

ASSESSMENT OF ACUTE LIVER TOXICITY OF *TRIGONELLA FOENUM-GRÆCUM* (FENUGREEK) SEEDS AQUEOUS EXTRACT IN MALE MICE

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ABSTRACT

Data on acute toxicity and safety of fenugreek still not sufficient. Hence the objective of this study is to investigate the acute toxicity of fenugreek seeds aqueous extract (FSA) in vivo. Twelve male Swiss albino mice, were randomly divided into control (C), and three treatment (T₁, T₂, and T₃) groups (n = 3 each). T₁, T₂, and T₃ were given 3gm, 6gm, and 9gm/kg body weight FSA respectively. Intra-gastric divided doses of FSA were given as per OECD guidelines 425. Continuous observation of signs of acute toxicity and survival set up. Body weight was measured every 3 days, blood glucose level was measured after 6 hours, then on days 3, 7, and 14. Liver function test was measured on days 3 and 14 which is the day of sacrificing the mice. Liver was dissected and processed for histopathological examination. Administration of 9g/kg body weight of FSA showed 66.7% survival rate, while the lower FSA doses showed 100% survival. 3, 6, and 9g/kg body weight FSA failed to induce any signs of acute toxicity. Furthermore, no significant effect shown on mice body weight, blood glucose level and liver enzymes. Histologically, all FSA administered doses showed mild portal inflammation, mild mononuclear cell infiltration in hepatic parenchyma, in addition to mild bile stasis induced only in mice received 9g/kg of FSA. Conclusion: FSA showed minimum lethal oral dose and mild liver histopathological inflammatory changes.

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1. Introduction

Different pharmaceutical dosage forms of fenugreek are available these days as a herbal drug for medical uses in treatment of: bronchitis, abscess, diabetes, hypercholesterolemia, and as a protective drug for liver against lipid accumulation (Al-Ashban, Abou-Shaabab, & Shah, 2010), and for kidney against diabetic nephropathy (Shetty & Salimath, 2009). Due to its documented historical and traditional use as a spice and medicinal herb in various parts of the world, fenugreek has been granted “generally recognized as safe” (GRAS) status by the U.S. food and drug Administration (FDA)-SP/ESO, GRAS - 182.10, 182.20 (U.S. Food and Drug Administration, 2006). Significant clinical harmful adverse effects (in human), due to consumption of fenugreek as a food or medicinal supplement have not been reported (Basch, Ulbricht, Kuo, Szapary, & Smith, 2003). But data of safety of different fenugreek extracted forms, and the acute & chronic toxicity doses still not anticipating the increasing medical use of fenugreek. Various pharmacologically active compounds with different concentrations have been isolated from fenugreek seeds such as: Alkaloids, flavonoids, tannin like phenolic compounds, polyphenols, steroids, saponins, free amino acids, unusual

amino acid 4-hydroxyisoleucine, lipids, phospholipids, mucilaginous fibers, vitamins, and minerals (Lee, Bandara, Driedger, Acharya, & Thomas, 2011), some functions of these active compounds are known, but many still unknown. Even though no fenugreek adverse effects on human has been reported to date, but testing fenugreek toxicity in animal models is the first step to open the window for future clinical trials, to investigate safety of fenugreek for applied medical uses. Fenugreek seeds aqueous extract (FSA) has been tested for many therapeutic uses, therefore toxicological evaluation in laboratory animals is needed before FSA is recommended to be safe for human usage, hence this study aimed to investigate the FSA acute toxicity on liver in male mice model.

2 Materials and Methods

2.1 Animals

Twelve Swiss albino male mice were purchased from Sapphire enterprise (Selangor -Malaysia). Animals were aged 6 to 7 weeks, weighing 25 to 26 grams, housed as a single animal per cage, in temperature of 24°C, with relative humidity 60 ± 5%, with 12-hour dark / light cycle, and the mice had free access to water ad libitum and left for one week for acclimatization to the new environment before starting the

experiment. The experiment carried out through 14 days, and conducted according to OECD guide lines 425 (2008). The experimental protocol was approved by the International Islamic University Malaysia Institutional Animal Care and Use Committee (IACUC-IIUM) IIUM (No. of IACUC Approval: IIUM / IACUC Approval /2016 / (11) (68)).

