCl for 15 min at 37°C. Centrifugation was repeated. The cell pellet was suspended in chilled fixative (methanol acetic acid 3:1) by dropwise addition to avoiding clumping of cells. The fixed cells were centrifuged at 1000 rpm for 10 min. Supernatant was discarded, and cells were washed twice with fixative. Cells were then suspended in a small volume of fixative and mixed well. Drops of cell suspension were put on clean, chilled slides. Slides were air dried and stained with 5% Giemsa in 0.01 M phosphate buffer (pH 6.8) for 10 min. Analysis of structural chromosome aberrations was performed by a competent analyst, who was trained in the standard operating procedures at the test laboratory.

Statistical analysis

Statistical analysis was performed using SPSS analysis program (SPSS Inc., Chicago, Illinois, USA). All parameters' values were represented as mean ± standard deviation (SD) and analyzed using statistical methods. All the data were checked for normality. Data of each parameter were analyzed by one-way ANOVA followed by Dunnett's test to compare the difference between treated and control groups. The frequency of chromosome aberrations (aberration per cell) and percentage cells aberrated was analyzed using Cochran-Armitage trend test and Students *t*-test, respectively. The dose-dependent statistically significant increase in the number of structural chromosome aberrations and/or statistically significant and reproducible at any one concentration was considered to be a positive response. $P < 0.05$ was considered statistically significant.

RESULTS

Acute oral toxicity study

There were no mortality or treatment-related clinical signs of toxicity during the evaluation period. There was no weight loss during 14-day of the observation period. Finally, no treatment-related gross pathological changes were observed during necropsy. The results indicated the safety of single oral dose of IDM01 with median lethal dose (LD50) of >2000 mg/kg of body weight.

Repeated dose 90-day oral toxicity (subchronic) study

No mortality was observed in any of the groups during the 90-day evaluation period. There were no treatment-related clinical signs of toxicity or ophthalmoscopic or functional abnormalities during the evaluation period. There were no abnormalities in functional observation battery parameters during handling (piloerection, reaction to removal, reaction to handling, palpebral closure, eye examinations, lacrimation, salivation, mucous membrane) and open field test (appearance, gait, mobility, arousal, respiration, tonic or clonic or stereotype movement, vocalization, rearing, urination, defecation).

The average daily food consumptions in treatment groups did not show statistically significant differences as compared with respective VC groups during treatment or reversal period in male and female rats [Figure 1]. During the treatment period, body weights of male and female rats groups did not show a significant difference [Table 1]. However, male rats (but not female rats) from IDM01 reversal group (IDM01-1000-R group) showed a significant reduction in body weights as compared with VC-R ($P < 0.05$) during the reversal period.

Hematological [Tables 2 and 3] and biochemical observations [Tables 4 and 5] and urinalysis were recorded in male and female rats. All hematological and biochemical values were within normal biological and laboratory limits. The differences between the values were not consistent with treatment, doses, or period of observation (treatment/reversal) and so not treatment specific. For example, in male rats, IDM01-1000-R (but not any other treatment group) showed a significant increase ($P < 0.05$) in MCH values as compared with VC-R group. However, such increase was not found in female rats. On the other hand, IDM01-250 and IDM01-500 group showed a significant ($P < 0.05$) increase in MCHC in female but not in male rats. Similarly, IDM01-1000-R group showed a significant ($P < 0.01$) decrease in platelet count in female but not male rats.

In the case of liver function-related biochemical parameters, ALT values of IDM01-250 group from male (but not in female) rats showed

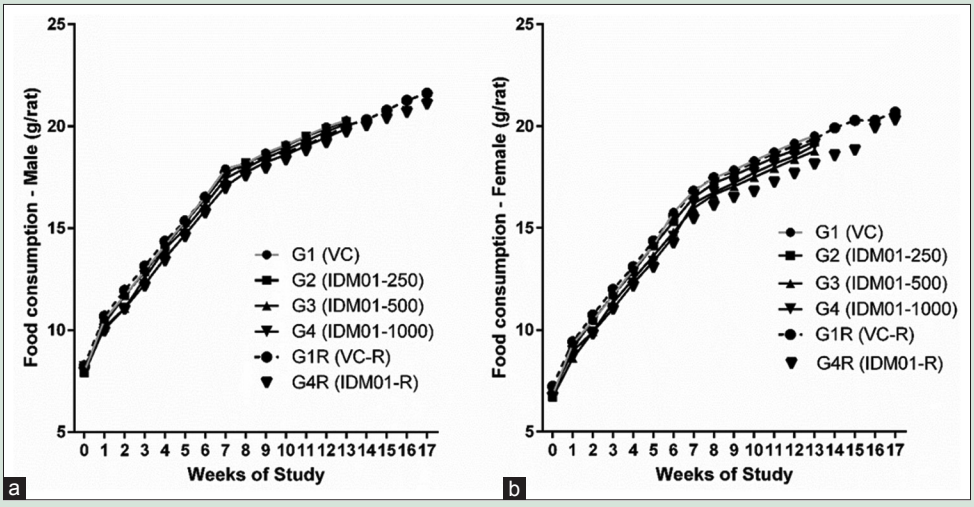


Figure 1: Effect of IDM01 on food consumption in (a) male and (b) female rats during 90-day repeated dose toxicity study. Data are expressed as mean ± standard deviation

Table 1: Effect of IDM01 on body weights of rats during 90-day repeated dose toxicity study

Weeks	Group					
	G1	G2	G3	G4	G1R	G4R
	VC	IDM01-250	IDM01-500	IDM01-1000	VC-R	IDM01-1000-R
Male						
1	93.09±8.30	92.58±7.51	93.65±7.48	94.34±7.31	92.00±7.78	94.82±7.07
12	376.61±13.57	376.36±16.47	385.85±16.30	376.42±23.04	380.03±12.10	381.32±22.37
13	380.88±12.00	381.09±16.56	390.99±15.43	381.38±22.75	385.34±11.68	386.10±21.91
17					404.42±8.20	390.38±14.52*
Female						
1	93.52±8.01	93.11±6.96	94.14±6.76	95.16±5.89	92.68±7.72	95.71±5.67
12	266.03±16.58	265.67±10.91	265.60±11.13	263.91±11.64	264.21±11.55	262.43±8.38
13	268.57±16.55	268.12±10.58	268.31±10.95	266.47±10.99	266.67±10.98	264.87±8.26
17					278.62±10.18	277.58±6.70

n=15 per group per sex. Data were represented as mean±SD. Data were analyzed by unpaired *t*-test. **P*<0.05 as compared with respective parameter value of respective VC group. SD: Standard deviation; VC: Vehicle control; VC-R: VC-reversal

Table 2: Effect of IDM01 on hematological parameters during 90-day repeated dose toxicity study (male rats)

