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Prenatal Developmental Oral Toxicity Evaluation of Defatted Fenugreek Seed Flakes (Fenuflakes™) in Laboratory Rats

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Abstract

Fenugreek seed-based ingredients showed potential health benefits towards female-specific conditions. The present work is aimed to assess the prenatal oral toxicity of fibers and protein rich defatted fenugreek seed flakes (Fenuflakes™). The acute oral toxicity and dose range-finding studies in non-pregnant and pregnant rats were conducted before the main study. The selected doses of Fenuflakes (500, 1000, and 2000 mg/kg) were orally gavaged to rats daily from day 0 to day 19 (one day before the expected day of parturition) post-conception with the concurrent vehicle control (VC) group. On the 20th day of gestation, the maternal and embryo-fetal toxicity parameters were recorded after the cesarean sections of dams. Results: Fenuflakes in tested doses exposure did not show significant toxicological changes in maternal (body weights, food intake, anogenital distance, or clinical observations) and embryo-fetal evaluations (number of corpora lutea, resorptions, and implantations, or fetus weights, sex ratio or incidence of anomalies) compared with VC. Conclusion, Oral prenatal exposure to Fenuflakes was found safe with no significant maternal and embryo-fetal toxicities. The "No Observed Adverse Effect Level" (NOAEL) of Fenuflakes (> 2000 mg/kg/day) can be used for risk assessment before human consumption in pregnant female population.

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Keywords
Defatted Fenugreek Seed Flakes; OECD No. 414; Prenatal Developmental Toxicity; Reproductive System.

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Introduction

Herbal medicines have been used for the past several decades for nutrition, food, and medicinal uses in animals and humans. With the growing usage of natural products,[1] safety is a significant issue for regulatory authorities worldwide.[2] A large body of scientific literature and regulatory authorities worldwide highlighted the importance of standardized nutritional content[3,4] and toxicology assessment[5,6] in developing better and safer nutritional products and food supplements.

Plant-based proteins and fibers are documented for many health and medicinal potential. Plant-based proteins[7] and dietary fiber intake[8] were reported to offer nutritional and medicinal benefits against metabolic syndrome and diabetes and gained interest among researchers and consumers.

Fenugreek (*Trigonella foenum graecum* L.) has been used as a natural food additive and traditional herbal medicine. Fenugreek seeds are a rich source of nutritional content such as proteins, fibers, vitamins, minerals, amino acids, and bioactive content such as antioxidants, and essential oils. The fenugreek seeds are consumed as a metabolic enhancer to add nutritional value to food preparations such as cereals and snacks[9] and as an ingredient for dietary supplements.[10] Fenugreek seeds comprise a high amount of proteins (20–30%)[11] with amino acids, especially methionine, histidine, isoleucine, lysine, glutamine, leucine, arginine, threonine, asparagine, and cysteine.[12] The major amino acid of fenugreek seeds, 4-hydroxyisoleucine (4HI), stimulates insulin secretion and reduces serum cholesterol levels.[13]

Fibers from fenugreek seeds have numerous applications in various food products because of their stability and long shelf life.[10] Fenugreek seed contains dietary fibers, nearly 50% of its weight, of which 30% are soluble fibers (galactomannan or oligosaccharides), and 20% are bulk-forming fibers.[10] The high amount of fibers in fenugreek seeds or its powder is thought to be responsible for its galactagogue,[14] antihyperglycemic,[15] insulin-sensitizing,[16] and antihyperlipidemic[17] benefits.

The inclusion of fenugreek seeds powder (1% to 50) in the diet is reported with numerous beneficial properties.[18-20] The defatting process enriches seeds with fibers and proteins and removes fats[21] and reduces the bitterness of fenugreek.[22] Therefore, defatted fenugreek seed flakes with high fiber- and protein can be excellent ingredients for functional food, nutritional additives, or supplements.

Recently, fenugreek seed-based ingredients showed potential for female-specific conditions as a galactagogue, lactational aid, libido enhancer and management option for polycystic ovary syndrome, post-menopausal problems such as dysmenorrhea, cognitive decline, and osteoporosis.[23] Therefore, fenugreek-based ingredient, such as defatted fenugreek seed flakes (Fenuflakes™) can be consumed by pregnant women for potential health benefits.