2.2 Preparation of fenugreek seeds aqueous extract (FSA)

A kilogram of fenugreek seeds was purchased from local market in Yemen (Rada'a market). The fenugreek seeds were identified and verified by Botanist at Biodiversity Unit, institute of Bioscience University Putra Malaysia in cooperation with Herbarium department Kulliyah of Pharmacy IIUM, voucher specimen identification was deposited at the Herbarium, Kulliyah of Pharmacy under voucher specimen No.: PIIUM 0226-2. Using a modified method of traditional medicinal practitioners, FSA extract was prepared (Khalki, M, Bennis, Chait, & Sokar, 2010). The prepared fenugreek seed powder was put in distilled water in a ratio of 1 gram of powder to 20 ml of distilled water, then stirred for 24 hours using magnetic stirrer at room temperature. Then, the aqueous extract was transferred into 50 ml falcon tubes and centrifuged at 10000 rpm for 5 minutes using centrifuge machine (Hettich UNIVERSAL 320R, Germany), the yield of the extract was then frozen at -80°C in (Haier ULT FREEZER, China). The extract then put in freeze dried machine for 7 days (Freeze Alpha 1-2LDplus CHRIST, Germany). Then FSA powder form, was stored in -20°C freezer (Haier freezer, China) until use.

2.3 Fenugreek seeds aqueous extract doses and administration

Based on: Evaluated maximum tolerated dose of fenugreek (MTD) (9.77 g/kg) (Al-Yahya, 2013), LD_{50} of fenugreek leaves aqueous extract (10 g/kg) (Abdel-Barry, Abdel-Hassan, & Al-Hakim, 1997), previously tested maximum safe dose of fenugreek (3g/kg) (Al-Ashban et al., 2010), and OECD guidelines 425 (OECD, 2008) for testing chemicals, the following doses of fenugreek seed aqueous extract were selected for acute toxicity study: 3g/kg, 6g/kg, and 9g/kg. There were three treatment groups of Swiss albino male mice (3 per group), and one control group. FSA was administered once in divided doses in the first day of the experiment, with continuous observation for 14 days post treatment. At the end of the experiment (14 days) all mice were euthanized and liver was dissected for histopathological processing after macroscopic examination.

2.4 Measurements, blood collection, biochemical tests, and histopathological processing

Survival rate measurement was recorded throughout the period of study, body weight for each animal was recorded every 72 hours, blood glucose was taken from mouse tail after 6 hours of the last FSA dose, and then on days 3, 7, and 14 of the experiment measured using glucometer (MEDISAFE MINI Blood Glucose Reader, Japan). On day 3 of the experiment; blood for measurement of liver function test (LFT), was collected from the facial vein. On the last day of

the experiment which is day-14 and under general anaesthesia, blood sample for LFT was collected by cardiac puncture, LFT was analysed by Flex[®] reagent cartridge, SIEMENS USA. The dissected liver was grossly examined for: surface smoothness, colour change, and any abnormal growth, and then immediately was fixed in 10% neutral buffer formalin for histopathological study. The histopathological processing was done according to the histopathology laboratory work procedure and safety guidelines of IIUM pathology department using Haematoxylin and Eosin staining (Mkri, Muda, & Kasmur, 2011).

2.5 Statistical analysis

The raw data of these results were analysed by SPSS statistic software version 15. The level of statistical significance was taken at 95% confidence interval, ($p < 0.05$). Results were represented as mean and standard deviation (SD). The mean comparisons were performed using analysis of variance repeated measures (ANOVA) for normally distributed data, and the differences between each pairs of the study groups were done by LSD Post-hoc test. The data that were not normally distributed were analysed by the nonparametric test Kruskal-Wallis test for mean rank and significance difference between the treated and the control groups. Data for survival rate were analyzed using Kaplan-Meier analysis followed by log rank (Mantel-Cox) test.