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	IDM01-250	IDM01-500	IDM01-1000	VC-R	IDM01-1000-R
Hb (g%)	15.56±1.30	15.67±0.82	15.17±1.06	15.97±0.76	14.90±0.69	15.24±1.31
RBC (×10^6/μL)	8.82±0.66	8.96±0.50	8.66±0.71	9.02±0.54	8.92±0.31	8.81±0.67
Reticulocytes (%)	1.59±0.55	1.47±0.52	1.55±0.51	1.53±0.50	1.72±0.90	1.38±0.53
HCT (%)	44.97±3.61	45.46±2.31	43.78±2.84	45.91±2.33	43.86±1.39	44.08±3.72
MCV (μm^3)	50.95±1.50	50.79±1.60	50.66±1.68	50.95±1.14	49.20±1.07	50.00±0.89
MCH (pg)	17.63±0.44	17.51±0.59	17.55±0.65	17.71±0.46	16.72±0.59	17.28±0.33*
MCHC (%)	34.61±0.38	34.47±0.32	34.65±0.47	34.79±0.34	33.98±0.69	34.60±0.51
Platelets (×10^3/μL)	424.33±96.43	410.80±82.29	447.67±72.08	460.53±70.81	320.20±70.60	296.80±47.69
TLC	12.89±3.39	10.79±4.48	10.91±2.55	14.90±3.46	11.86±1.69	9.30*±1.91
PT (s)	14.47±3.31	15.00±3.63	14.40±3.48	13.93±3.73	14.60±3.05	15.80±4.09
DLC						
N (%)	21.00±3.27	21.33±3.75	21.07±3.17	21.20±3.59	21.00±3.39	20.80±4.15
L (%)	75.53±3.00	75.80±3.69	75.73±3.13	75.47±3.44	75.60±3.51	75.60±3.85
E (%)	1.07±0.88	1.00±0.85	1.07±0.88	1.00±0.76	1.20±0.84	1.40±0.89
M (%)	2.40±0.83	1.87±0.99	2.13±0.83	2.33±0.82	2.20±0.84	2.20±0.84
B (%)	0	0	0	0	0	0

n=15 per group. Data were represented as mean±SD; data were analyzed by unpaired *t*-test. **P*<0.05 as compared with respective parameter value of respective VC group. VC: Vehicle control; VC-R: VC-reversal; SD: Standard deviation; Hb: Hemoglobin; RBC: Red blood corpuscles; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; TLC: Total leukocyte count; PT: Prothrombin time; DLC: Differential leukocyte count; N: Neutrophils; L: Lymphocytes; E: Eosinophils; M: Monocytes; B: Basophils

Table 3: Effect of IDM01 on hematological parameters during 90-day repeated dose toxicity study (female rats)

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	IDM01-250	IDM01-500	IDM01-1000	VC-R	IDM01-1000-R
Hb (g%)	15.22±1.63	15.18±0.93	14.90±0.93	14.71±0.67	14.12±1.14	14.22±0.83
RBC (×10^6/μL)	8.28±0.67	8.27±0.64	8.09±0.44	8.07±0.45	7.90±0.63	7.81±0.53
Reticulocytes (%)	1.59±0.53	1.50±0.46	1.51±0.44	1.48±0.48	1.46±0.44	1.40±0.43
HCT (%)	43.61±4.34	43.01±2.63	42.20±2.56	42.22±2.05	41.18±2.52	41.02±2.30
MCV (μm^3)	52.65±1.81	52.05±1.62	52.11±1.02	52.34±1.56	52.36±4.65	52.54±0.69
MCH (pg)	18.40±0.80	18.36±0.59	18.41±0.41	18.25±0.63	18.02±2.01	18.18±0.36
MCHC (%)	34.91±0.46	35.29±0.27*	35.32±0.36*	34.87±0.36	34.32±1.04	34.58±0.60
Platelets (×10^3/μL)	405.27±70.95	417.73±108.50	394.20±73.93	399.60±79.44	444.40±60.69	360.80±48.11**
TLC	13.59±2.63	11.73±4.50	11.55±5.15	14.24±4.86	8.80±3.08	9.30±3.00
PT (s)	15.20±3.43	15.40±3.27	14.53±3.56	14.53±3.44	15.00±3.87	14.80±3.11
DLC						
N (%)	21.13±3.85	21.00±3.57	21.07±3.43	20.87±4.24	22.40±3.21	21.20±3.03
L (%)	75.60±3.33	75.87±3.60	75.53±3.11	75.87±3.66	74.60±3.05	75.20±2.77
E (%)	1.13±0.74	1.07±0.80	1.07±0.80	1.07±0.88	0.80±0.84	1.20±0.84
M (%)	2.13±0.74	2.07±0.88	2.33±0.72	2.20±0.86	2.20±0.84	2.40±0.89
B (%)	0	0	0	0	0	0

n=15 per group. Data were represented as mean±SD; data were analyzed by unpaired *t*-test, **P*<0.05, ***P*<0.01 as compared with respective parameter value of respective VC group. VC: Vehicle control; VC-R: VC-reversal; SD: Standard deviation; Hb: Hemoglobin; RBC: Red blood corpuscles; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; TLC: Total leukocyte count; PT: Prothrombin time; DLC: Differential leukocyte count; N: Neutrophils; L: Lymphocytes; E: Eosinophils; M: Monocytes; B: Basophils

Table 4: Effect of IDM01 on blood chemistry on during 90-day repeated dose toxicity study (male rats)

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	IDM01-250	IDM01-500	IDM01-1000	VC-R	IDM01-1000-R
Liver function test						
ALT (IU/L)	42.60±5.30	36.40±6.46*	39.60±5.53	39.67±5.85	36.00±8.09	40.40±7.70
AST (IU/L)	63.73±4.67	62.33±4.79	62.40±4.29	62.20±4.57	62.80±7.09	61.60±2.30
ALP (IU/L)	70.93±5.95	70.53±5.48	71.67±5.60	108.07±10.66**	70.60±6.84	71.60±5.08
GGT (U/L)	16.27±3.97	16.60±4.15	17.73±4.17	14.80±4.20	15.20±5.07	16.20±2.77
Bilirubin (mg%)	0.67±0.08	0.70±0.08	0.69±0.08	0.64±0.06	0.61±0.05	0.68±0.05*
Total protein (g%)	7.23±0.68	7.69±0.42	7.65±0.58	7.66±0.49	7.51±0.45	7.29±0.42
Albumin (g%)	3.66±0.31	3.73±0.24	3.78±0.16	3.64±0.30	3.58±0.32	3.54±0.20
Kidney function test						
Creatinine (mg%)	0.99±0.14	0.93±0.08	0.92±0.09	0.94±0.10	0.94±0.04	0.94±0.07
CK (IU/L)	63.07±5.85	61.13±4.19	59.20±3.86	59.20±5.94	61.20±4.32	58.80±6.18
BUN (mg%)	35.07±3.73	33.07±4.65	35.07±3.71	35.00±5.17	38.40±6.43	38.80±4.60
Serum electrolytes						
Ca (mg%)	2.33±0.09	2.48±0.10**	2.37±0.10	2.23±0.11*	2.37±0.11	2.49±0.08*
P (mg%)	4.09±0.56	4.12±0.42	4.10±0.37	4.33±0.37	4.02±0.36	4.18±0.47
Na (mmol/l)	131.03±1.52	132.55±1.30**	133.21±0.98**	132.96±0.88**	128.52±1.61	128.27±0.97
K (mmol/l)	4.72±0.34	4.55±0.54	4.23±0.53*	4.21±0.32**	4.14±0.38	4.50±0.35
Cl (mmol/l)	103.13±1.20	101.24±1.00**	102.97±1.87	102.89±1.24	108.59±2.13	108.67±1.00
Metabolic function test						
FPG (mg%)	95.27±9.32	94.13±8.69	101.07±8.00	91.67±7.31	94.40±9.96	102.20±7.46
Cholesterol (mg%)	63.47±5.57	64.67±5.60	63.73±4.96	62.20±3.91	65.80±2.95	62.00±3.81*
Triglyceride (mg%)	111.87±9.05	103.33±8.81*	110.60±8.21	113.27±8.15	105.00±9.97	110.80±6.76
LDH (IU/L)	368.13±26.83	353.47±32.11	364.73±31.52	368.00±25.47	371.00±19.01	374.20±31.60

n=15 per group. Data were represented as mean±SD; data were analyzed by unpaired *t*-test, **P*<0.05, ***P*<0.01 as compared of respective parameter value of respective VC group. VC: Vehicle control; VC-R: VC-reversal; SD: Standard deviation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltransferase; BUN: Blood urea nitrogen; Ca: Calcium; P: Phosphorus; Na: Sodium; K: Potassium; Cl: Chlorine; FPG: Fasting plasma glucose; LDH: Lactate dehydrogenase; CK: Creatine kinase