In the past, systematic review on toxicological literature concluded overall safety of oral fenugreek seeds, seed powder and many extracts for oral consumption.[24] Few isolated studies on extracts of fenugreek seed[25,26] or to fenugreek decoction, intraperoneal 0.8, 1.6 and 3.2 g/kg/bw)[27] in pregnant female animals were reported. However, toxicological data on fenugreek seed or powder per se on oral prenatal exposure in female animals is limited. Oral administration of Fenugreek powder (200 mg/kg/day for 30 days)[28] or 30% level in diet[20] in female rats and rabbit respectively, were reported to cause changes in female harmonal levels and fetal developments[20,28] with proliferative changes in endometrium.[20]

However, these reports cannot be comparable for toxicological risk assessment because of inconsistencies in fenugreek seed-based ingredients, especially diversity in compositions, sources, and processing of fenugreek seeds, dosage, and duration of exposure, route of administration (as gavage or feed), the extraction solvent, different parts or whole of fenugreek plants (leaves, seeds), authentication procedures, and adherence standard guidelines for toxicological studies.[24] Therefore, separate toxicological evaluation using specific composition, e.g. Fenuflakes, with prenatal exposure during organogenesis period of pregnancy, in compliance with international guidelines and comprehensive set of evaluations is crucial for risk assessments for human use. Therefore, the present study aimed at the toxicity assessment of

Fenuflakes on single (acute) or repeated-dose oral exposure during pregnancy (period of gestation) in laboratory rats using relevant guidelines.

Material and Methods

Design

The study was performed as per guidelines issued by "Organisation for Economic Co-operation and Development" (OECD) for "Acute oral toxicity, Test No. 423[29] and "Prenatal Development Toxicity Study, Test No. 414" (as adopted on 25th June 2018].[30] The compliance with the necessary guidelines for laboratory animal facilities, as recommended by the 'Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA)[31] and the "National Research Council, USA" were maintained.[32] All study protocols (study no. 19645, 19646, 19647 and 19648) were approved by the "Institutional Animal Ethics Committee" of organization, where experiments were conducted.

Animals

The experiments were performed on the rats procured from Charles River Labs Inc., USA, through Indian licensee Hylasco Bio-Technology (India) Pvt. Ltd, Telangana, India. The animals were maintained in polypropylene cages with necessary attachments (grill, bedding, water and feed attachments) and room conditions following CPCSEA guidelines.[31]

The Test ingredient

The test food ingredient, Fenuflakes (beige color, free flowing flakes), was obtained from Indus Biotech Limited (Pune, India), and was prepared as follows. Fenugreek seeds were procured from Rajasthan, India, and authenticated by an expert taxonomist. Fenugreek seeds with less than 7% moisture content were soaked in purified water at room temperature for 24 h. The soaked seeds were flaked in a Roller flaker. The flakes were then defatted with solvent (ethanol: water in ratio 80:20), which is 2.5 times of volume as that of fenugreek seeds, at a temperature of 62– 65 °C and filtered using a polypropylene filter cloth fitted in the extractor. The resultant flakes were washed with ethanol, filtered, and dried in a stainless steel-made fluidized bed dryer at a temperature of up to 80° C till loss on drying (LOD) of less than 10 % was achieved. Dried flakes (71% yield) are then passed through a metal detector to prevent metal contamination (size - 0.5 mm ferrous, 0.8 mm non-ferrous, and 1.0 mm of stainless steel) and packed under vacuum before storage in a polyline bag.

The content of dietary fibers (total, soluble and insoluble) and available carbohydrates in Fenuflakes was estimated using AOAC methods 991.43 using the Available Carbohydrates/ Dietary Fiber Assay Kit (Cat No. K-ACHDF, Megazyme, Bray, Ireland). The fat content and protein content were measured using the "AOAC Official Method" 920.39 (2005) and 984.13 (2005, Kjeldahl method). The Fenuflakes sample had 70.2% total dietary fibers (33.6% insoluble and 36.6% soluble fibers) with 26% proteins, 3.6% fat, and 0.2% available carbohydrates. The uniform viscous suspension of Fenuflakes in corn oil was prepared daily to obtain the suitable dose level at a volume of 4 mL/kg.