3. Results

3.1 Acute toxicity effect of FSA- mice survival analysis

No significant difference between treatment and control groups in survival rate ($p = 0.180$). Observation throughout the experimental period of this work (14 days) showed the following results: the overall survival rate was 91.7%. It was 100% in T_1 (treatment group 1 which received single dose of 3g/kg body weight FSA), and T_2 (treatment group 2 which received single dose of 6g/kg body weight FSA), and 66.7% survival rate in group T_3 (treatment group 3 which received single dose of 9g/kg body weight FSA) (Table 1).

Table 1. Summary of survival percentage of mice in experimental groups.

Groups	Total	N _B of events	Censored	
			N	Percent
Control	3	0	3	100.0%
T_1 (3g/kg)	3	0	3	100.0%
T_2 (6g/kg)	3	0	3	100.0%
T_3 (9g/kg)	3	1	2	66.7%
Overall	12	1	11	91.7%

3.2 Acute toxicity effect study of FSA- mice body weight analysis

No significant difference in the body weight of mice in the treatment groups when compared to control group throughout 14 days of experiment (Table 2). Group T_3 showed lower mean body weight as compared to the control and other treatment groups but it was statistically non-significant (T_1 (3g/kg) versus control $p = 0.336$, T_2 (6g/kg) versus control $p = 0.958$, and T_3 (9g/kg) versus control $p = 0.100$).

Table 2. Effect of acute toxicity of fenugreek seeds aqueous extract on body weight throughout 14 days.

Group Day	Control			T_1 (3g/kg)			T_2 (6g/kg)			T_3 (9g/kg)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Day 1	3	31.00	1.73	3	30.33	3.51	3	33.67	3.21	3	27.50	2.12
Day 3	3	33.00	1.00	3	30.00	2.65	3	33.67	2.08	2	28.00	0.00
Day 6	3	34.00	1.00	3	30.33	3.51	3	32.00	3.00	2	28.50	0.71
Day 9	3	34.00	2.00	3	31.33	4.04	3	34.00	3.61	2	32.00	0.00
Day12	3	34.00	1.73	3	32.67	3.51	3	34.67	3.51	2	29.00	1.41
Day14	3	34.00	1.73	3	32.67	3.51	3	34.67	3.51	2	29.00	1.41

Table 3. Effect of acute toxicity of fenugreek seeds aqueous extract on blood glucose level 6 hours after treatment, days 3, 7, and 14 of the experiment.

Group Day	Control			T ₁ (3g/kg)			T ₂ (6g/kg)			T ₃ (9g/kg)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
6 hours after treatment	3	9.33	0.93	3	9.63	0.64	3	8.50	2.05	2	7.10	0.99
Day 3	3	9.23	1.17	3	8.63	0.42	3	7.63	1.67	2	7.20	3.11
Day 7	3	6.67	2.20	3	7.77	2.33	3	7.30	1.01	2	7.90	0.00
Day 14	3	6.57	0.46	3	7.23	0.49	3	7.17	1.00	2	4.75	1.62

3.3 Acute toxicity effect study of FSA- biochemical analysis

3.3.1 Blood glucose

The present study showed no statistically significant difference in blood glucose level between the treatment and the control groups after administration of single dose of fenugreek seeds aqueous extract at doses of 3g/kg, 6g/kg, and 9g/kg to the groups T₁, T₂, and T₃, respectively. Post Hoc LSD test showed no significant statistical difference (T₁ (3g/kg) versus control $p = 0.675$, T₂ (6g/kg) versus control $p = 0.731$, and T₃ (9g/kg) versus control $p = 0.237$) as demonstrated in table 3.

3.3.2 Serum level of liver enzymes

No statistically significant difference in serum liver enzymes, between the treatment and the control groups $p > 0.05$, table 4.

Table 4. Effect of fenugreek seeds aqueous extract on liver enzymes in the serum on days 3 and 14 of the experiment.