Table 5: Effect of IDM01 on blood chemistry on during 90-day repeated dose toxicity study (female rats)

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	IDM01-250	IDM01-500	IDM01-1000	VC-R	IDM01-1000-R
Liver function test						
ALT (IU/L)	42.00±5.54	41.27±4.73	39.07±5.42	41.07±4.13	37.40±4.34	35.40±3.36
AST (IU/L)	64.60±4.61	62.40±5.32	62.93±3.28	59.80±5.12*	58.80±4.15	63.20±4.97
ALP (IU/L)	72.33±5.72	71.27±5.34	71.20±5.75	110.07±8.00**	69.80±10.03	72.00±5.66
GGT (U/L)	15.93±4.40	16.13±4.84	14.27±5.12	15.47±4.56	17.00±6.28	13.40±3.21
Bilirubin (mg%)	0.68±0.06	0.68±0.08	0.64±0.06	0.66±0.08	0.69±0.08	0.63±0.04
Total protein (g%)	7.25±0.77	7.56±0.64	7.14±0.66	7.49±0.72	7.46±0.48	7.56±0.58
Albumin (g%)	3.73±0.22	3.70±0.27	3.60±0.29	3.59±0.31	3.66±0.32	3.86±0.18
Kidney function test						
Creatinine (mg%)	0.95±0.12	0.94±0.11	1.00±0.13	0.97±0.12	1.03±0.12	0.93±0.03
CK (IU/L)	59.93±4.48	61.20±4.97	62.07±5.60	56.80±4.38	62.40±3.78	59.20±3.63
BUN (mg%)	34.00±5.35	32.67±3.27	33.87±3.38	35.20±3.86	34.80±5.93	31.80±3.42
Serum electrolytes						
Ca (mg %)	2.46±0.10	2.44±0.08	2.50±0.12	2.53±0.15	2.35±0.08	2.35±0.21
P (mg%)	4.17±0.44	3.96±0.54	4.19±0.39	4.09±0.43	4.22±0.39	4.34±0.42
Na (mmol/l)	132.38±1.09	134.57±1.12**	133.48±1.45*	125.58±4.79**	136.04±1.02	135.41±0.55
K (mmol/l)	4.44±0.43	4.60±0.42	4.43±0.45	4.59±0.50	3.83±0.43	3.15±0.39**
Cl (mmol/l)	101.26±0.82	101.60±0.94	100.61±1.20	103.78±3.41**	102.07±0.78	102.85±0.75
Metabolic function test						
FPG (mg%)	94.73±10.34	91.60±10.13	92.73±11.29	92.33±11.57	93.20±10.08	99.60±8.85
Cholesterol (mg%)	63.47±4.64	64.07±6.12	61.87±5.55	61.27±3.81	63.40±3.36	66.60±3.36
Triglyceride (mg%)	111.20±6.30	113.27±5.89	108.87±9.54	107.87±8.98	109.60±7.70	116.00±3.67*
LDH (IU/L)	358.33±23.19	363.27±29.59	362.93±25.69	365.60±24.51	374.80±11.61	379.00±12.12

n=15 per group. Data were represented as mean±SD; data were analyzed by unpaired *t*-test. **P*<0.05; ***P*<0.01 as compared of respective parameter value of respective VC group. VC: Vehicle control; VC-R: VC-reversal; SD: Standard deviation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltransferase; BUN: Blood urea nitrogen; Ca: Calcium; P: Phosphorus; Na: Sodium; K: Potassium; Cl: Chlorine; FPG: Fasting plasma glucose; LDH: Lactate dehydrogenase; CK: Creatine kinase

a significant decrease. ALP values of IDM01-1000 group showed a significant increase from their VC group in male and female rats. Serum electrolyte data showed significant changes in Ca (IDM01-250, IDM01-1000, and IDM01-1000-R group but not IDM01-500 group vs. VC) in male rats whereas differences in female rats were not significant. Serum Na levels showed a significant increase in IDM01-250,

IDM01-500, and IDM01-1000 groups (vs. VC group) in male but not female rats. In female rats, serum Na levels of IDM01-250 and IDM01-500 group showed a significant increase (vs. VC group), but IDM01-1000 group showed a significant decrease (vs. VC group). Serum K levels of IDM01-500 and IDM01-1000 showed a significant decrease in male rats but not in female rats. On the other hand, serum K levels of IDM01-1000-R group showed a significant decrease (vs. VC-R group) in female but not in male rats. Serum cholesterol levels from IDM01-1000-R showed a significant decrease (vs. VC-R group) in male but not in female rats. Serum triglyceride levels of IDM01-250 group showed a significant decrease (vs. VC group) in male but in female rats. On the other hand, serum triglyceride levels from IDM01-1000-R showed a significant increase (vs. VC-R group) in female rats but not in male rats. However, these changes did not show correlation with treatment or dose changes except elevated levels of ALP in IDM01-1000 group in male and female rats.

Urine analysis conducted at the end of the dosing period in week 13 (on day 85, 86, 89, 90, and 91) and at the end of the recovery period. There was no significant difference observed between IDM01 treatment and control animals revealed no abnormality attributable to the treatment.

No treatment-related gross pathological changes were observed in any organ of the test animals during necropsy. The data of weights of organs in IDM01 treated rats during treatment and reversal period in male and female rats are presented Tables 6 and 7. None of the IDM01 treated group showed significant difference during treatment or reversal period as compared with corresponding VC group.

The findings from histological examination of sections of vital organs and reproductive system organs are shown in Figures 2 and 3. Histopathological examination revealed minimal to mild, focal lymphocytic infiltration and/or minimal focal necrosis in the liver; minimal focal lymphocytic infiltration and/or necrosis in the kidneys; minimal to mild pneumonitis, congestion, alveolar hemorrhages and/or histiocytosis in the lungs; minimal to mild lymphocytic infiltration in trachea; minimal to mild focal lymphocytic infiltration or necrosis in the brain; minimal vacuolation, sinusoidal dilatation or accessory adrenocortical tissue present in adrenals; minimal focal hemorrhages or lymphoid necrosis in thymus; minimal focal hemorrhages in the lymph nodes; mild focal cysts in pituitary; minimal to mild, focal to multifocal, ultimobranchial cysts in thyroid; minimal focal glandular dilatation in the stomach or minimal to mild eosinophilic infiltration and/or dilatation in the uterus in male or female animals from VC and IDM01-1000 group. None of the IDM01-treated group showed significant difference during treatment or reversal period as compared with corresponding VC group. The finding from histological examination of sections of organs is presented in Table 8 and representative photomicrographs of the section from vital and reproductive organs are presented in Figures 2 and 3. The changes observed in both VC and IDM01-1000 treatment groups were similar and comparable in both sexes and hence considered as incidental, congenital, and spontaneous. Based on the results of the present study, the no observed adverse effect level (NOAEL) is 500 mg/kg/day.