Acute Oral Toxicity (AOT) in Normal Rats

As the first step, AOT of Fenuflakes was evaluated in compliance with OECD guidelines Acute Toxic Class Method, No.423.[29] The 8-week old, healthy, young, non-pregnant, and nulliparous female Wistar rats, weighing 150-161 g, were used. A single dose was orally administered to the group of three female Wistar rats in a stepwise manner. In the first step, only one rat was dosed. In the second step, two more rats were dosed. A total of 5000 mg/kg was administered in two divided doses (2500 mg/kg each). For each step, the mortality and incidence of abnormalities in clinical signs were observed for 14 days, then sacrificed for necropsy examination.

Dose-Range Finding Study in Non-Pregnant Rats

As second step, the dose range finding study (14-day repeated dose) in non-pregnant females was carried out, as safety data on oral consumption of Fenuflakes in regular (non-pregnant) animals was not available. Such study helps to remove possible interference of factors, such as treatment un-related fatal loss and avoids unnecessary animal handling. This study was conducted in non-pregnant healthy, adult, nulliparous, female Wistar rats (11 to 16 weeks old, 5 rats per group).

The oral gavage administration of either vehicle (corn oil, 4 mL/kg) or Fenuflakes (625, 1250, or 2500 mg/kg, daily, in two equally divided doses) for 14 days to rats was done. The measurements were performed daily for clinical examination, morbidity and mortality, and signs of toxicity. On initiation (Day 0), Day 8, and

Day 15 of treatment, food consumption and body weights were measured. All rats were sacrificed for a detailed necropsy on Day 15, and organ weights (kidneys, liver, adrenals, spleen, brain, ovaries, and heart) were recorded.

Prenatal Developmental Toxicity in Pregnant Rats

As third step, the "dose-range finding study" on pregnant rats was performed females Wistar rats, as per OECD guidelines, Test No. 414.[30] Mating was done using forty male Wistar rats (adult, healthy, proven fertile, aged 14-15 weeks.). The females rats were assigned a temporary identification number. Single males and 1-2 females were housed in a cage during mating. After successful mating, the single female rat was housed per cage. After mating was confirmed (day 0), a unique identification number, specified on the individual cage tag and individual tail, was assigned to the pregnant female rats. After mating was confirmed (Day-0), the rats were randomly assigned to any of 4 groups (control or treatments groups)

The dose finding study involved the oral administration of either vehicle (corn oil, 4 mL/kg) or Fenuflakes (625, 1250, and 2500 mg/kg/day, oral gavage, in two equally divided doses, i.e., 312.5, 625, and 1250 mg/kg, twice daily) to 4 groups of 7 rats each, from day-0 to day-19 of gestation day was done. The weight changes or clinical abnormalities of dams were done during the gestational period. On 20th day of gestation, dams undergo a cesarean section for pregnancy status, maternal examinations, and embryo-fetal defects. The fetuses were identified, sexed, weighed, and examined for any external anomalies. The number of corpora lutea was recorded. Uterus was examined for its gravid status, a number of implants, live and dead fetuses, and resorption sites. The dose-range finding studies indicated the safety of subacute oral repeated-dose administration of Fenuflakes without developmental toxicities in non-pregnant and pregnant rats.

As last step, "the main study" was performed with groups of 25 pregnant rats. Oral gavage treatments were given to each rat, from gestation day-0 to gestation day-19, at approximately 4-6 h apart in the morning and afternoon (i.e., 250, 500, 1000 mg/kg, twice a day, dose volume of 4 mL/kg). A concurrent vehicle control (VC) treated with corn oil to 25 pregnant rats was maintained.

Each dam's body weight and food consumption were recorded in the gestation period. All the rats were euthanized on gestation day 20 by CO_2 asphyxiation. The abdomen was incised and examined for the maternal and fatal parameters as per recommendations in guidelines.[30,33,34]

All live fetuses were examined for external abnormalities. The fetuses specimens were examined for microscopic examinations using reported procedures for skeletal,[35] or visceral[34] malformation or variations.

