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Evaluation of the developmental toxicity of the aqueous extract from *Trigonella foenum-graecum* (L.) in mice

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ABSTRACT

Aim of the study: The use of medicinal plant products to treat various ailments is a common practice in many developing countries. However, a lack of information on the adverse effects of these plants raises questions on their safety and possible adverse side effects. This study was undertaken to evaluate the potential toxic effects of fenugreek seeds on pregnant mice and foetal development.
Materials and methods: Lyophilized aqueous extract from fenugreek seeds (LAE-FS) was administered to mated female mice during the entire period of pregnancy, at doses of 500 and 1000 mg/kg/day. Females were examined for standard parameters of reproductive performance. Foetuses were weighed and examined for externally visible malformations.
Results: In pregnant females, there were no obvious symptoms of toxicity, LAE-FS-related deaths or macroscopic abnormalities. Developmental toxicity in offspring included an increase in the foetal death rate, a decrease in the litter size, and a reduction in the foetal body weight. In addition there was an increase in the incidence of morphological abnormalities.
Conclusions: Based on these results, it was concluded that fenugreek seeds extract may have deleterious toxic effects on reproductive performance and potential teratogenic effects in foetuses.

1. Introduction

Trigonella foenum-graecum L. (fenugreek) is an annual plant belonging to the family of Papilionaceae. It is extensively cultivated in India and northern Africa. Seeds and leaves of this plant have been used for centuries not only as food but also as an ingredient in traditional medicine (Petropoulos, 2002). Fenugreek seeds are known for various medicinal purposes. In Morocco, it is used as a tonic, as a remedy against stomach disorders, diabetes, fever, anaemia, constipation, as a galactogogue and for stimulation of appetite (Bellakhdar, 1997). Many of these traditional uses of fenugreek are supported by data from pharmacological studies. Fenugreek is reported to have anti-diabetic (Jayadev et al., 2001; Thakran et al., 2004; Kumar et al., 2005), hypocholesterolaemic (Abdel-Barry et al., 1997), antifungal (Haouala et al., 2008), anti-bacterial (Sudar and Kirti, 2006), immunomodulatory (Ramesh et al., 2002; Bin-Hafeez et al., 2003), anti-inflammatory and antipyretic (Ahmadiani et al., 2001) effects.

However, fenugreek seeds have also been identified as potentially allergenic (Kruse Fæste et al., 2009) and have an anti-fertility effect in rabbit (Kassem et al., 2006). Cases of pronounced congenital malformations such as hydrocephalus, anencephaly and spina bifida were found among women who consumed fenugreek seeds during pregnancy in Morocco (Skalli, 2006).

The present study was undertaken to evaluate foeto-toxic and teratogenic effects of aqueous extract from *Trigonella foenum-graecum* L. seeds in female albino mice and foetal development.

2. Materials and methods

2.1. Plant collection and identification

Trigonella foenum-graecum seeds were collected in the area of Settat, Morocco, in November 2008. The plant material was identified in the Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University. A voucher specimen ($n = 5511$) was deposited at the herbarium of the mentioned faculty.

2.2. Preparation of extract

Using a modified method of traditional medicinal practitioners, an aqueous extract was prepared. Seeds were ground to fine powder using a grinding machine. Powdered seeds were agitated in distilled water (1 g powder/20 ml water) for 10 h. The aqueous extract obtained was centrifuged and the supernatant was

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lyophilized. The yield of the extract was 20% (w/w in terms of powder material). The lyophilized aqueous extract was stored at −20 °C until further use.

2.3. Experimental animals

Male and female Swiss mice raised in the Animal facility of the Faculty of Science were used. The average weight was 29 ± 2 g. The mice were kept under constant conditions of ambient temperature (22 ± 2 °C) under a 12 h light/12 h dark cycle, with access to food and water *ad libitum*.

Animal procedures were conducted in strict compliance with approved institutional protocols and in accordance with the provisions for animal care and use described in the Scientific Procedures on Living Animals ACT 1986 (European Council directive: 86/609 EEC). Care was taken to minimize the number of animals used for the experiments.