Mutagenicity study

The bacterial background lawn was comparable with that of the respective VC plate up to the highest concentration of 5000 μg/plate. No substantial increases in the revertant colony count in any of the five strains were reported at any of the test concentrations in the presence or absence of

Table 6: Effect of IDM01 on relative organ weights (g) of during 90-day repeated dose toxicity study (male rats)

Organ	G1	G2	G3	G4	G1R	G4R
	VC	IDM01-250	IDM01-500	IDM01-1000	VC-R	IDM01-1000-R
Brain	0.567±0.038	0.582±0.030	0.554±0.030	0.572±0.027	0.563±0.027	0.567±0.019
Liver	2.981±0.265	2.984±0.254	3.101±0.390	3.113±0.290	3.138±0.295	3.311±0.234
Kidneys	0.723±0.081	0.757±0.091	0.769±0.074	0.775±0.066	0.793±0.053	0.826±0.026
Adrenals	0.0139±0.0024	0.0127±0.0017	0.0140±0.0023	0.0128±0.0021	0.013±0.002	0.014±0.002
Testes	0.868±0.090	0.896±0.051	0.851±0.078	0.886±0.075	0.852±0.057	0.881±0.094
Heart	0.332±0.039	0.337±0.026	0.338±0.036	0.343±0.033	0.343±0.033	0.366±0.042
Spleen	0.373±0.073	0.359±0.086	0.381±0.095	0.426±0.080	0.354±0.052	0.319±0.062
Lungs	0.470±0.054	0.476±0.064	0.487±0.071	0.481±0.070	0.501±0.092	0.480±0.067
Thymus	0.072±0.028	0.069±0.027	0.072±0.025	0.078±0.021	0.056±0.013	0.062±0.013
Epididymis	0.348±0.035	0.374±0.034	0.372±0.042	0.351±0.025	0.349±0.020	0.376±0.039

n=15 per group. Data were represented as mean±SD, data were analyzed by unpaired *t*-test, None of the values were significant for G2, G3, and G4 versus G4R and G1R. SD: Standard deviation; VC: Vehicle control; VC-R: VC-reversal

Table 7: Effect of IDM01 on relative organ weights (g) of during 90-day repeated dose toxicity study (female rats)

Organ	G1	G2	G3	G4	G1R	G4R
	VC	IDM01-250	IDM01-500	IDM01-1000	VC-R	IDM01-1000-R
Brain	0.792±0.034	0.787±0.032	0.807±0.024	0.806±0.035	0.783±0.034	0.809±0.045
Liver	3.053±0.247	2.897±0.239	3.055±0.306	3.172±0.309	3.007±0.115	3.042±0.154
Kidneys	0.745±0.057	0.729±0.064	0.739±0.067	0.724±0.067	0.716±0.032	0.714±0.071
Adrenals	0.0272±0.0040	0.0253±0.0028	0.0262±0.0047	0.0267±0.0055	0.023±0.003	0.027±0.003
Ovaries	0.0338±0.0036	0.0299±0.0076	0.0308±0.0079	0.0306±0.0052	0.0260±0.0045	0.0219±0.0059
Heart	0.364±0.029	0.346±0.026	0.356±0.036	0.366±0.032	0.374±0.017	0.377±0.031
Spleen	0.433±0.083	0.387±0.068	0.387±0.074	0.421±0.131	0.412±0.092	0.436±0.082
Lungs	0.580±0.063	0.576±0.067	0.640±0.082	0.602±0.101	0.656±0.098	0.636±0.055
Thymus	0.100±0.027	0.099±0.030	0.098±0.029	0.109±0.022	0.081±0.019	0.086±0.012
Uterus	0.182±0.044	0.190±0.053	0.184±0.049	0.197±0.043	0.179±0.041	0.181±0.047

n=15 per group. Data were represented as mean±SD, data were analyzed by unpaired *t*-test, None of the values were significant for G2, G3, and G4 versus G4R and G1R. SD: Standard deviation; VC: Vehicle control; VC-R: VC-reversal

Table 8: Effect of IDM01 on incidence and severity of histopathological findings of the animals from the control and high-dose groups (1000 mg/kg)

Group	Sex			
	Male		Female	
	VC (mg/kg)	IDM01-1000 (mg/kg)	VC (mg/kg)	IDM01-1000 (mg/kg)
Number of animals	15	15	15	15
Adrenals				
Vacuolation	1	NAD	NAD	1
Dilatation, sinusoidal	NAD	NAD	NAD	1
Aorta	NAD	NAD	NAD	NAD
Brain				
Lymphocytic infiltration	2	4	NAD	NAD
Necrosis	NAD	NAD	1	1
Cecum	NAD	NAD	NAD	NAD
Colon	NAD	NAD	NAD	NAD
Duodenum	NAD	NAD	NAD	NAD
Epididymis	NAD	NAD	X	X
Eyes	NAD	NAD	NAD	NAD
Heart	NAD	NAD	NAD	NAD
Ileum	NAD	NAD	NAD	NAD
Jejunum	NAD	NAD	NAD	NAD
Kidneys				
Lymphocyte infiltration	1	4	1	2
Necrosis	1	1	NAD	NAD
Liver				
Lymphocyte infiltration	9	9	8	10
Necrosis	0	2	2	1
Lungs				
Pneumonitis	15	15	14	15
Hemorrhages	4	3	1	1
Congestion	NAD	NAD	1	0
Histiocytosis	0	2	NAD	NAD
Lymph node				
Hemorrhages	NAD	1	NAD	NAD
Ovaries	X	X	NAD	NAD
Skin	NAD	NAD	NAD	NAD
Esophagus	NAD	NAD	NAD	NAD
Pancreas	NAD	NAD	NAD	NAD
Pituitary				
Cysts	NAD	1	NAD	NAD
Prostate	NAD	NAD	X	X
Rectum	NAD	NAD	NAD	NAD
Sciatic nerve	NAD	NAD	NAD	NAD
Seminal vesicles	NAD	NAD	X	X
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	1	NAD	NAD	NAD
Testes	NAD	NAD	X	X
Trachea				
Lymphocytic infiltration	5	6	4	5
Thymus				
Necrosis, lymphoid	1	NAD	NAD	NAD
Hemorrhages	NAD	1	NAD	NAD
Thyroid/parathyroid				
Ultimobranchial cysts	1	2	4	2
Urinary bladder	NAD	NAD	NAD	NAD
Uterus	X	X		
Eosinophilic infiltration			15	15
Dilatation			NAD	1

n=15 per group. X: Organs not subjected to histopathological examination due to sex difference; NAD: No abnormality detected; VC: Vehicle control

metabolic activation (S9 mix). Positive controls resulted in significant increases in the revertant count. The spontaneous reversion rates in the negative and positive control were within the range of historical data. There was no biologically relevant increase in the revertant counts in any of the five tester strains preincubated with the test item. The results of these investigations suggest that under the experimental conditions,