Statistical Analysis

The data was analyzed with the help of statistical software, SPSS (Version 23.0. IBM, NY, USA). The parametric data (continuous numbers, such as weights, distance, and % loss) was represented as mean ± standard deviation (SD), which was analyzed by one-way analysis of variance, followed by Dunnett's test. The discrete data, such as ratios and numbers, were subjected to Kruskal–Wallis test, with the Mann–Whitney test. The frequency data (e.g., number of pups, incidences of malformations) were analyzed using Fisher's test. The comparisons between the groups (Fenuflakes v/ VC) were made and considered significant at 5% level ($p < 0.05$).

Results

AOT in Normal Rats

Acute administration of Fenuflakes did not cause death or significant changes in body weight or any gross pathological variations in their organs or tissues throughout the observation period of 14 days at a dose of 5000 mg/kg. Therefore, the acute oral median lethal dose (LD50) of Fenuflakes was assigned as greater than 5000 mg/kg. Fenuflakes can be categorized as "unclassified" for the obligatory labeling requirement for oral toxicity based on the "Globally Harmonised System" (GHS) for the classification of chemicals, and "OECD Guidelines for the Testing of Chemicals",[36] European Commission Dangerous Substances Directive (Directive 2001/59/EC).[37]

Dose Range-Finding Studies in Normal and Pregnant Rats

In non-pregnant female rat, 14-day repeated oral administration of Fenuflakes did not produce mortality, clinical abnormalities, gross pathological alterations in tissues, or changes in body weight, organ weights (absolute and relative) or food consumption (versus VC group).

Fenuflakes (625, 1250, and 2500 mg/kg, oral) treatment during the gestational period showed no mortality, abnormal clinical signs (systemic toxicity), changes in body weight, or gross abnormalities in tissues or organs (except for one dam from 2500 mg/kg group, exhibited distended uterine horns with black colored fluid). All maternal pregnancy-related observations like confirmed pregnancy (numbers), litters (numbers), live implants, dead implants, resorptions, and pregnancy rate did not show signs of abnormalities (versus VC). Similarly, all uterine parameters, such as uterus weights, and numbers of corpora lutea, implantations, or resorptions and implantation losses (%), did not show significant difference from VC.

Fetal examination of litters of the sacrificed females, treated with Fenuflakes, showed an absence of adverse effects and did not reveal any remarkable alterations in a number of litter or fetuses, anogenital distance, sex ratios, fetal weights, or external anomalies (malformations) as compared with VC group.

Dose selection for Main Study

Because of the highly viscous nature of Fenuflakes suspension in corn oil, the maximum dose of 2000 mg/kg/day was selected as the maximum dose for the main study. The final doses of 2000 mg/kg/day (Maximum), 1000 mg/kg/day (1/2 of maximum), and 500 mg/kg/day (1/4th of maximum) in two equally divided parts per day were used for the main study.

Table 1: Fenuflakes- Maternal findings

Findings	VC	Fenuflakes (mg/kg)		
		500	1000	2000
Rats per group (number)	25	25	25	25
Confirmed pregnancy[$] at necropsy (number)	22	23	25	23
Pregnancy rate (%)	88	92	100	92
Mortality (number)	Nil	Nil	Nil	Nil
Litters available for evaluation (number)	20	23	24	23
Maternal data				
Body weight (g) - Initial on GD0	293.50 ± 19.38	294.87 ± 19.36	296.68 ± ± 14.16	294.26 ± 20.44
Body weight (g) - Final	396.77 ± 55.5	417.09 ± 40.31	397.48 ± 50.65	399.87 ± 44.58
Body weight change (g)	101.86 ± 47.88	122.22 ± 31.42	100.80 ± 51.89	105.61 ± 42.94
Corrected Body weight (g)	323.37 ± 5.91	334.30 ± 38.10	321.64 ± 33.21	318.47 ± 34.92
Anogenital Distance (mm) male	1.80 ± 0.60	1.66 ± 0.08	1.64 ± 0.07	1.63 ± 0.06
Anogenital Distance (mm) female	1.50 ± 0.80	1.25 ± 0.12	1.20 ± 0.08*	1.2 ± 0.09*

Maternal data are expressed as Mean (g) ± SD.[$] Confirmed pregnancy, positive sperm status. * $p < 0.05$ (significant, v/s VC).

