

Study of Sub-Acute Toxicity Profile of Fenugreek (Trigonellafaeenum-Graecum) Seeds in Kidney Tissues of Albino Rat: A Randomized Control Trial

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Abstract

Background : The objective of the present study was to evaluate the sub-acute toxic effects of ethanol extract of fenugreek seeds on kidney tissues of albino rats.

Materials and methods: The present study was conducted on 30 albino rats divided into Group A, B, C, D and R. Group A (Control group) was treated with distilled water. Group B, Group C and Group D were treated orally with ethanol extract of fenugreek seeds at a dose of 1.25 gm/kg/day, 2.5 gm/kg/day and 5 gm/kg/day respectively for 90 consecutive days. Group R (Recovery group) was treated with seed extract of 5 gm/kg/day for 90 days followed by no treatment for next 28 days to observe any toxic effect if present in highest dose whether reversible or not. On 91st day all the animals of Group A, Group B, Group C and Group D were sacrificed and Group R was sacrificed on 119th day. Blood sample was collected from all the rats by cardiac puncture before sacrifice for measurement of biochemical and hematological parameters, and kidneys were collected for histopathological examination after sacrifice.

Results: Upto 2.5 gm /kg/day dose level, all parameters of all experimental groups were statistically not significant when compared with control group. At 5 gm/kg/ day dose level the seeds extract produced statistically significant change in biochemical parameters such as serum creatinine and blood urea. In gross microscopic examination of kidney, at the highest dose level showed the sign of cell injury like cellular swelling, hemorrhage, inflammatory cells infiltration, necrosis etc. Interestingly, these biochemical and microscopic findings at the highest dose level were absent in recovery group.

Conclusion: Finally, the study revealed that ethanol extract of fenugreek seeds was non toxic up to 2.5 gm/kg/day dose level and produce renal toxicity at 5 gm/kg/day dose level in sub acute toxicity test. So, if needed to use in higher therapeutic dose the toxicity profile of seeds extract must be re-evaluated.

Key words

Ethanol extract of fenugreek seeds; Sub-acute toxicity; Adverse drug reaction; Safety profile.

INTRODUCTION

The medicinal properties of Fenugreek locally called Methi have been known for a long time. A number of studies have been conducted to evaluate its pharmacological effects. Different animal and human trials have evaluated the possible hypoglycaemic and hypolipidaemic property of oral fenugreek seeds¹⁻⁶. Again this beneficial effect had also been demonstrated in Type-I and Type-II diabetic subjects⁵⁻⁹.

Presently, there is an ongoing world-wide “green” revolution which is mainly premised on the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs⁹. A World Health Organization (WHO) survey indicated that about 70% - 80% of the world populations rely on non conventional medicine mainly of herbal sources in their primary health care¹¹.

There is a wide spread perception amongst the publics that herbal medicines being natural are entirely safe. Gaillard and Pepin analyzed 40 toxic medicinal plants that were responsible for human death throughout the world e.g Foxglove (*Digitalis*) deadly nightshade (*Belladonna*)¹². So, it is necessary to carryout pre-clinical toxicity studies to explore the biological activities of the medicinal compounds in terms of its safety before starting to investigate it in human. To receive a marketing authorization, herbal medicines are required to meet safety, quality and efficacy criteria in a similar manner to any licensed medicine too¹³⁻¹⁴.

Drugs for hyperglycaemia and dyslipidaemia are either too expensive or have undesirable side effects and contra-indications¹⁵. So, in developing country like Bangladesh with little Health Service Research (HSR) Fenugreek seeds can offer effective health protective measure by its hypoglycaemia and hypolipidaemic properties. But the suggested dose of Fenugreek seeds for diabetic and hyperlipidaemic patient is 30mg/day/person has however been much higher than the quantities normally consume in diet¹⁶. Further these patients are advised to consume these seeds for prolong period.

Acute toxicity study of ethanol extract of Fenugreek seeds on experimental animal found safe¹⁷. Sub-acute and chronic effects are often detected over an extended period of time during which exposure may be continuous or intermittent, though obviously at levels which are too low to produce an acute toxic effect. So it is necessary to evaluate properly of toxicological properties of Fenugreek seeds in animal model before this is recommended to be safe for long-term human consumption and in quantities larger than what is normally consumed.