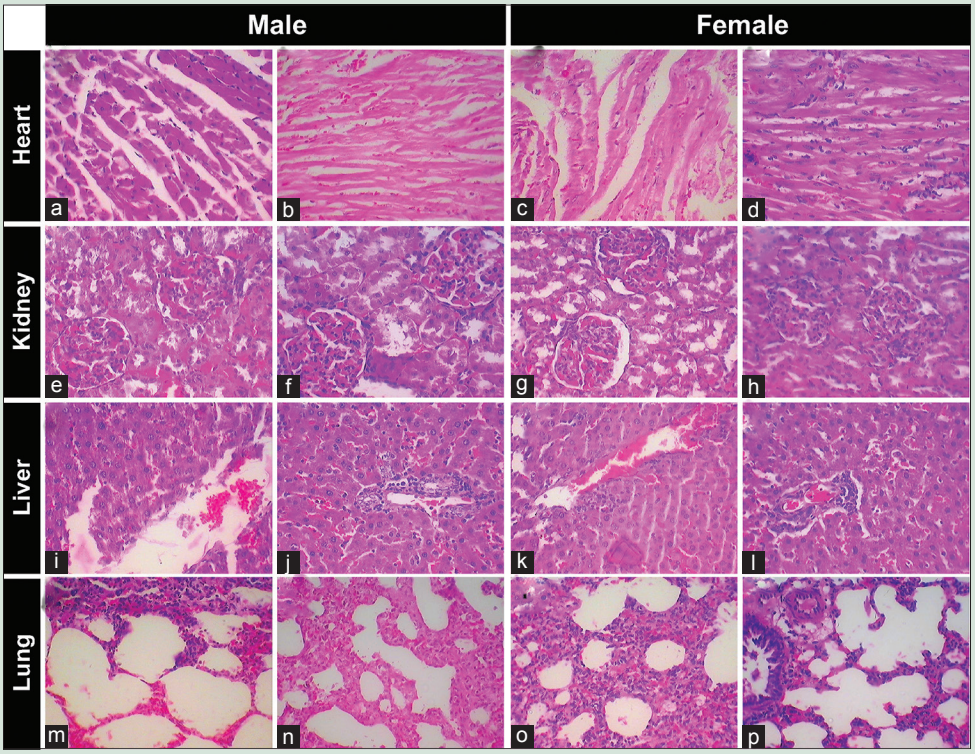


Figure 2: Histology of vital organs namely heart (a-d), kidney (e-h), liver (i-l), and lung (m-p) tissues in rats during 90-day repeated dose toxicity study. Photomicrographs from representative rats from respective groups: VC (a, e, i, m, c, g, k, and o), IDM01-1000 (b, f, j, n, d, h, l, and p) (×40)

Table 9: Effect of IDM01 on Ames test in *Salmonella typhimurium* TA1535, TA97, TA98, TA100, and TA102, with (+S9) and without (−S9) metabolic activation (Experiment 1)

Treatment	Dose (μg/plate)	S9	Mean number of revertants				
			TA1535	TA97a	TA98	TA100	TA102
IDM-01	5000	Negative	9.00±1.00	125.67±3.06	26.33±1.53	151.00±8.00	265.00±4.00
		Positive	11.33±0.58	146.33±11.37	35.33±1.53	167.67±3.51	292.67±5.51
	1666.67	Negative	10.00±1.00	134.00±3.61	34.67±4.04	161.67±5.69	274.00±6.00
		Positive	12.33±0.58	152.33±6.03	46.00±2.00	179.67±4.16	300.33±3.06
	555.55	Negative	11.67±1.53	141.67±4.04	39.67±2.08	168.33±6.11	284.67±9.71
		Positive	13.33±0.58	158.67±5.51	57.67±1.53	189.00±2.65	309.00±5.00
	185.18	Negative	13.67±1.53	150.33±3.51	47.33±2.52	177.33±3.06	292.33±7.77
		Positive	15.00±1.00	169.33±4.04	65.67±2.08	200.33±7.09	322.00±5.57
	61.72	Negative	14.67±1.53	160.67±1.53	60.33±2.52	190.33±4.04	298.00±6.56
		Positive	16.33±1.53	177.00±5.57	75.00±3.61	214.00±3.61	330.33±3.51
Distilled water (solvent control)	100 μl	Negative	12.00±1.00	140.00±3.00	39.33±2.52	168.33±3.79	281.00±6.24
		Positive	15.00±1.00	163.67±5.51	50.67±2.08	187.33±4.51	311.33±2.52
MMS (μl/plate)	1.0	Negative	-	-	-	2155.00±223.22	5530.00±74.32
4 NQNO	0.5	Negative	-	528.67±56.75	292.67±24.11	-	-
Sodium azide	0.5	Negative	1128.00±132.53	-	-	-	-
2-AF	10.0	Positive	-	1551.00±52.85	1304.33±159.43	1259.33±170.77	-
Danthron	30.0	Positive	-	-	-	-	1321.33±149.00
2-AA	0.5	Positive	207.33±44.79	-	-	-	-
Positive control			9.00±1.00	125.67±3.06	26.33±1.53	151.00±8.00	265.00±4.00

n=3, data were represented as mean±SD. Data were analyzed by unpaired *t*-test. SD: Standard deviation, 4 NQNO: 4-nitroquinolene-N-oxide; MMS: Methyl methanesulfonate; 2-AF: 2-aminofluorene; 2-AA: 2-aminoanthracene

IDM01 did not induce gene mutation by pair changes or frameshifts in the genome of the strains used [Tables 9 and 10].

Genotoxicity study (chromosomal aberrations test)

The mitotic indices at 25 mg/culture concentration were 2.45, 2.50, and 2.45, 2.58 in the solvent control group without and with metabolic activation, respectively. Thus, the mitotic activity at 25 mg/culture concentration did not reduce and was comparable with solvent control. The number of chromosome aberrations per cell was 0.005, 0.01, and 0.005 at 25 mg/culture, 12.5 mg/culture, and 6.25 mg/culture concentrations whereas it was 0.005 and 0.005 in negative and solvent control. The percentage cells aberrated were 0.5, 1.0, and 1.5 at 25 mg/culture, 12.5 mg/culture, and 6.25 mg/culture concentrations, respectively, whereas it was 0.5 and 0.5 in negative and solvent control.

Table 10: Effect of IDM01 on Ames test in *Salmonella typhimurium* TA1535, TA97, TA98, TA100, and TA102, with (+S9) and without (−S9) metabolic activation (Experiment 2)

Treatment	Dose (µg/plate)	S9	Mean number of revertants				
			TA1535	TA97a	TA98	TA100	TA102
IDM-01	5000	Negative	9.00±1.00	133.33±3.21	21.33±2.52	163.00±5.00	243.33±6.66
		Positive	12.00±1.00	142.33±2.52	33.00±3.61	175.00±4.00	271.67±3.51
	1666.67	Negative	10.33±0.58	142.00±4.58	27.00±2.65	169.00±3.61	251.33±5.86
		Positive	13.00±1.00	153.67±4.16	40.67±2.52	184.00±4.00	290.00±10.15
	555.55	Negative	12.00±1.00	151.00±3.61	34.67±2.52	181.00±4.36	260.33±7.02
		Positive	14.33±0.58	166.67±3.51	46.00±4.58	198.67±4.51	295.33±6.51
	185.18	Negative	13.67±1.53	168.67±4.16	42.67±6.03	191.33±7.37	269.33±7.23
		Positive	16.67±1.53	187.67±5.51	55.67±5.13	214.33±8.33	306.00±7.00
	61.72	Negative	15.33±1.53	187.33±2.52	48.00±5.00	202.67±7.57	282.67±4.51
		Positive	18.33±2.08	200.67±2.52	64.67±3.51	221.00±4.58	313.33±4.04
Distilled water (solvent control)	100 µl	Negative	13.00±1.00	143.67±4.04	30.33±1.53	175.00±4.58	260.00±7.00
		Positive	16.33±1.53	156.67±3.51	43.00±3.00	192.33±6.03	304.33±18.18
MMS (µl/plate)	1.0	Negative	-	-	-	2352.00±116.05	4632.33±589.23
4 NQNO	0.5	Negative	-	559.33±53.91	334.00±39.34	-	-
Sodium azide	0.5	Negative	1119.00±139.47	-	-	-	-
2-AF	10.0	Positive	-	1463.33±128.13	1352.33±146.41	1278.00±91.41	-
Danthron	30.0	Positive	-	-	-	-	1267.67±47.04
2-AA	0.5	Positive	168.00±20.52	-	-	-	-
Positive control			9.00±1.00	133.33±3.21	21.33±2.52	163.00±5.00	243.33±6.66

n=3, data were represented as mean±SD. Data were analyzed by unpaired *t*-test. SD: Standard deviation; NQNO: 4-nitroquinolene-N-oxide; MMS: Methyl methanesulfonate; 2-AF: 2-aminofluorene; 2-AA: 2-aminoanthracene