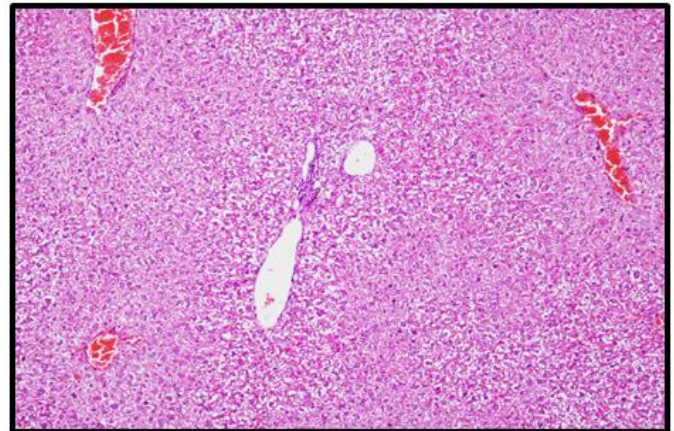
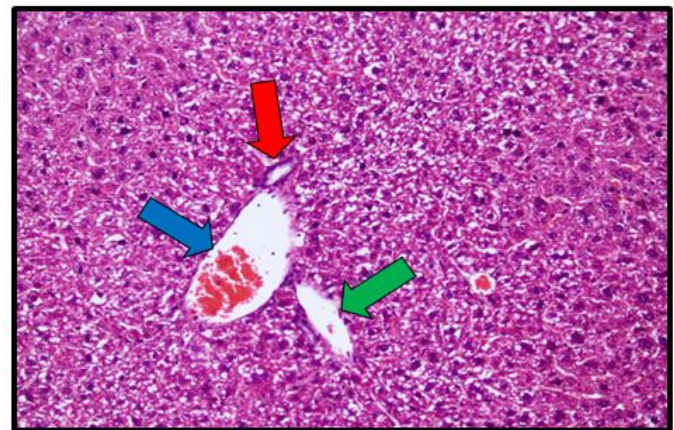
Parameters		Animal groups			
		Control	T ₁	T ₂	T ₃
Day 3	ALT	64.00±26.67	101.33±75.58	162.67±105.14	89.00±65.05
	AST	178.00±62.22	229.00±114.89	379.67±106.25	156.00±186.68
	ALP	167.33±36.89	167.33±65.68	121.50±60.10	116.67±94.36
	GGT	4.17	7.17	3.50	5.83
Day 14	ALT	47.00±30.61	86.67±10.69	71.33±17.24	70.50±57.28
	AST	231.33±83.73	228.67±40.82	187.33±37.99	280.50±77.07
	ALP	126.67±19.60	129.67±53.59	122.67±51.05	84.50±81.32
	GGT	3.33	7.83	6.17	7.00

3.4 Acute toxicity effect study of FSA- on liver histology

Table 5; summarizes the histopathological findings in the liver of the experimental animals. Group T₁ (received FSA dose 3g/kg body weight) and group T₂ (received FSA dose 6g/kg body weight) when compared to the control (Figure 1A and Figure 1B), showed mild portal inflammation, with minimal hepatic parenchymal mononuclear cell infiltration as shown in figures (1C to 1F).

Group T₃ (received FSA 9g/kg body weight) when compared to the control, showed mild portal inflammation, mild liver parenchymal mononuclear cell infiltration, with

mild bile stasis, however no steatosis was observed (Figure 1G and Figure 1J).

**Figure 1A. Photomicrograph of liver histology from control group (Hematoxylin and Eosin stain, 100x magnification).****Figure 1B. Photomicrograph of liver tissue from control group. It shows portal triad (red arrow - hepatic artery, blue arrow - portal vein, green arrow - bile duct. (Hematoxylin and Eosin stain, 200x magnification).****Table 5. Acute toxicity effects of fenugreek seeds aqueous extract on liver histopathology.**

Histopathological change Group	Portal inflammation	Mononuclear cell infiltrate	Steatosis	Bile stasis
Control	Absent	Absent	Absent	Absent
T ₁ (3g/kg)	Mild (+1)	Mild (+1)	Absent	Absent
T ₂ (6g/kg)	Mild (+1)	Mild (+1)	Absent	Absent
T ₃ (9g/kg)	Mild (+1)	Mild (+1)	Absent	Mild (+1)