2.4. Mating procedure

Virgin female mice at pro-oestrus phase were kept with male in the ratio of 2:1 overnight (12 h). The following morning, the presence of a vaginal plug indicated that mating had occurred. Confirmation corresponded to day 0 of pregnancy. This mating procedure was repeated until enough mated females were available, which were then assigned to the experimental groups in random order.

2.5. Treatment of the animals

The pregnant mice were divided into three groups of five animals each. The control group was administered distilled water and the treated groups received 500 and 1000 mg/kg/day of a 25% extract solution of LAE-FS. The aqueous extract was administered orally by gavages once daily during gestational period.

2.6. Maternal observation

Mice were observed at least once daily for changes in behaviour, general health condition and signs of pharmacological effects. Mortality, morbidity, abortion or premature delivery and body weight were recorded daily from day 0 until delivery. The presence of vaginal bloodstains on day 13 of gestation was examined to detect any signs of abortion and/or foetal expulsion.

2.7. Postnatal development evaluation of the offspring

All females were allowed to give birth. Foetal parameters quantified were: litter size, foetal viability, body weight and weight gain on day 0, 7, 14, and 21 of development. Each pup was checked for signs of malformations and abnormal morphological changes. For each group the following indices were calculated: female pregnancy index (number of pregnant females/number of vaginal plug positive females × 100), delivery index (number of females delivering/number of pregnant females × 100), live-birth index (number of live offspring/number of offspring delivered × 100), viability index (number of live offspring at lactation day 4/number of live offspring delivered × 100) and weanling index (number of live offspring at day 21/number of live offspring born × 100).

2.8. Brain weight

At postnatal day 28, mice were anesthetized with ether and perfused intracardially with 4% paraformaldehyde in PBS. The brain was removed, weighed, and stored in 4% paraformaldehyde solution.

2.9. Statistical analysis

The results were presented as mean values ± S.E.M. Statistical significance between groups was performed by one-way analysis of variance (ANOVA) follow by Student–Newman–Keuls multicomparison test. Proportions were analyzed by the *t*-test. Statistical evaluation was performed using Sigma Stat programs, and a difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Prenatal evaluation of maternal parameters

No death or treatment-related signs of abnormal behavioural changes were observed in the females. None of the pregnant mice showed vaginal bleeding or expulsions of foetal products. There were no significant differences in the body weight gains of dams treated with LAE-FS at the doses of 500 and 1000 mg/kg/day compared to the control group ($F_{(2,14)} = 0.701$; ns) (Table 1). In addition, the period of gestation did not differ ($F_{(2,14)} = 0.142$; $P = 0.869$) between control and Fenugreek seed treated groups. However, the ratio of pregnant per vaginal plug positive females (pregnancy index) showed a significant dose-depend decrease between the higher dose group and the control ($t = 3.038$; $P = 0.01$) (Table 1).

3.2. Reproductive index of mice exposed to plant extract and pups body weight

Delivery, viability and weaning index of LAE-FS treated groups did not differ compared to the control group (Table 2). On the other hand, the live-birth index was significantly reduced

Table 1
Fertility outcomes following exposure of pregnant mice to fenugreek seeds aqueous extract during pregnancy.

Parameters	Fenugreek seeds aqueous extract (mg/kg/day)		
	Control	500	1000
Plug vaginal positive females, *n*	7	8	10
Pregnant females number	5	5	5
Aborted mice number	0	0	0
Pregnancy index (%)[a]	71	63	50**
Maternal weight day 0 (g)	29.50 ± 2.56	26.28 ± 1.62	26.30 ± 1.34
Maternal weight day 20 (g)	43.74 ± 9.62	44.1 ± 1.75	44.96 ± 1.64
Maternal weight gain (g), day 0–20 (g)	14.23 ± 6.47	17.82 ± 5.88	18.25 ± 5.26
Length of pregnancy (days)	20.2 ± 0.83	20.25 ± 1.7	19.8 ± 1.64

Data are given as means ± S.D.
*Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; $P < 0.05$.
[a] (Number of pregnant females/number of vaginal plug positive females) × 100.
** Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; $P < 0.01$.