Table 11: Effect of IDM01 on mitotic index in structural chromosome aberration analysis, with (+S9) and without (−S9) metabolic activation

Treatment	Concentration (mg/culture)	S9	Total Number of cells	Number of dividing cells	Mitotic index
Duration of exposure (4 h)					
Negative control	-	Negative	2015	53	2.63
		Positive	2020	52	2.57
Solvent control	-	Negative	2010	53	2.64
		Positive	2000	50	2.50
IDM01	12.5	Negative	2002	50	2.50
		Positive	2014	49	2.43
	25	Negative	2011	50	2.49
		Positive	2019	50	2.48
	50	Negative	2000	43	2.15
		Positive	2009	41	2.04
Duration of exposure (24 h)					
Negative control	-	Negative	2001	51	2.55
		Positive	2012	51	2.53
Solvent control	-	Negative	2000	49	2.45
		Positive	2004	48	2.39
IDM01	12.5	Negative	2007	48	2.39
		Positive	2014	50	2.48
	25	Negative	2006	52	2.59
		Positive	2001	49	2.45
	50	Negative	2002	42	2.10
		Positive	2007	44	2.19

The aberrations induced were of chromatid-type only. Hence, IDM01 did not induce polyploidy and endoreduplication [Tables 11 and 12].

DISCUSSION

The present study evaluated IDM01, a composition from fenugreek seeds for acute and subchronic, mutagenicity, and genotoxicity potential in rats. The safety studies on natural food supplements help determine the dose levels for their safe human use as food or dietary supplement over a prolonged period.[26,28,29] Typically, safety studies consist of AOT study which is used to establish target organ toxicity on single oral administration at the very high dose.[39] The subacute (28-day repeated dose) or subchronic (90-day repeated dose studies) studies are being utilized to evaluate the toxicity of test substance over a long period.[29] Repeated dose studies help anticipate the adverse effects of a test substance qualitatively and quantitatively in laboratory animals.[40] The guidelines issued by OECD are considered as standard for toxicity assessments of chemicals and well-accepted by regulatory agencies worldwide. Therefore, the present study followed OECD guidelines.

In the present investigation, single oral administration of IDM01 showed median lethal dose (LD_{50}) of more than 2000 mg/kg during AOT study. Hence, we conducted subchronic 90-day repeated dose toxicity study using dose levels of 250, 500, and 1000 mg/kg. In the OECD guidelines, for subchronic oral toxicity assessment, the adverse effect of the test substances has been determined using various hematological, biochemical, and histopathological analysis which provides the insight

Table 12: Effect of IDM01 on chromosome break analysis in structural chromosome aberration analysis, with (+S9) and without (−S9) metabolic activation

Treatment	Concentration (mg/culture)	S9	Total number of meta-phases analyzed	Total number of aberrations	Type of aberrations			Total number of cells with aberrations	Aberrations per cell[b]	Percentage cells with aberrations[c]
					Gap[a]	Chromatid	Chromosome			
Duration of exposure (4 h)										
Negative control	0.0	Negative	200	0	0	0	0	0	0	0
		Positive	200	1	0	1	0	1	0.005	0.5
Solvent control	100 µl	Negative	200	0	0	0	0	0	0	0
		Positive	200	2	1	2	0	2	0.01	1.0
IDM01	12.5	Negative	200	1	1	1	0	1	0.005	0.5
		Positive	200	2	0	2	0	2	0.01	1.0
	25	Negative	200	2	3	2	0	2	0.01	1.0
		Positive	200	1	1	1	0	1	0.005	0.5
	50	Negative	200	1	1	1	0	1	0.005	0.5
		Positive	200	1	1	1	0	1	0.005	0.5
Positive control benzo(a) pyrene	2 µg	NA	190	13	7	13	0	12	0.068**	6.8**
Duration of exposure (24 h)										
Negative control	0.0	Negative	200	1	0	1	0	1	0.005	0.5
		Positive	200	1	0	1	0	1	0.005	0.5
Solvent control	100 µl	Negative	200	1	2	1	0	1	0.005	0.5
		Positive	200	0	0	0	0	0	0	0
IDM01	12.5	Negative	200	0	0	0	0	0	0	0
		Positive	200	2	1	2	0	2	0.01	1.0
	25	Negative	200	2	2	2	0	2	0.01	1.0
		Positive	200	2	1	2	0	2	0.01	1.0
	50	Negative	200	2	2	2	0	2	0.01	1.0
		Positive	200	1	1	1	0	1	0.005	0.5
Positive control EMS	1200 µg	NA	177	15	9	15	0	15	0.085**	8.5**

[a]Not considered for calculations of aberrations; [b]Analysis by Cochran-Armitage trend test; [c]Analysis by Student's *t*-test; **$P<0.01$ (vs. solvent control). NA: Not available; EMS: Early mortality syndrome

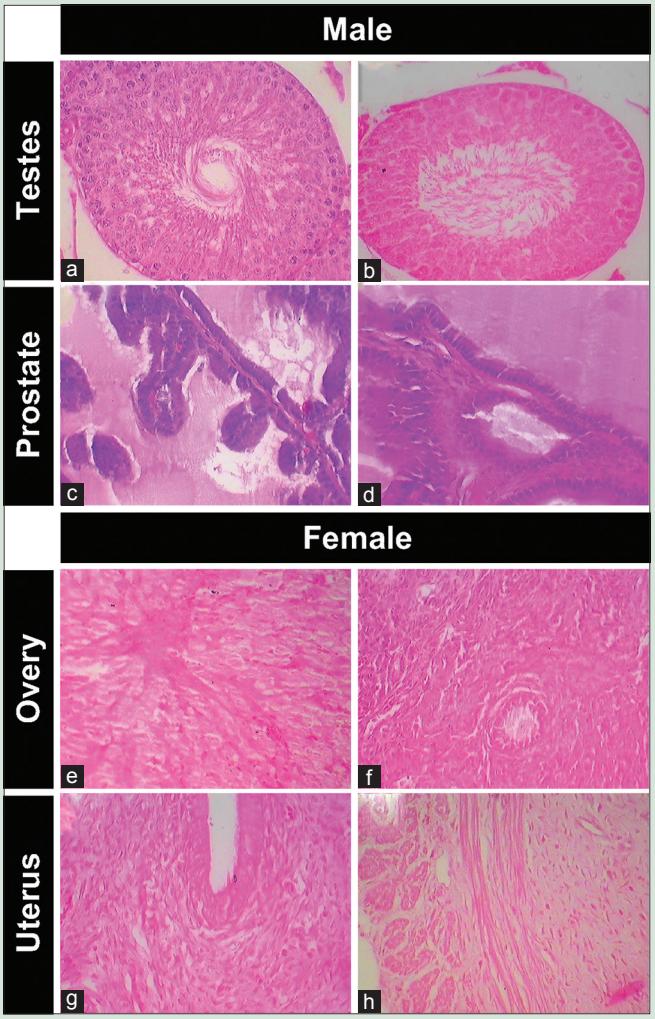


Figure 3: Histology of reproductive organs namely testes (a and b) and prostate gland tissues (c and d) in male rats and ovary (e and f) and uterus (g and h) tissues in female rats during 90-day repeated dose toxicity study. Photomicrographs from representative rats from respective groups: VC (a, c, e, and g), IDM01-1000 (b, d, f, and h) (×40)