Considering the above factor, in present study, the concern was on the evaluation of the sub-acute toxicity (90 days) of Fenugreek seeds (*Methi*) in kidney tissues of Albino rats orally at different doses.

MATERIALS AND METHODS

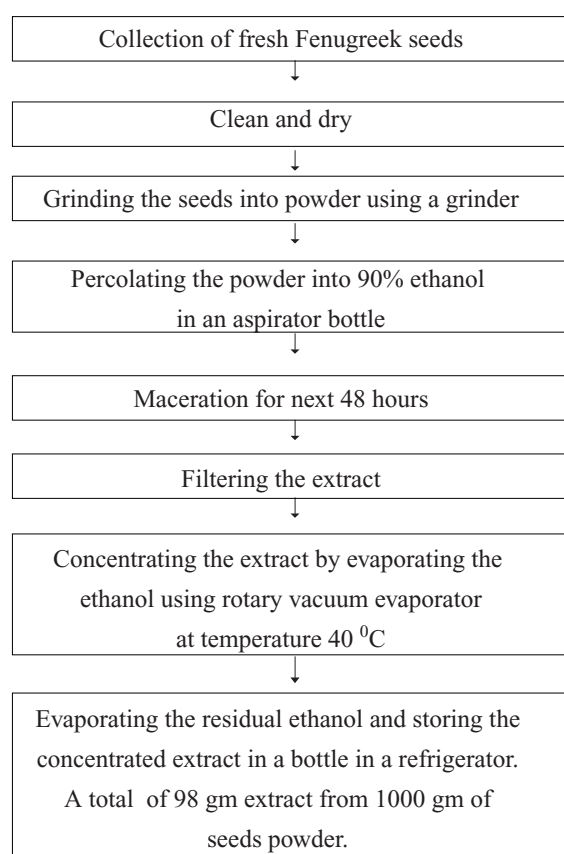
The study was conducted in the Pharmacology and Therapeutics Department, Chattogram Medical College, in collaboration with the Bangladesh Council of Scientific and Industrial Research Laboratory (BCSIR) Chattogram, during the period, 1st January, 2008 to 31st December, 2008.

Animals

The whole experiment was carried out on a total number of 30 male Wistar Albino rats, 3-4 months of age, weight 180-200 gm for sub-acute toxicity test. They were obtained from the animal house of BCSIR laboratory, Chattogram. They were housed in standard size plastic cages at room temperature in well ventilated room, in hygienic condition. All the animals were allowed free access to standard rat diet and water ad libitum before and throughout the experiment. For the purpose of identification, each rat was marked with colored fluid on legs after their selection for the experiment.

Preparation of Seed Extract

The chronological steps involved in the extract preparation of Fenugreek seeds have been shown in following flow chart:

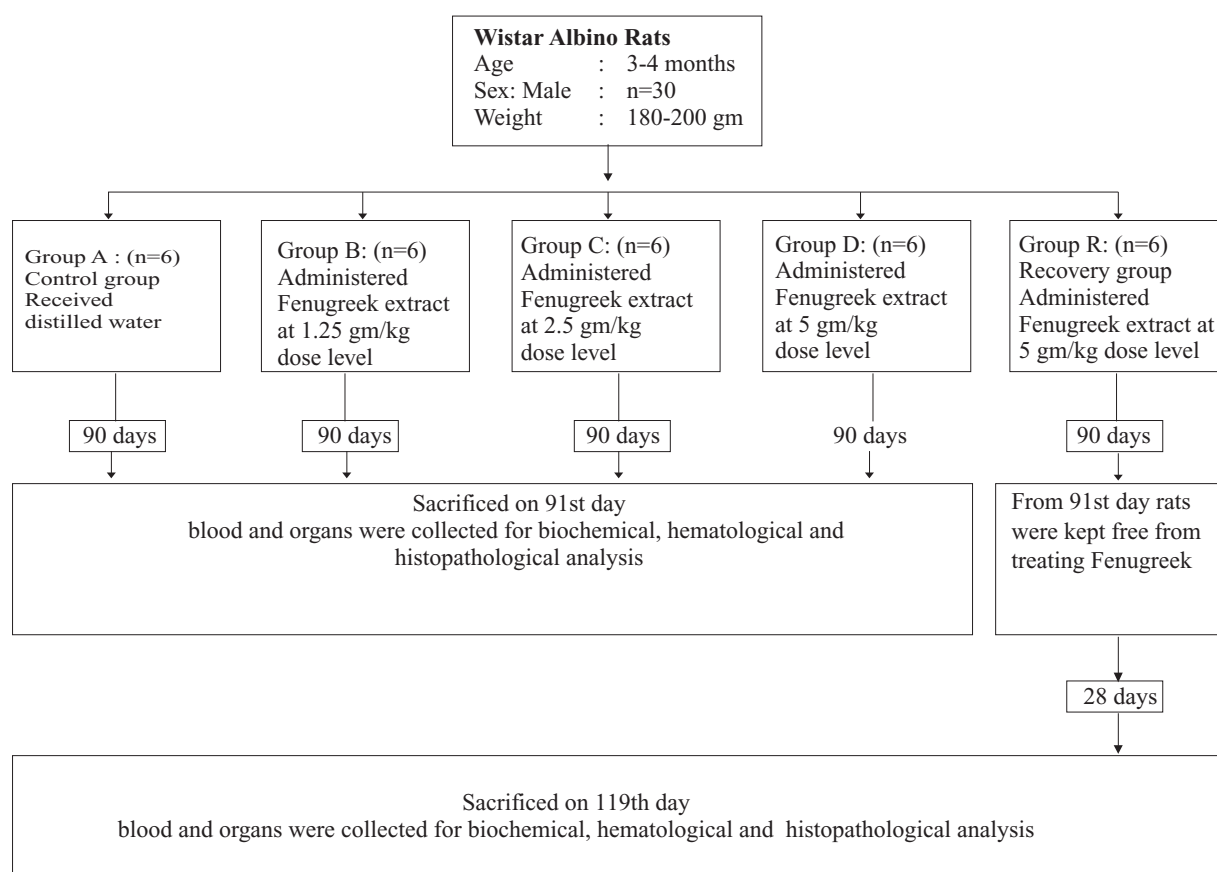


Dose Calculation^{18,19}

Suggested therapeutic dose of Fenugreek seeds powder for diabetic and dislipidaemic patient is 30 gm/day/person. So, in an average 60 Kg human being the dose will be .5 gm /Kg/day. In the experiment 10% Ethanol extract of Fenugreek seeds was obtained. So the dose of the extract will be .05 gm/kg/day. 100 times of this extract dose is 5 gm/kg/day, 50 times of this extract dose is 2.5 gm/kg/day. 25 times of this extract dose is 1.25 gm /kg/day.

Experimental Design of Sub-Acute Toxicity Study

30 rats were allocated into five group as-



Collection of Blood and Separation of Serum

On 91st day and 119th day (Recovery group) after keeping fasting for 16-18 hours all the animals were anaesthetized with diethyl ether. Blood was collected by cardiac puncture and then all the rats were sacrificed. Collected blood was preserved into two pre-labeled test tube for each rat. Half of the blood was taken in a tube containing anticoagulant, Ethylene Dichloro Tetra Acetic acid (EDTA) for hematological examination and the remaining blood was collected in another glass test tube and allowed to clot form at room temperature for 20 minutes. Then the glass tubes were centrifuged at 2000 r.p.m for 10 minutes. Then clean supernatant plasma and serum were pipetted out & collected into pre-labeled Wintrobe tubes. Collected serum & plasma was then used for different Biochemical tests.

Collection and Preservation of Kidneys

Chest & abdomen of the rats were opened. Both kidneys of each rat were carefully removed and cleaned. Then all were weighted in digital balance and immersed separately into pre-labeled 10% formalin containing specimen container for histopathological examination.