Table 2
Reproductive index from dams treated with fenugreek seeds aqueous extract during pregnancy and body weight of pups.

Parameters	Fenugreek seed aqueous extract (mg/kg/day)		
	Control	500	1000
No dams	5	5	5
Delivery index (%)[a]	100	100	100
Live-birth index (%)[b]	100	100	87.5**
Viability index (%)[c]	100	100	100
Weanling index (%)[d]	100	100	100
No pups/litter	11.2 ± 1.9	8.75 ± 0.5**	4.8 ± 1.3**

Data are given as means ± S.D.
*Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; $P < 0.05$.
[a] (Number of females delivering/number of pregnant female) × 100.
[b] (Number of females delivered/number of pregnant animal) × 100.
[c] (Number of live offspring at lactation day 4/number of live offspring delivered) × 100.
[d] (Number of live offspring at day 21/number of live offspring born) × 100.
** Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; $P < 0.01$.

($t = 3.65$, $P < 0.001$) at the highest dose. A significant reduction of litter size was noted at both LAE-FS doses of 500 mg and 1000 mg/kg/day ($F_{(2,14)} = 27.63$, $P < 0.001$). Pup body weights of the treated foetuses (500 and 1000 mg/kg/day) at birth were significantly reduced (31% and 27%, respectively) compared to the control group ($F_{(2,122)} = 457.314$; $P < 0.001$). This difference between groups remains more important until the 28th-day post-partum ($F = 1493.688$; $P < 0.001$) with a decrease average 42% and 43%, respectively (Table 3).

3.3. Foetal examination

The total number of foetuses available for morphological evaluations in the control, LAE-FS 500, and 1000 mg/kg/day groups were respectively 56, 43 and 24. No obvious external malformations were observed in the control group. However, foetuses from treated dams presented 3 cases of external malformations: one at the dose of 500 mg/kg/day, its an aplasia of external ear and 2 from dams treated with 1000 mg/kg/day; bump on the head (Fig. 1A) and median cleft of the lower lip (Fig. 1B).

3.4. Brain weight

After 28 days of the birth, the brain weight values (mean ± S.E.) for the control, 500 and 1000 mg/kg/day LAE-FS groups, were respectively: 0.426 ± 0.009 g, 0.363 ± 0.005 g, and 0.37 ± 0.002 g. The absolute brain weight of prenatal treated mice showed a significant reduction (15% and 13%, respectively) compared to control group ($F = 154.299$; $P < 0.001$) (Fig. 2). The brain to body weight ratio was significantly higher (34% and 36%, respectively) in treated groups than control ($F = 27.981$; $P < 0.001$).

Table 3
Pups body weight following aqueous extract fenugreek treatment seed maternal.

Pups body weight (g)	Fenugreek seeds aqueous extract (mg/kg/day)		
	Control	500	1000
Day 0	4.01 ± 0.23	2.74 ± 0.19**	2.93 ± 0.24**
Day 7	4.34 ± 0.4	2.98 ± 0.44***	3.03 ± 0.89***
Day 14	6.96 ± 0.64	5.31 ± 0.29***	5.14 ± 0.64***
Day 21	12.22 ± 1.1	6.58 ± 1.13***	6.43 ± 0.28***
Day 28	16.35 ± 0.99	9.38 ± 0.23***	9.31 ± 0.38***

Data are given as means ± S.D.
** Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; $P < 0.01$.
*** Significant parameters were obtained statistically from one-way ANOVA and Dunnett's multicomparison test; $P < 0.001$.