about NOAEL.[41] During our study, on subchronic (90 days repeated dose) exposure of IDM01, all rats survived until the scheduled euthanasia and no gross pathological alterations were found in the internal organs. IDM01-treated rats did not show significant changes in food consumption, functional observation battery parameters, or gross pathological abnormalities during treatment or reversal period. Reversal groups (G1R and G4R) were incorporated to assess the reversibility of any potential toxicity that can progress after cessation of administration. G4R group did not show abnormalities or deviations from normal in physiological values after cessation of treatments in reversal groups. Taken together, IDM01 showed potential for development as a safe food supplement.

During hematological and biochemical parameters, measurements or histological findings did not have deviations from normal physiological limits in IDM01 treated groups (G2, G3, and G4 and G4R) compared to respective VC groups at dose levels up to 1000 mg/kg with all values (except ALP levels in IDM01-1000 group) were within normal physiological range for rats.[42,43] Therefore, the dose level of 500 mg/kg was considered as no-observed adverse effect level (NOAEL) for the subchronic administration of IDM01 in rats.

The reverse mutation assay (Ames test) developed the detection of potential mutagens and was routinely used for screening of chemicals for mutagenicity.[44] In the present study, IDM01 over a broad concentration range (61.72–5000 μg/plate), did not show mutagenicity either in presence or absence of a metabolic activator.

Abnormal cell proliferation, differentiation, and genomic instability are some of the silent features of cancer cells.[45] Genomic instability resulted in chromosome aberrations that include limited DNA sequence alterations such as large chromosomal changes, smaller deletions, and rearrangements are found in many tumor suppressor loci.[45] This genetic alteration accelerates the carcinogenesis.[45] Therefore, chromosome aberration assay is reliable and wee-accepted test system to determine the genotoxicity and cancer risk.[46] Breakdown of double-stranded DNA with missing repair is an important tool in this CA test.[47] In the present study, the genotoxicity of IDM01 was evaluated using peripheral blood lymphocytes in chromosome aberration assay both in the presence and absence of metabolic activation (S9 fraction) at the concentrations of 25 mg/culture, 12.5 mg/culture, and 6.25 mg/culture. IDM01 could induce few aberrations that were of chromatid-type only indicating a lack of genotoxicity for IDM01 in the present study.

Toxicological studies in animals on plant-based products and/or phytochemicals can be used to determine the correlation between the efficacy and safety.[48,49] During the past publications, the efficacy of subacute (twice a day for 28 days) oral administration of IDM01 at 60 mg/kg[23,24] and 100 mg/kg, once a day[20,21] had been demonstrated in an animal model of insulin resistance and DM respectively. These preclinical efficacy dose levels were much lower than NOAEL (500 mg/kg) found in the present study, indicating a wide safety margin of IDM01.

CONCLUSIONS

The present study demonstrated the preclinical safety of IDM01 during acute and subchronic (90-day repeated dose) administration in laboratory rats *in vivo* without mutagenicity or genotoxicity *in vitro*. The median lethal dose of IDM01 was more than 2000 mg/kg and NOAEL for 90-day repeated dose administration of 500 mg/kg in rats. The present study indicated wide safety margin of IDM01, which can be developed for clinical management of chronic disorders after appropriate clinical studies.

Acknowledgement

We would like to acknowledge the Indian Institute of Toxicology, Pune, Maharashtra, India, for their contract research services.

Financial support and sponsorship

Indus Biotech Private Limited, Pune, Maharashtra, India.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Perera PK, Li Y. Functional herbal food ingredients used in type 2 diabetes mellitus. Pharmacogn Rev 2012;6:37-45.
2. Carai MA, Colombo G, Loi B, Zaru A, Riva A, Cabri W, *et al.* Hypoglycemic effects of a standardized extract of *Salvia miltiorrhiza* roots in rats. Pharmacogn Mag 2015;11 Suppl 4:S545-9.
3. Yimam M, Lee YC, Moore B, Jiao P, Hong M, Nam JB, *et al.* UP1304, a botanical composition containing two standardized extracts of curcuma longa and morus alba, mitigates pain and inflammation in adjuvant-induced arthritic rats. Pharmacognosy Res 2016;8:112-7.
4. Geil P, Shane-McWhorter L. Dietary supplements in the management of diabetes: Potential risks and benefits. J Am Diet Assoc 2008;108 4 Suppl 1:S59-65.
5. Kibiti CM, Afolayan AJ. Herbal therapy: A review of emerging pharmacological tools in the management of diabetes mellitus in Africa. Pharmacogn Mag 2015;11 Suppl 2:S258-74.