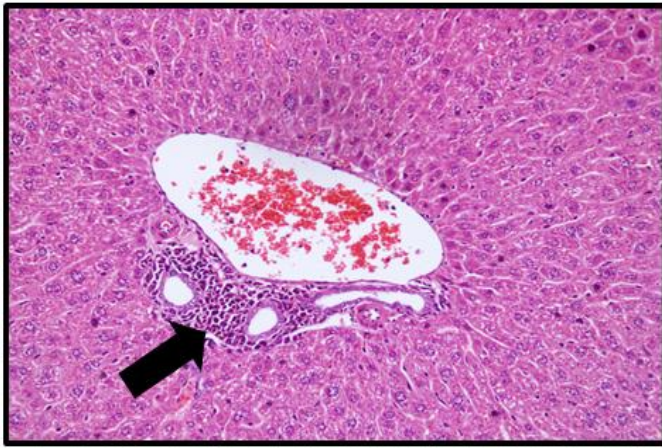


Figure 1C. Photomicrograph of liver tissue from treatment group T₁ (received Fenugreek Seeds Aqueous extract 3g/kg body weight), shows mild portal inflammation as seen in the portal triad and indicated by the arrow. (Hematoxylin and Eosin stain, 200x magnification).

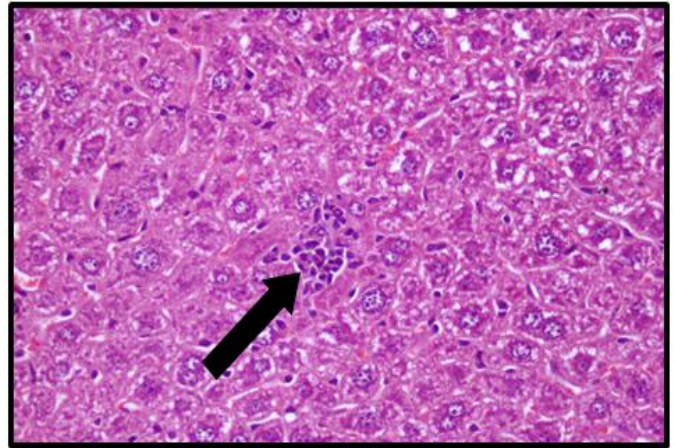


Figure 1F. Photomicrograph of liver tissue from treatment group T₂ (received Fenugreek Seeds Aqueous extract 6g/kg body weight), shows mild mononuclear cell infiltrate in hepatic parenchyma as indicated by the arrow. (Hematoxylin and Eosin stain, 400x magnification).

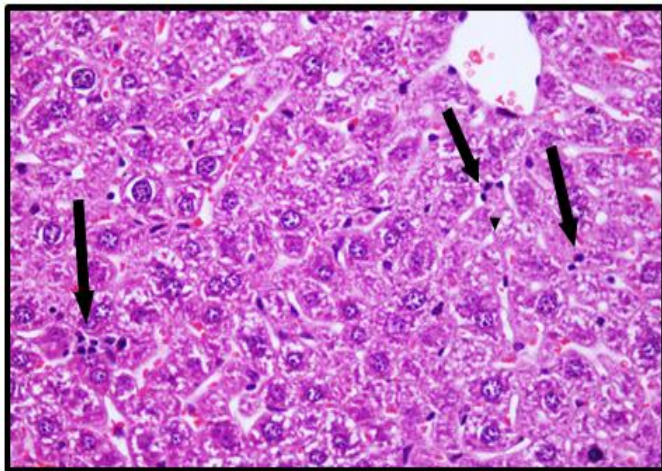


Figure 1D. Photomicrograph of liver tissue from treatment group T₁ (received Fenugreek Seeds Aqueous extract 3g/kg body weight), shows mild mononuclear cell infiltrate in hepatic parenchyma as indicated by the arrows. (Hematoxylin and Eosin stain, 400x magnification).