Statistical Analysis

All the results had been expressed as the mean \pm Standard Errors of Mean (SEM). Statistical analysis was performed by using the statistical analysis software- "Microsoft Excel-2008". All data's from each control and treated group were analyzed by using Student's "t" test. Differences with a p-value less than 0.05 were considered to be statistically significant.

Parameters Studied

The following parameters were studied for each animal and comparative studied were made between control and experimental groups.

Macroscopic Parameters

- i) Body weight: Body weights of all rats were measured at the first day and then every 7 days interval.
- ii) Organ weight: Weight of the kidneys and of all rats were measured after sacrifice.

Biochemical Parameters and Procedure of Estimation

Estimation of blood urea by using enzymatic end point Berthelot method.

Kit- Human Gesellschaft for Biochemica and DiagnosticambH. Wiesbaden,Germany.

Estimation of serum creatinine by using Mod. Jaffe. Kinetic method.

Kit- Human Gesellschaft for Biochemica and DiagnosticambH. Wiesbaden,Germany.

Hematological Parameters and Procedure of Estimation

Estimation of Hematological parameter includes HB, PCV, and Differential Leukocyte count 20 (Khaleque, 1982). Estimation of Prothrombin time by using Manual and Automated Determination of Prothrombin Time (PT, QUICK TEST). Kit used Human Gesellschaft for Biochemica and DiagnosticambH. Wiesbaden, Germany.

Microscopic Parameters and Histopathological Analysis

Microscopic examination of the renal tissues of rats was done. At first, gross section of the tissues of the Kidneys (Preserved in 10% formalin containing bottle) was taken. Then the tissues were cut in longitudinal and transverse pieces and processed for preparation of slide¹⁷.

RESULTS

Effect of Fenugreek Seeds Extract on Weekly Weight Gain (gm) and Weight of Kidneys of Albino Rats

After 90 days study, mean body weight of only group D treated with 5 gm/kg/day Fenugreek seeds extract showed a significant (p<0.05) decrease in body weight while comparing to control group. On the other hand, mean weight gain of rats of the Group R on 118th days of study was 79.5 ± 10.5 gm. But there was no significant difference (p>0.05) in the overall weight gain of this group compared to control group. The results of the total weight gain of albino rats and the weight of kidney are shown in Table-I.

Table I : Effect of Fenugreek seeds extract on body weight gain of albino rats and weight of kidneys

Group n=30 (6 in each group)	Day 0	Day 90	Weight gain (gm)		
			Day 118	Total Weight gain (gm)	Weight of Kidneys (gm)
Group A: Control (Distilled water)	181.72 ± 3.8	275.16 ± 6.8	-	93.44 ± 1.9	1.49 ± 0.06
Group B Fenugreek extract 1.25 gm/kg	NS 178 ± 4.9	NS 270.75 ± 6.25	-	NS 92.75 ± 2.2	NS 1.66 ± 0.07
Group C Fenugreek extract 2.5 gm/kg	NS 180 ± 5.8	NS 271.87 ± 7.0	-	NS 91.87 ± 1.6	NS 1.49 ± 0.05
Group D Fenugreek extract 5 gm/kg	NS 185.12 ± 4.3	NS 246.12 ± 5.9	-	* 61 ± 1.2	NS 1.45 ± 0.04
Group R Fenugreek extract 5 gm/kg for 90 days & Distilled water for next 28 days	NS 189 ± 5.5	NS 250.54 ± 7.5	NS 268.5 ± 11.5	NS 79.5 ± 10.5	NS 1.42 ± 0.07

Here, values are expressed as MEAN ± SEM.
*Significant (t-test, p<0.05) and NS= Not significant (p>0.05) compared with control group.

Effect on Serum Creatinine and Urea Level of Albino Rats (mg/dl)

Among the three experimental groups, only the Group D (treated with 5 gm/kg/day for 90 days) showed a significant different (t-test, p<0.05) in mean serum creatinine and urea level when compared with control group. Again in Recovery group (Gr-R) the mean value of serum creatinine was 0.83 ± 0.02 and the blood urea level was 47.3 ± 0.59 mg/dl which was not statistically significant (t-test, p>0.05) when compared with control group. The results are shown in table II.