Maternal Examinations

All dams were examined daily for any abnormal clinical signs, with no mortality, treatment-related clinical abnormalities, or abortion, except one dam each from VC, 1000 and 2000 mg/kg group showed abdominal breathing, ruffled skin, emaciation, vocalization, and hypoactivity. The difference was found non-significant in the mean food consumption in Fenuflakes treated groups (20.9 g, 20.2 g, and 19.8 g in 500, 1000, and 2000 mg/kg dose groups,

respectively) as compared with the VC group (20.7 g) during the study period. As shown in Table 1, the pregnancy data and maternal data (except anogenital distances in females) in the Fenuflakes group did not have significant differences from the VC group.

Table 2: Fenuflakes- Embryo-fetal reproductive findings

Findings	VC	Fenuflakes (mg/kg)		
		500	1000	2000
Gravid uterus weight (g)	73.4 ± 31.4	77.5 ± 20.8	75.8 ±24.7	81.4 ± 16.7
Corpora lutea (no.)	13.6 ± 3.0	14.0 ± 2.9	13.2 ± 3.5	13.9 ± 2.7
Implantations per female (no.)	13.0 ± 3.3	13.3 ± 2.8	13.1 ± 3.5	13.7 ± 2.6
Early resorptions (no.)	1.4 ± 2.3	1.2 ± 3.3	0.6 ± 0.9	0.2 ± 0.4
Late resorptions (no.)	0.0 ± 0.2	0.0 ± 0.0	0.1 ± 0.4	0.00 ± 0.2
Pre-implantation loss (%)	4.3 ± 11.6	3.5 ± 11.7	1.2 ± 3.5	1.7 ± 3.5
Post-implantation loss (%)	15.1 ± 29.2	8.2 ± 18.9	10.9 ± 22.6	3.1 ± 5.4
Litter size (no.)	12.9 ± 3.4	12.2 ± 3.6	12.9 ± 3.3	13.5 ± 2.7
Live fetuses (no.)	12.9 ± 3.4	12.2 ± 2.8	12.8 ± 3.4	13.4 ± 2.9
Dead fetuses (no.)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.6	0.1 ± 0.4
Live male fetuses (no.)	5.8 ± 3.4	6.3 ± 2.9	7.5 ± 2.5*	7.0 ± 2.2
Live female fetuses (no.)	7.1 ± 1.9	5.9 ± 2.6	5.4 ± 2.3	6.4 ± 2.6
Male/female sex ratio (no.)	0.9 ± 2.6	1.4 ± 1.4	1.5 ± 0.8	1.5 ± 1.3
Average fetal weight (g)	4.0 ± 0.6	4.11 ± 0.8	4.01 ± 0.4	3.95 ± 0.6
Average fetal |weight (g) - Male	4.12 ± 0.7	4.20 ± 0.8	4.09 ± 0.4	4.06 ± 0.6
Average fetal weight (g) - Female	3.90 ± 0.6	4.01 ± 0.7	4.08 ± 1.5	4.46 ± 2.6

Values are expressed as Mean ± SD. * $P < 0.05$ v/s. VC.

Reproductive Findings

Table 2 shows the effects of treatments on embryo-fetal reproductive findings. The differences between the Fenuflakes treated groups and VC groups for the numbers (corpora lutea, live fetuses, pre- and post-implantation loss, implantation sites, and resorptions) were not significant. The number of male fetuses in the Fenuflakes (1000 mg/kg) treated group was significantly more ($p < 0.05$) than that of the VC groups.

Fetal Examination (External)

Each fetus was examined externally for the incident of any abnormal findings (malformation/ variation) with skeletal and visceral abnormalities, as presented in Table 3. The observations on length, cranium, eyes, palate, limbs, tail, genitals, and sex were considered for external abnormalities. Oral treatment of Fenuflakes in the tested doses or VC did not show any significant external malformations in any of the litters (Table 3). The total number of litter examined for external examination was 20 in the VC group, 23, 24, and 23 in the Fenuflakes groups (500, 1000, and 2000 mg/kg), respectively. Three litters from VC, two litters from Fenuflakes (1000 mg/kg), and four litters from Fenuflakes (2000 mg/kg) were observed with undersized (runt) fetuses. The Fenuflakes (1000 and 2000 mg/kg) group showed four and two undersized and autolyzed dead fetuses, respectively. A minor anomaly (agenesis of the tail) was seen in one fetus in Fenuflakes (2000 mg/kg) treated rats. These are 'normal variants' of solitary nature and so considered incidental.