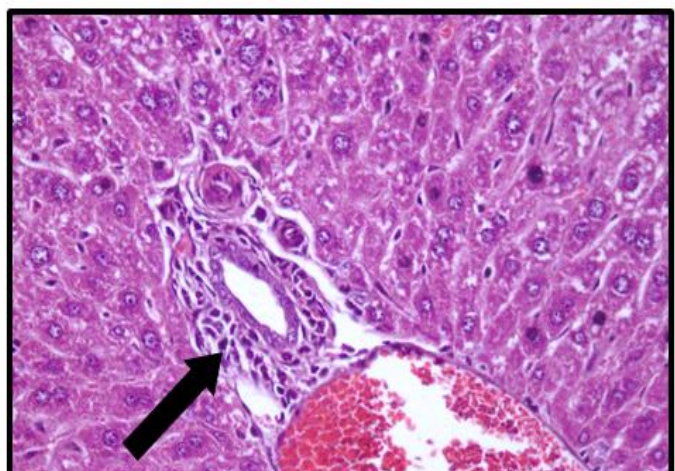


Figure 1G. Photomicrograph of liver tissue from treatment group T₃ (received Fenugreek Seeds Aqueous extract 9g/kg body weight), shows mild portal inflammation as indicated by the arrow. (Hematoxylin and Eosin stain, 400x magnification).

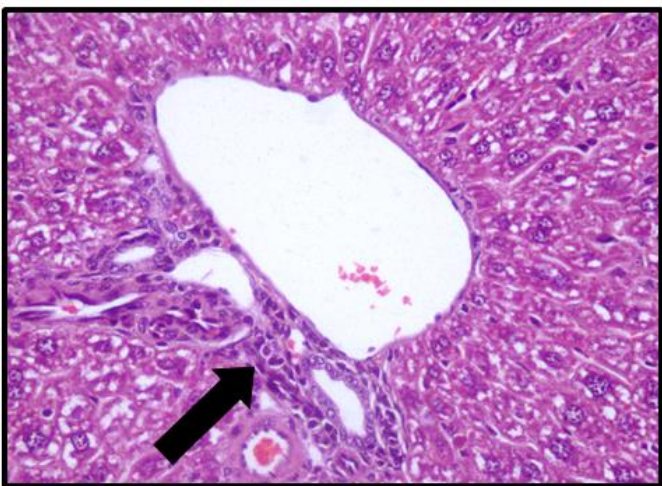


Figure 1E. Photomicrograph of liver tissue from treatment group T₂ (received Fenugreek Seeds Aqueous extract 6g/kg body weight), shows mild portal inflammation as indicated by the arrow. (Hematoxylin and Eosin stain, 400x magnification).

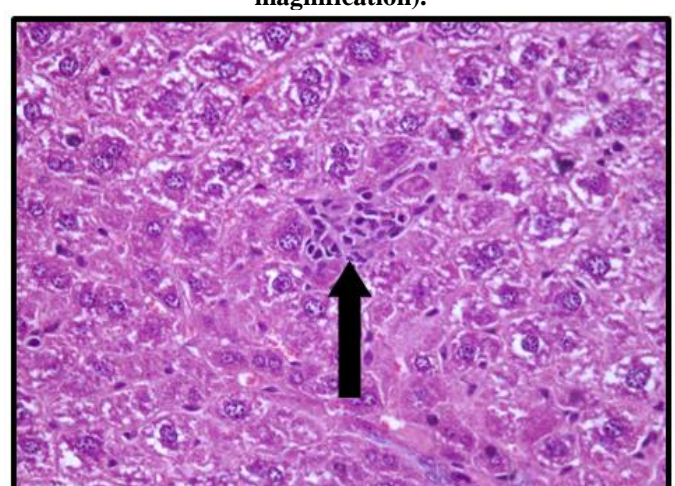


Figure 1H. Photomicrograph of liver tissue from treatment group T₃ (received Fenugreek Seeds Aqueous extract 9g/kg body weight), shows mild mononuclear cell infiltration in liver parenchyma as indicated by the arrow. (Hematoxylin and Eosin stain, 400x magnification).