Table II : Effect of Fenugreek extract on some important serum creatinine and urea level of albino rats

Groups n=30(6 in each group)	Creatinine (mg/dl)	Blood Urea (mg/dl)
Group-A Control (Distilled water)	0.84 ± 0.02	47.5 ± 1.16
Group-B Fenugreek extract 1.25 gm/kg/day	NS 0.82 ± 0.03	NS 48.3 ± 1.40
Group-C Fenugreek extract 2.5 gm/ kg /day	NS 0.83 ± 0.02	NS 46.2 ± 2.2
GROUP-D Fenugreek extract 5 gm/kg/day	* 1.31 ± 0.12	* 53.2 ± 1.47
GROUP-R Recovery group 5 gm/kg for 90 days & Distilled water for next 28 days	NS 0.83 ± 0.02	NS 47.3 ± 0.59

Here, values are expressed as MEAN ± SEM. * Significant (t-test, p<0.05) and NS= Not significant (t-test, p>0.05) compared with control group.

Hematological Parameters

The results of all hematological parameters in all experimental groups were statistically not significant compared to control group in this experiment. It was an indication of absence of any haematotoxic potency of fenugreek seeds up to the highest dose level. The results are shown in Table III.

Table III : Effect of Fenugreek seeds extract on some hematological values of albino rats

Groups n=30 (6 in each group)	Hematological Values				
	Total RBC Count (x 10 ⁹ /mm ³)	Platelet count (x 10 ⁵ /mm ³)	Hemoglobin (gm/dl)	PCV (%)	Prothrombin Time [Sec]
Group-A Control (Distilled water)	5.12 ± 0.05	3.29 ± 0.05	13.40 ± 0.5	44.67 ± 0.8	18.0 ± 0.93
Group-B Fenugreek extract 1.25 gm/kg/day	NS 5.07 ± 0.03	NS 2.98 ± 0.13	NS 13.25 ± 0.36	NS 44.17 ± 0.83	NS 16.0 ± 0.63
Group-C Fenugreek extract 2.5 gm/ kg /day	NS 5.28 ± 0.03	NS 3.16 ± 0.08	NS 13.70 ± 0.29	NS 44.50 ± 0.56	NS 17.0 ± 0.72
Group-D Fenugreek extract 5 gm/kg/day	NS 5.05 ± 0.02	NS 2.97 ± 0.12	NS 13.17 ± 0.33	NS 46.17 ± 1.07	NS 14.83 ± 0.70
GROUP-R Recovery group 5 gm/kg for 90 days & Distilled water for next 28 days	NS 5.13 ± 0.02	NS 3.14 ± 0.06	NS 13.43 ± 0.22	NS 44.42 ± 0.6	NS 15.92 ± 0.52

Here, values are expressed as MEAN±SEM. NS= Not significant (t-test, p>0.05) compared with control group.

Effect of Fenugreek Seeds Extract on Total and Differential Count of W.B.C.

Effect on Total WBC count ($\times 10^3/\text{mm}^3$)

Results of all the experimental groups did not showed any significant change (t-test, p>0.05) in mean Total WBC count when compared with control group. The results are shown in Table IV.

Table IV : Effect of Fenugreek seeds extract on total and differential counts of WBC

Groups n=30 (6 in each group)	Total WBC Count ($\times 10^3/\text{mm}^3$)				
	Neutrophil	Eosinophil	Lymphocyte	Monocyte	
Group-A (Control) (Distilled water)	8.57 ± 0.98	40.67 ± 4.5	4.64 ± 0.49	53.00 ± 4.8	2.17 ± 0.4
Group-B Fenugreek seed extract (1.25 gm/kg)	NS 8.33 ± 0.90	NS 42.00 ± 3.5	NS 5.17 ± 0.41	NS 51.17 ± 3.8	NS 1.67 ± 0.22

Group-C Fenugreek seed extract (2.5 gm/kg)	NS 9.33 ± 0.78	NS 43.67 ± 3.1	NS 5.00 ± 0.36	NS 49.17 ± 3.08	NS 2.33 ± 0.21
Group-D Fenugreek seed extract (5 gm/kg)	NS 6.43 ± 0.81	NS 35.33 ± 2.23	NS 5.17 ± 0.54	NS 57.50 ± 2.12	NS 1.67 ± 0.22
Group-R Recovery group 5 gm/kg for 90 days & Distilled water for next 28 days	NS 7.88 ± 0.46	NS 42.83 ± 2.4	NS 5.11 ± 0.37	NS 53.33 ± 1.43	NS 2.00 ± 0.18

Here, values are expressed as MEAN±SEM. NS= Not significant (t-test, p>0.05) compared with control group.