Fetal Examination (Visceral)

As presented in Table 3, none of the litters of dams treated with Fenuflakes showed remarkable or significant alterations in the soft tissues of any of the litters. One minor anomaly each (lateral ventricle

dilation in the brain in Fenuflakes (2000 mg/kg) group, bilateral hydronephrosis of kidney in one litter (4.35%) from Fenuflakes (500 mg/kg) group, the hypoplastic testes, epididymitis, and discoloration of thymus in one litter (5.00%) of VC group) was found and was considered as a normal variant. These were considered incidental and unrelated to the treatment without dose- or treatment dependencies.

Table 3. Fenuflakes- fatal findings (malformations and variations)

Findings	VC	Fenuflakes (mg/kg)		
		500	1000	2000
No. of fetuses (litters)				
External	258(20)	280 (23)	308 (24)	308 (23)
Visceral	123 (20)	135 (23)	145 (24)	150 (23)
Skeletal	135 (20)	145 (23)	163 (24)	158 (23)
External malformation	Nil	Nil	Nil	Nil
Visceral variations				
Kidney: Hydronephrosis (Bilateral)	Nil	2	Nil	Nil
Epididymis and Testis: Hypoplastic	1	Nil	Nil	Nil
Thymus- Discoloration- Normal Variant	1	Nil	Nil	Nil
Brain: Head-External- Dome-shaped; Internal- Slight hydrocephalus of lateral ventricles	Nil	Nil	Nil	1
Skeletal variations				
Skull ossification (Poor ossification / Scrambled ossification/ / not ossified/Incomplete ossification)	Nil	3	1	1
Sternebra Variations				
Incomplete / Poor ossification	14	14	13	17
Un-ossified	11	15	8	9
Rudimentary, Split, Dumbbell / Asymmetrically dumbbell shaped, Displaced and Misshapen	3	23	21	23
Rib Variations				
14th (right /left/both)- Rudimentary	19	15	22	7
Extra / Accessory (right /left/both)	3	1	2	1
Asymmetrical/wavy/absent/ Undulated,/nodulated/, short	3	4	2	3
Vertebrae variations				
Sacral: No/ Poor ossification	1	Nil	Nil	Nil
Lumbar: Incomplete / Poor ossification	Nil	2	Nil	Nil
Sacral: Fusion	Nil	Nil	1	0
Cervical: No/ Poor ossification	3	Nil	Nil	Nil
Thoracic vertebra centra: Dumbbell/spilt/ asymmetrical/misshapen	20	20	24	21
Limb Variations				
Forelimb: Fingers misshapen	Nil	Nil	Nil	1

a The incidence of individual defect is presented as the number of fetuses (number of litter)

Fetal Examination (Skeletal)

As shown in Table 3, none of the dams treated either with Fenuflakes or vehicle show significant and/or life-threatening malformations, which can be considered as teratological importance except for one incidence of normal variants or minor anomalies in each dam. Abnormalities of the normal variant were observed in three litters from the Fenuflakes (500 mg/kg) and one litter in each Fenuflakes (1000 and 2000 mg/kg) treated group. Such normal variants are known to be consistently present and distributed in large percentages throughout the rodent population without directly affecting the fetus's survival.[38,39] Therefore, these abnormalities were incidental without toxicological considerations.

Discussion

The prenatal toxicity evaluation of defatted fenugreek seed flakes (Fenuflakes) was performed in the present study. The oral administration of Fenuflakes during the gestational period in female rats at tested doses (250, 500, and 1000 mg/kg, twice a day) did not show any abnormalities or signs of maternal or fetal developmental toxicities. The frequency of two times a day was used in the present study based on recommendations for the medicinal properties of fenugreek seed or powder in the traditional and modern scientific literature.[10,11,40]

The developmental toxicology studies provide information on the effects of xenobiotics on prenatal exposure on pregnant animals and developing embryos and fetuses, such as malformation, growth retardation, and death,[30] and found predictive of effects in human prenatal development.[41] Prenatal testing is in vivo protocol that is used to evaluate reversible or irreversible malformations, structural variations, resorptions, and growth in litters of pregnant rats or rabbits with test compound exposure, during organogenesis.[41]