6. Yadav UC, Baquer NZ. Pharmacological effects of *Trigonella foenum-graecum* L. in health and disease. Pharm Biol 2014;52:243-54.
7. World Health Organization. WHO Monographs on Selected Medicinal Plants. Geneva: World Health Organization; 2007.
8. Kalshetti PB, Alluri R, Mohan V, Thakurdesai PA. Effects of 4-hydroxyisoleucine from fenugreek seeds on depression-like behavior in socially isolated olfactory bulbectomized rats. Pharmacogn Mag 2015;11 Suppl 3:S388-96.
9. Singh AB, Tamarkar AK, Narender T, Srivastava AK. Antihyperglycaemic effect of an unusual amino acid (4-hydroxyisoleucine) in C57BL/KsJ-db/db mice. Nat Prod Res 2010;24:258-65.
10. Shah S, Bodhankar SL, Badole SL, Kamble HV, Mohan V. Effect of trigonelline: An active compound from *Trigonella foenum graecum* linn. in alloxan induced diabetes in mice. J Cell Tissue Res 2006;6:585-90.
11. Gupta A, Gupta R, Lal B. Effect of *Trigonella foenum-graecum* (fenugreek) seeds on glycaemic control and insulin resistance in type 2 diabetes mellitus: A double blind placebo controlled study. J Assoc Physicians India 2001;49:1057-61.
12. Madar Z, Abel R, Samish S, Arad J. Glucose-lowering effect of fenugreek in non-insulin dependent diabetics. Eur J Clin Nutr 1988;42:51-4.
13. Jelodar GA, Maleki M, Motadayen MH, Sirus S. Effect of fenugreek, onion and garlic on blood glucose and histopathology of pancreas of alloxan-induced diabetic rats. Indian J Med Sci 2005;59:64-9.
14. Raju J, Gupta D, Rao AR, Yadava PK, Baquer NZ. *Trigonella foenum graecum* (fenugreek) seed powder improves glucose homeostasis in alloxan diabetic rat tissues by reversing the altered glycolytic, gluconeogenic and lipogenic enzymes. Mol Cell Biochem 2001;224:45-51.
15. Bera TK, Ali KM, Jana K, Ghosh A, Ghosh D. Protective effect of aqueous extract of seed of *Psoralea corylifolia* (Somraji) and seed of *Trigonella foenum-graecum* L. (Methi) in streptozotocin-induced diabetic rat: A comparative evaluation. Pharmacognosy Res 2013;5:277-85.
16. Patil R, Patil R, Ahirwar B, Ahirwar D. Current status of Indian medicinal plants with antidiabetic potential: A review. Asian Pac J Trop Biomed 2011;1 2 Suppl: S291-8.
17. Bawadi H, Maghaydah S, Tayyem R, Tayyem R. The postprandial hypoglycemic activity of fenugreek seed and seeds' extract in type 2 diabetics: A pilot study. Pharmacogn Mag 2009;5:134-8.
18. Roberts KT. The potential of fenugreek (*Trigonella foenum-graecum*) as a functional food and nutraceutical and its effects on glycemia and lipidemia. J Med Food 2011;14:1485-9.
19. Hannan JM, Ali L, Rokeya B, Khaleque J, Akhter M, Flatt PR, *et al*. Soluble dietary fibre fraction of *Trigonella foenum-graecum* (fenugreek) seed improves glucose homeostasis in animal models of type 1 and type 2 diabetes by delaying carbohydrate digestion and absorption, and enhancing insulin action. Br J Nutr 2007;97:514-21.
20. Kulkarni C, Bodhankar SL, Ghule AE, Mohan V, Thakurdesai PA. Antidiabetic activity of *Trigonella foenum-graecum* L. seeds extract (IND01) in neonatal streptozotocin-induced (n-STZ) rats. Diabetol Croat 2012;41:29-40.
21. Shah S, Bodhankar SL, Bhonde R, Mohan V. Combinative therapeutic approach for better blood sugar level control in alloxan diabetic mice. Int J Diabetes Metab 2006;14:104-5.
22. Shitole P, Badole S, Bodhankar SL, Mihan V, Bhaskaran S. Anti-hyperglycaemic activity of IND01 and its interaction with glyburide and pioglitazone in alloxan induced diabetic mice. Int J Diabetes Metab 2009;17:21-6.
23. Thakurdesai P, Mohan V, Kandhare A, editors. Synergistic Composition of Fenugreek Seed Extract (Sugaheal®) Ameliorates High-fat Diet-induced Insulin Resistance in C57BL/6 Mice Via Modulating the Expression of Leptin, Glut-2, Glut-4, IRS-2 and SREBP-1c. 2nd International Conference on Biotechnology and Bioinformatics (ICBB-2015); 2015 Febuary 6-8, 2015; Pune, India. International Center for Stem Cells, Cancer and Biotechnology (ICSCCB), Pune; 2015.
24. Kandhare A, Bodhankar S, Mohan V, Thakurdesai P, editors. Therapeutic Potential of Synergistic Composition from Fenugreek Seeds (Sugaheal®) Against Nonalcoholic Steatohepatitis in C57BL/6 mice. 2nd International Congress of Society for Ethnopharmacology (SFEC-2015) 2015; Nagpur. Society for Ethnopharmacology; 2015.
25. Chandel HS, Pathak AK, Tailang M. Standardization of some herbal antidiabetic drugs in polyherbal formulation. Pharmacognosy Res 2011;3:49-56.
26. Middha SK, Goyal AK, Lokesh P, Yardi V, Mojamdar L, Keni DS, *et al*. Toxicological evaluation of *Emblica officinalis* fruit extract and its anti-inflammatory and free radical scavenging properties. Pharmacogn Mag 2015;11 Suppl 3:S427-33.
27. Ajazuddin S, Saraf S. Legal regulations of complementary and alternative medicines in different countries. Pharmacogn Rev 2012;6:154-60.
28. Ilyas UK, Katare DP, Aeri V. Comparative evaluation of standardized alcoholic, hydroalcoholic, and aqueous extracts of *Phyllanthus maderaspatensis* Linn. against galactosamine-induced hepatopathy in albino rats. Pharmacogn Mag 2015;11:277-82.
29. Olorunnisola OS, Afolayan AJ, Adetutu A. Sub-chronic administration of methanolic whole fruit extract of *Lagenaria breviflora* (Benth.) roberty induces mild toxicity in rats. Pharmacogn Mag 2015;11 Suppl 4:S516-21.
30. Werner SM. Patient safety and the widespread use of herbs and supplements. Front Pharmacol 2014;5:142.
31. Govindaraghavan S, Sucher NJ. Quality assessment of medicinal herbs and their extracts: Criteria and prerequisites for consistent safety and efficacy of herbal medicines. Epilepsy Behav 2015;52(Pt B):363-71.
32. Morani AS, Bodhankar SL, Mohan V, Thakurdesai PA. Ameliorative effects of standardized extract from *Trigonella foenum-graecum* L. seeds on painful peripheral neuropathy in rats. Asian Pac J Trop Med 2012;5:385-90.
33. OECD. Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure. Paris, France: OECD Publishing; 2008.
34. OECD. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. Paris, France: OECD Publishing; 1998.
35. Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. Mutat Res 1983;113:173-215.
36. OECD. Test No. 471: Bacterial Reverse Mutation Test. Paris, France: OECD Publishing; 1997.
37. Ong T, Mukhtar M, Wolf CR, Zeiger E. Differential effects of cytochrome P450-inducers on promutagen activation capabilities and enzymatic activities of S-9 from rat liver. J Environ Pathol Toxicol 1980;4:55-65.
38. OECD. Test No. 473: *In vitro* Mammalian Chromosome Aberration Test. Paris, France: OECD Publishing; 1997.
39. Sim KS, Sri Nurestri AM, Sinniah SK, Kim KH, Norhanom AW. Acute oral toxicity of *Pereskia bleo* and *Pereskia grandifolia* in mice. Pharmacogn Mag 2010;6:67-70.
40. Joshua Allan J, Damodaran A, Deshmukh NS, Goudar KS, Amit A. Safety evaluation of a standardized phytochemical composition extracted from *Bacopa monnieri* in Sprague – Dawley rats. Food Chem Toxicol 2007;45:1928-37.
41. Organisation for Economic Co-operation and Development. Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris: OECD Publishing; 1998. p. 1.
42. Pavelka M, Ellinger A. Localization of binding sites for concanavalin A, *Ricinus communis* I and *Helix pomatia* lectin in the Golgi apparatus of rat small intestinal absorptive cells. J Histochem Cytochem 1985;33:905-14.
43. Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, *et al*. Reference range data base for serum chemistry and hematology values in laboratory animals. J Toxicol Environ Health 1986;18:161-88.
44. Mortelmans K, Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res 2000;455:29-60.
45. Wang J, van der Heijden R, Spruit S, Hankermeier T, Chan K, van der Greef J, *et al*. Quality and safety of Chinese herbal medicines guided by a systems biology perspective. J Ethnopharmacol 2009;126:31-41.
46. Bonassi S, Hagmar L, Strömberg U, Montagud AH, Tinnerberg H, Forni A, *et al*. Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health. Cancer Res 2000;60:1619-25.
47. Natarajan AT, Palitti F. DNA repair and chromosomal alterations. Mutat Res 2008;657:3-7.
48. Delaney B. Strategies to evaluate the safety of bioengineered foods. Int J Toxicol 2007;26:389-99.
49. Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, *et al*. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol 2000;32:56-67.