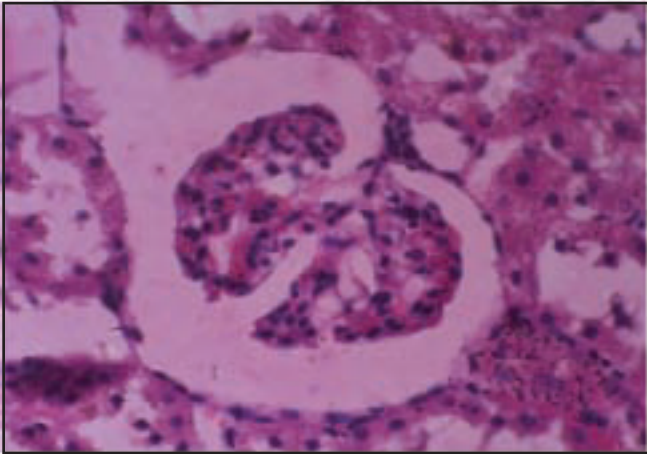
Microscopic Parameters

During gross microscopic examination the following findings were recorded. Histopathological changes of left and right kidney are shown in Table 5.

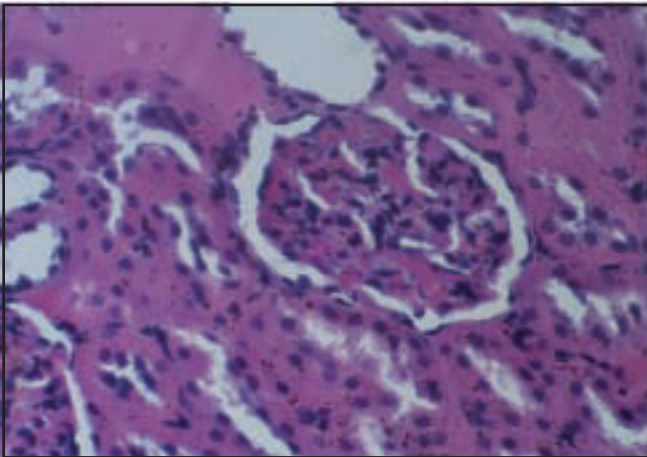
Table V : Summary of changes observed during gross microscopic examination within the Left Kidney (L/K) and Right Kidney (R/K) of albino rats receiving Fenugreek seeds extract for 90 days

Groups n=30 (6 in each group)	Histopathological findings															
	Congestion		Infiltration of mixed inflammatory cells		Hemorrhage		Cellular Swelling		Ischemic Necrosis		Focal Necrosis		Fatty Change		Focal Fibrosis	
	L/K	R/K	L/K	R/K	L/K	R/K	L/K	R/K	L/K	R/K	L/K	R/K	L/K	R/K	L/K	R/K
Group-A Control Group Distilled water	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Group-B Fenugreek extract 1.25 gm/kg/day	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Group-C Fenugreek extract 2.5 gm/kg/day	3	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Group-D Fenugreek extract 5 gm/kg/day	3	4	0	1	3	2	2	1	0	0	3	2	0	0	3	2
Group-R Recovery Group 5 gm/kg for 90 days & Distilled water for next 28 days	2	1	0	0	0	0	0	0	0	0	0	0	0	0	2	2