In the past, the defatted fenugreek seed powder was reported safe in toxicological literature,[24] but reports on fenugreek seed extracts have been inconsistent. A few incidences of maternal or fetal developmental toxicities were reported on prenatal oral exposure to fenugreek seed extract during pregnancy.[42] In contrast, many other reports demonstrated the safety of fenugreek seed extract or its constituents.[43-46] Many of these reports have inconsistent compositions with unstandardized test materials or ingredients, i.e., crude and whole extracts, without standardization to marker compounds. Because of such inconsistencies, each fenugreek-based product must be evaluated for standardized content for reliable toxicological risk assessment before human usage. The present study provided the female-specific toxicological information of oral administration of Fenuflakes during prenatal exposure in the embryofoetal development stage and can form a basis of risk assessment for safe human use.

The present developmental prenatal toxicity evaluation of Fenuflakes in pregnant rats was done using internationally accepted guidelines, OECD Guideline No. 414.[30] The modest increase in protein and fiber together was reported to achieve metabolic, and weight reduction benefits[47] and significantly improve HDL-C levels to manage dyslipidemia in overweight or obese individuals with T2DM.[48] Therefore, the prenatal safety information of Fenuflakes from the present study will be valuable for safer nutritional supplement development with better health benefits.

The exposure of Fenuflakes at a daily oral dose > 2000 mg/kg during the gestational period in rats could not induce reproductive toxicities in dams or pups. However, doses more than 1000 mg/kg twice a day, i.e., 2000 mg/kg/per day, could not be evaluated as the soluble fibers of fenugreek seeds increase viscosity, which is in line with an earlier report.[49] Therefore, the “no-observed-adverse-effect-level” (NOAEL) for prenatal exposure of Fenuflakes is > 1000 mg/kg/day twice a day, i.e., 2000 mg/kg/day. The “human equivalent dose” (HED) of Fenuflakes can be calculated from the formula suggested by “USFDA guidance for Industry” as > 19.2 g.[50] However, based on NOAEL of 2500 mg/kg, which was found in the dose-range finding experiment in pregnant rats of the present study, the HED of > 24.0 g/day can be considered a safe dose during the gestational period.

Fenugreek has received the status of an approved food ingredient in the USA, Australia, Canada, and India under various regulatory regulations. The 25 g of fenugreek (methi) is recommended in the sample menu plan for an adult man doing moderate activity in the report on “Nutrient requirements

and recommended dietary allowances (RDA) for Indians."[51] The review published on the toxicological properties of fenugreek[42] reported the calculated and suggested limit dose of 21.16 g per adult human, weighing 60 kgs, calculation based on body surface area, formulae provided by USFDA.[50] Moreover, 30 g of dried seeds per day is a recommended limit dose in various scientific and regulatory documents, such as the monograph on fenugreek seed published by Health Canada,[52] which is based on the British herbal compendium.[53] the Ayurvedic Pharmacopoeia of India[54] and some books.[55,56] Based on the daily recommended dose of 30 g. seeds and the obtained yield of Fenuflakes (71%) from fenugreek seeds, during the present study, a dose of more than 21.3 g/ day. Fenuflakes can be recommended for safe long-term human consumption. This is first step as pre-clinical study. It must be further examined with big number of volunteer in well-designed human clinical studies in compliance with international standards.

Conclusions

In conclusion, the oral daily prenatal exposure of Fenuflakes during the gestational period in rats, did not show significant maternal or embryofoetal developmental toxicity at NOAEL > 1000 mg/kg, twice a day (> 2000 mg/kg per day). These results can be helpful for risk assessment of Fenuflakes as nutritional ingredients and need more clinical studies for use as dietary supplements for human usage.

Contribution of Authors

PT and PD were involved in the conception, design, revising, and approval of the manuscript. RP was involved in the interpretation of data, with drafting, revising, and approval of the manuscript. SG and MP were involved in the study design, with data acquisition, analysis, and interpretation, with revision and approval of the manuscript.

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Conflict of Interest

Indus Biotech Limited, Pune, India, funded the study but do not have role in data collection, acquisition, analysis, and interpretation of data. The authors declare no conflict of interest during these studies.

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