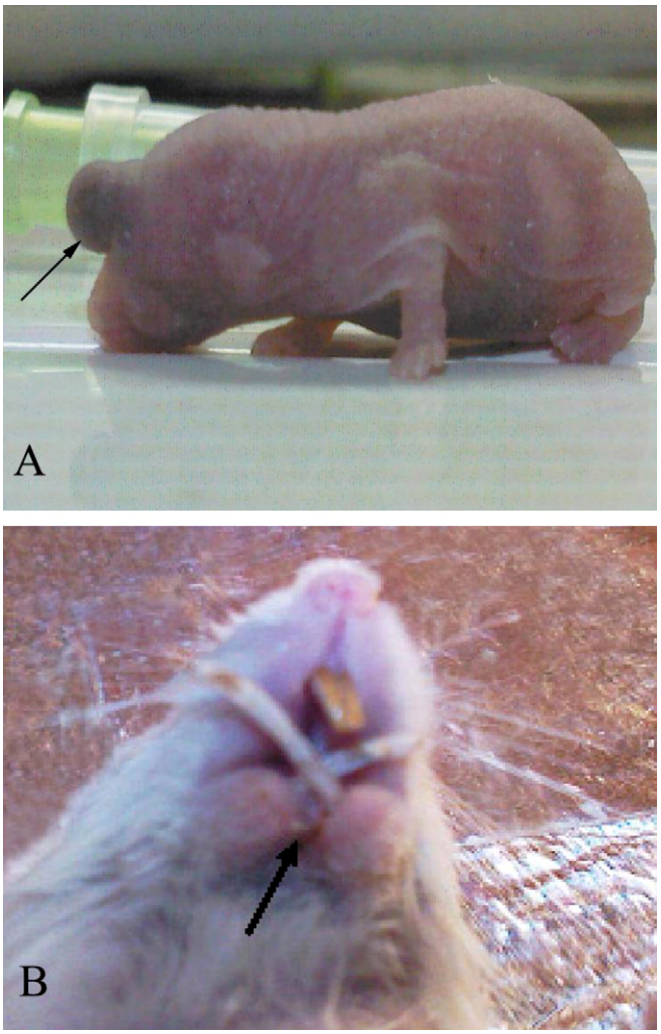

Fig. 1. Photographs of pup (PND 7) with a bump on head (A) and pup (PND 28) with median cleft of the lower lip (B) both prenatally treated with aqueous extract from fenugreek seeds by gavage at 1000 mg/kg/day daily during gestational period.

4. Discussion

The widespread use and importance of the *Trigonella foenum-graecum* in folk medicine, as well as its potential pharmacological properties underline the relevance of this toxicological study. Oral administration of aqueous extract of *Trigonella foenum-graecum* seeds did not provoke any observable clinical signs at the doses of 500 and 1000 mg/kg body weight per day in pregnant mice. No deaths occurred in any group. These results are similar to those obtained by Muralidhara et al. (1999), who showed that fenugreek powder administered intragastrically to mice and albinos rats of both sexes failed to induce any signs of toxicity or mortality up to a maximum practical dosage of 2 and 5 g/kg body weight, respectively. Thus, any toxic effects of the LAE-FS in foetus will not be due to an eventually maternal toxicity.

However, our results showed that fertility was affected by continuous treatment with aqueous extract from *Trigonella foenum-graecum* during pregnancy. In fact, this extract at the doses of 500 and 1000 mg/kg/day orally administered, produced a significant dose-dependent reduction in the number of foetuses per litter but failed to produce complete infertility. Furthermore, sim-