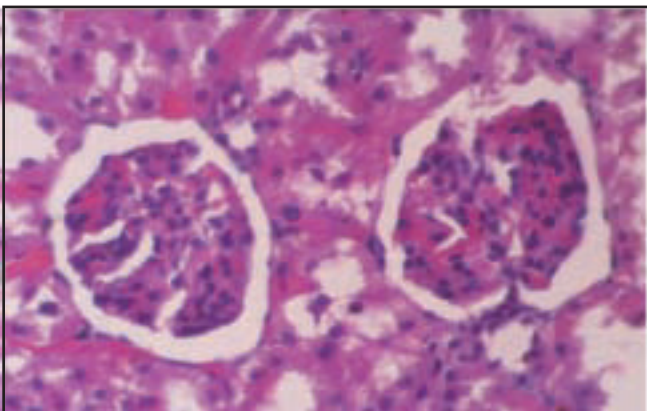
Microscopic findings of renal tissues are shown in photomicrograph no.1,2, 3, 4, 5.



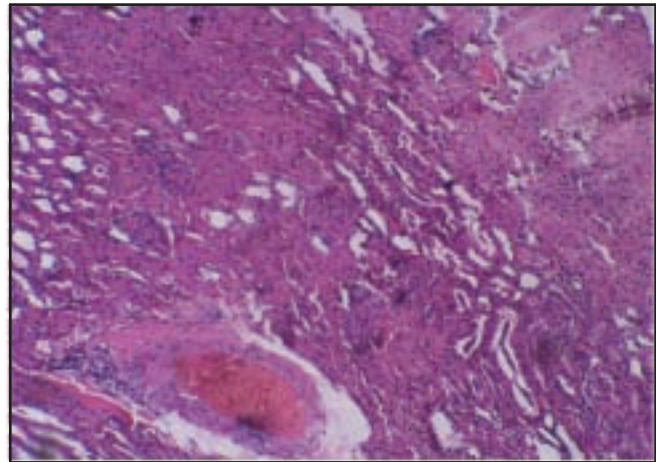
Photomicrograph 1 : Showing renal tissue of rat (GAR-4) of Control group in Sub acute toxicity test revealing no abnormalities. Stain : H & E. 22 X



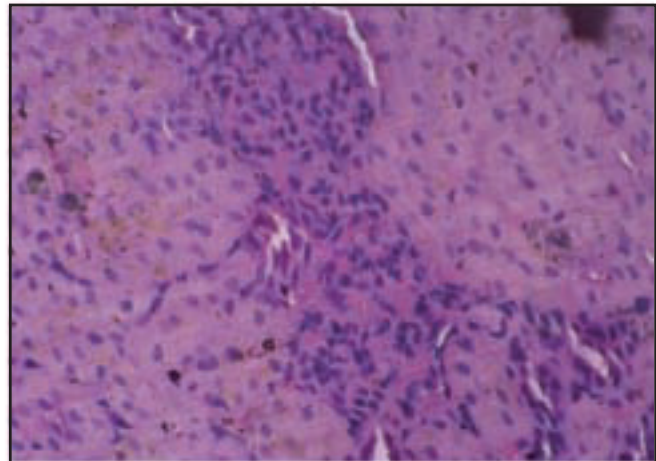
Photomicrograph 2 : Showing renal tissue of rat (GAR-3) treated with 1.25 gm per Kg. per day of Fenugreek seeds extract in sub acute toxicity test revealing area of congestion & mild hemorrhage. Stain : H & E. 220 X



Photomicrograph 3 : Showing renal tissue of rat (GAR-4) treated with 2.5 gm per Kg. per day of Fenugreek seeds extract in sub acute toxicity test revealing no abnormalities. Stain : H & E. 220 X



Photomicrograph 4 : Showing renal tissue of rat (GAR-3) treated with 5 gm per Kg. per day of Fenugreek seeds extract in sub acute toxicity test revealing necrosis, congestion & mild Hemorrhage. Stain : H & E. 220 X



Photomicrograph 5 : Showing renal tissue of rat (GAR-1) treated with 5 gm per Kg. per day of Fenugreek seeds extract for 90 days followed by no treatment in sub acute toxicity test revealing area of focal fibrosis. Stain : H & E. 220 X

DISCUSSION

The medicinal properties attributed to Fenugreek seeds are extensive. The safety is important in relation to its medicinal use. This is the first study conducted by using such a high doses (100 times, 50 times & 25 times greater than suggested therapeutic dose for human) for toxicity assessment of the seeds in rats.