4. Discussion and Conclusion

Measurement of survival rate is a corner stone in any toxicity study according to (OECD, 2008) guidelines. In the current study none of the mice were died in the experimental group treated with 3g/kg body weight dose level. This finding is in a good agreement with early acute toxicity study conducted on fenugreek seeds ethanol extract which reported that no lethality at dose of 3g/kg body weight in mice (Al-Ashban et al., 2010). Same results also were reported by Mowla and his co-workers who were used ethanol extract of fenugreek seeds, which indicate reproducibility of safety of fenugreek at 3g/kg body weight in experimental animals as far as the survival is the concern (Mowla, et al. 2009). Also no death was recorded in mice treated with 6g/kg body weight, whereas in the group of mice treated with 9 g/kg body weight, one mouse out of three was died in the third day of experiment. These results are supported by a study which was done on fenugreek leaves aqueous extract showed that LD₅₀ of oral dose was 10g/kg body weight (Abdel-Barry et al., 1997), also supported by another investigation documented the maximum tolerated dose of fenugreek was 9.77g/kg body weight (Al-Yahya, 2013), hence, the present study can consider that the minimum lethal dose (MLD) of FSA is 9g/kg body weight. However our findings on animal survival rate were different from results obtained by Kandhare et.al (2015) where they studied acute toxicity on mice using glycosides based standardized fenugreek seed extract at a dose of 5g/kg body weight, they reported 40% mortality rate and concluded median lethal dose (LD₅₀) is more than 4350mg/kg body weight (Kandhare, Bodhankar, Mohan, & Thakurdesai, 2015a).

According to the present study, fenugreek seeds aqueous extract may affect the survival rate at dose of 9g/kg body weight as an oral dose. This could be due to toxic effect of fenugreek on the liver viability. Kandhare et al. (2015) reported that mortality was attributed to toxic effects of characterized glycosides of fenugreek on the liver according to their biochemical and histological findings. In the current study, the aqueous extract of fenugreek as a natural mixture appeared to have a wide safety range, and less toxic in the given doses.

Present study was conducted to assess the effect of FSA on male mice body weight because it is a vital indicator of toxicity effect as stated by OECD guidelines (OECD, 2008). The observed body weight pattern in the current experimental study indicated that all tested fenugreek seeds aqueous extract oral doses have no statistical significant effects on male mice body weight. However, mean body weight is observed to be the lowest in the animals treated with the highest fenugreek dose when compared to the control and other treated groups. This indicate accumulative effect of fenugreek dose which altered body weight due to reduction in the food intake.

Earlier study conducted by Muralidhara et al. (1999) showed no effect on body weight when tested fenugreek powder dose of 2g/kg body weight. This supports our consideration that, fenugreek seeds aqueous extract has no acute effect on body weight at a higher doses. Current study results are not agreed with the results published by Kandhare et al.(2015) which showed a significant reduction in body weight when mice treated with a single oral dose 5g/kg body weight (which is lower than our tested doses) of characterized glycosides extract of fenugreek. In their explanation of mechanism of action, they attributed the reduction in body

weight to the effect of glycosides in reducing food intake (Kandhare et al., 2015).

Reduction in body weight due to fenugreek has been approved by many studies but the duration of all of those studies were sub-acute and chronic toxicity studies using lower fenugreek doses. The mechanisms in those cases were recognized to the bioactive ingredients of fenugreek in reducing food intake (Etsuko Muraki, et al. 2011).

Present acute toxicity study showed no statistical significance effect of FSA in high doses on male mice blood glucose level. None of the treated groups were exhibited a significant dropping in blood glucose level when compared to the control. Mowla et al. ,(2009) was studied the effect of fenugreek on the glycaemic control and was found that the fenugreek effect is non-dose dependent effect (Mowla et al., 2009). They found that 1g/kg body weight is more effective in lowering blood glucose levels than dose of 3g/kg body weight. These findings support results which reported in the present work. Another study showed the effective dose of ethanol extract of fenugreek seeds to decrease blood glucose level was 0.5g/kg body weight and was taken as the pharmacologically active dose of fenugreek seeds ethanol extract. Same study (Al-Ashban et al., 2010), showed that a fenugreek dose of 3g/kg was not as effective as 0.5g/kg or 1g/kg body weight (Al-Ashban et al., 2010) which further support the results of the present study.