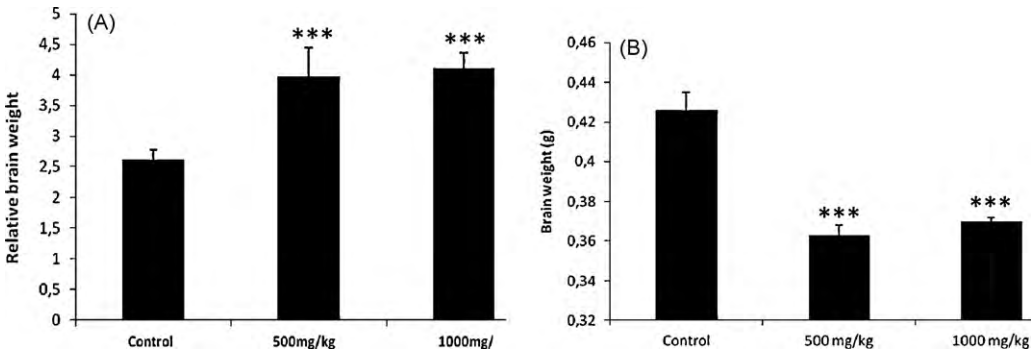

Fig. 2. Brain weight in pups (PND 28) treated prenatally with aqueous extract from fenugreek seed. Absolute brain weights were significantly decreased in the treated groups (A). Brain to body weights ratios were significantly increased in the same groups (B). ***$P < 0.001$.

ilar observations have been reported by Kassem et al. (2006) who observed a significant reduction of developing foetuses as assessed by a reduction of both foetal and placental weights at 20 days of gestation and of litter size during a reproductive screening of fenugreek seed extract. These results suggest an embryo-foetal toxicity effect of the LAE-FS. This is consistent with the fact that some plant extracts, such as Savin essential oil extract and *Acanthus montanus* aqueous extract affect fertility either by promoting anti-implantation (Chamorro et al., 1990) or through embryonic loss or re-absorption (Asonglem et al., 2008).

At the two doses (500 and 1000 mg/kg/day) of LAE-FS, adverse effects in the pups included increased pup mortality and reduced body weights. The postnatal observations showed a retarded growth at least up to the 28th day. It has been clearly shown that during organogenesis, the effects of chemicals or drugs may be expressed as abortions, malformations or delayed development (Sullivan, 1993). Moreover, even if the brain weight decreases by exposure to LAE-FS, this decrease is less important than body weight. Thus, the brain to body weight ratio was greater in both treatment groups than the control group. These results suggest that fenugreek seed extract may cause intrauterine growth retardation and alter brain development both at doses of 500 and 1000 mg/kg/day. This growth retardation continues beyond birth, brain growth remains more rapid and even earlier. So, these data suggest that, at least in the postnatal period, the brain is relatively less affected by fenugreek extract than the whole body.

Usually, mice may spontaneously develop malformations (Kotwani et al., 1995). However, there were no cases of malformation in the control group and the number of malformations was significantly different between groups, suggesting a probably teratogenic effect of the extract. This is in agreement with work that showed that rats receiving a single intraperitoneal injection (0.8 g/kg, 1.6 g/kg, and 3.2 g/kg) of a decoction from fenugreek leaves on day 10 after mating showed a decrease of foetal size (ear diameter to ear diameter) and an increase in foetal mortality rate (Araee et al., 2009).

Moreover, phytochemical screening of *Trigonella foenum-graecum* seeds reveals tannic acid, fixed and volatile oils and a bitter extract, diosgenin, the alkaloids trigonelline, trigocoumarin, trigomethyl coumarin, and steroidal saponins such as gitogenin and traces of trigogenin and vitamin A (Petit et al., 1995). Steroidal saponins and alkaloids have been shown to be teratogenic. Recently, Araee et al. (2009) has considered that in view of the presence of the steroidal saponin diosgenin in fenugreek seeds, it is likely that the administration of fenugreek in high doses adversely influences bone marrow cell proliferation. Similarly, Incardona et al. (1998) demonstrated a teratogenic effect of alkaloids from *Veratrum californicum* in chick embryos. Accordingly, we suggest that these compounds could be responsible for the observed teratogenic effects in mice in our investigation.

In conclusion the present study provides experimental evidence that aqueous seeds extract of *Trigonella foenum-graecum* affects reproduction in mice and shows teratogenic and foetotoxic effect. Given the prevalent use of this plant during pregnancy in Morocco, further epidemiological and experimental investigations of the toxic potential of this spice should be conducted.

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