A previous study on toxicity profile of Fenugreek seeds is free from acute toxicity though the studies were done with different doses and methodology^{16, 21}. Acute toxicity study done by Alam SR 2008 suggested that ethanol extract of Fenugreek seed is nontoxic to animals upto 5gm/kg. Usually in shorter term test upto about 3 months of duration, the aim must be to push the dose as high as possible to determine the end organ toxicity¹⁸. There is no need to selection of such a high dose that animals die prematurely in toxicity studies of upto 3 months duration. Such

premature death of animal do not provide better information on the drug effects than dose evaluation of animals that reach the termination of study. So the dosage schedule followed in the work was calculated carefully considering the available information^{16,18,19}. The doses were 1.25 gm/kg/day, 2.5 gm/kg/day and 5 gm/kg/day.

In rodents, a decrease in weight gain is an important sign of deterioration of health. In this experiment, absence of any statistically significant change in weekly weight gain of the rats of the experimental group up to 2.5 gm/kg/day dose level indicate this dose was well accepted by the rats. But statistically significant reduction in weight gain was seen in the group at the highest dose level, which was an indication of adverse effect. This finding was not similar to the previous study. It may be because in previous study, the dose regimens were not so high¹⁶. Again in this study, instead of high dose, the results of the weights of kidneys were statistically not significant and this finding was consistent with the previous report¹⁶.

Blood urea and serum creatinine are two important biochemical tests of renal function²². Again Kidney is the main organ involved in drug elimination and therefore particularly exposed to the toxic effect of exogenous compound²³. In this study, the results of Blood urea and Serum creatinine test were statistically not significant up to 2.5 mg/kg/day dose level. Statistically significant difference in these two biomarkers at 5gm/kg/day dose produced renal toxicity in comparison to control group. But the rats of the recovery group did not show any significant difference when compared with control group. It was a sign of auto recovery of the rats from toxic effects produced at 5 gm/kg/day dose levels. Literature survey did not reveal any parallel report.

The results of all hematological parameters in all experimental groups were statistically not significant compared to control group indicates that no haematotoxic potency of Fenugreek seeds up to the highest dose level. Previous studies also reported this seeds as a non haematotoxic agent^{16,21}.

Microscopic examination of renal tissues of the rats revealed congestion and hemorrhaged both in control & all experimental groups. So any conclusive remark about renal toxicity cannot be made by these two findings. But the presence of other signs of cell injury such as inflammatory cells infiltration, cellular swelling, and necrosis within the renal tissue of the rats only at 5gm/kg /day dose level was an indication of renal toxicity. These microscopic findings also co-relate with the biochemical

findings of renal functions tests (Blood urea, Serum creatinine) in this experiment. No parallel report was available in favor or against this finding. Again almost absence of toxic findings of cell injury in recovery group was an indication of auto recovery of the rats from the toxic effects at the highest dose level.

The present study revealed that long term use of high dose (100 times higher than therapeutic dose) ethanol extract of Fenugreek seeds has nephrotoxic effects in albino rats.

CONCLUSION

This sub-acute toxicity study revealed that Fenugreek seeds extract free from toxic effect up to 2.5 gm/kg/day dose level and induced renal toxicity at 5 gm/kg/day dose level. There was tendency of auto-recovery of the rats from the toxic effects produced at 5gm/kg/day dose level without any fetal ending during the extract free period.

RECOMMENDATIONS

- i) When intended for use at higher therapeutic doses for better efficacy, toxicity profile of the seeds require re-evaluation by further sub-acute toxicity study, using different experimental animals and methodology
- ii) Chronic toxicity studies (More than 6 months) should be performed before starting clinical toxicity study
- iii) Specific type of preclinical toxicity tests like teratogenicity test, reproductive test, carcinogenicity test should be performed before starting clinical toxicity test
- iv) Study should be done to find out the active constituents within the seeds or its metabolites that were responsible to produce toxicity in this study.

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DISCLOSURE

All the authors declared no competing interests.

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