In a comparative study between fenugreek seeds aqueous extract and the methanol extract; they found that 0.5 and 1g/kg body weight in case of aqueous extract caused a significant hypoglycaemic effect, however the effective hypoglycaemic dose for methanol extract was 1g/kg body weight when administered orally in normoglycemic mice (Zia, Hasnain, & Hasan, 2001) .

Investigation of present study comes also in a good agreement with a report published by Kandhare, et al. in 2015 they were used glycoside based fenugreek seeds extract at a dose of 5g/kg body weight and showed no significant effect on blood glucose at this high tested dose (Kandhare, et al. (2015). The possible mechanisms by which fenugreek seeds can reduce blood glucose is by interference of FSA with carbohydrate digestion, furthermore by direct effect on digestive enzymes and by reducing glucose absorption (Zia et al., 2001). In the view of acute toxicity studies reduction in blood glucose can be due to acute liver injury (Andreu et al., 1998).

Assessment of liver is crucial in toxicity studies because of its physiological as well as its pathological importance, since the liver is the first organ exposed to any absorbed substance (Barrett, et al., 2010). Biochemical analysis of liver function by measurement of transaminases is considered as the main indicator of liver status, in addition to liver histopathological assessment (Erejuwa et al., 2011). Surveys of the present study did not report a statistical significant difference in biochemical liver function between the treatment and control groups. However, all treatment groups showed high mean transaminases (ALT and AST) when compared to the control group which might be indicated some sort of liver cell injury. The opposite response was observed in mean ALP, where the treatment group which were received the highest dose of fenugreek extract showed the lowest level of ALP in comparison to the control and the other two treatment groups. On the other hand, GGT enzyme which is the sensitive indicator of biliary system injury showed high mean levels in

dose dependent pattern which may indicate hepatic biliary system involvement.

Histologically the current study showed evidence of mild hepatic inflammation in the form of portal inflammation and mononuclear cell infiltrate, in all treatment groups, in dose dependent pattern. In addition to the mentioned findings, the treatment group which received dose of 9g/kg fenugreek extract showed very mild bile stasis. The biochemical results of the present study correlate with the mild histopathological finding for the evidence of mild toxic effect of high doses of fenugreek seeds aqueous extract on the liver. A previous study (Kandhare et al., 2015) tested acute toxicity of fenugreek seeds in a serial doses from 55mg/kg body weight to 5000mg/kg body weight as a single dose and they concluded no significant statistical difference of liver enzymes and this supports the results of the current study.

However, Kandhare et al., (2015) found a significant biochemical and histopathological liver changes on repeated dose 28 days (sub-acute study) with oral administration of 1000mg/kg body weight glycoside based fenugreek extract (Kandhare et al., 2015). Another earlier study of acute toxicity used 2g/kg fenugreek powder showed no biochemical or histopathological abnormality in liver (Muralidhara, et al., 1999).

The results of the present study showed possible mild toxic effect on liver by doses of 3g, 6g, and 9g/kg body weight of FSA extract in male mice which could be due to induction of hepatitis, or might be due to altered gene expression induced cell injury (P Sharma, Singh, & Bhat, 2014).

Conclusion

In the present study, administration of fenugreek seeds aqueous extract at doses of 3g, 6g, and 9g/kg body weight resulted in mild (but not significant) elevation of liver enzymes which was supported histopathologically by the presence of mild portal inflammation, mononuclear cell infiltration in liver parenchyma, and mild bile stasis. On the other hand, our study concluded that, fenugreek seeds aqueous extract in all given doses showed no significant effects on survival rate, body weight, blood glucose level, in male Swiss albino mice.

Finally, the current study also concluded that, the lowest observed adverse effects level (LOAEL) of FSA extract could be 3g/kg body weight, the no observed adverse effects level (NOAEL) is below 3g/kg body weight, the maximum tolerated (MTD) dose is 9g/kg body weight, and minimum lethal dose (MLD) is 9g/kg body weight. Based on the current study it is useful to calculate the human equivalent dose (HED) in future study from doses lower than 3g/kg body weight. Thus sub-acute (28 days), sub-chronic (90 days), and chronic studies (more than 90 days) with large sample size are recommended, with further molecular level studies to explore the mechanisms of fenugreek toxicity.

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7. Conflict of Interest

None declared.

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