Evaluation of teratogenic effects of orally administered Fenugreek aqueous extract in pregnant rats

Hind Brogi, Najat EL Amrani, Siham Amsaguine, Driss Radallah*

Laboratory of Biology and health, Research Unit Associate CNRST - URAC 34, Faculty of Sciences Ben M’Sik, Hassan II-Casablanca University, Casablanca, Morocco

Article history:
Received: 17 May, 2016
Accepted: 22 May, 2016
Available online: 09 July, 2016

Keywords:
Fenugreek seeds, gestation, teratogenic effects, weight gain, hydrocephalus

Corresponding Author:
Driss R.*
Professor
Email: driss.radallah (at) gmail (dot) com

Brogi H.
PhD student

Najat EL A.
Professor

Amsaguine S.
Professor

Abstract
Fenugreek (Trigonella foenum-graecum L) plays a very important role in the culinary and healing traditions among the majority of people in developing countries, such as Morocco. However, health risks due to ignorance of this plant and the lack of mastery of a defined dosage encourage awareness of the dangers to the consumption of seeds of this plant, especially in pregnant women. The aim of our study was to assess the teratogenic effect of the aqueous extract of fenugreek seeds used in traditional Moroccan pharmacopoeia in two groups of female Wistar rats receiving 450 and 900 mg/kg/day, respectively until parturition. Our results showed that the aqueous extract of fenugreek seeds increased the body weight of pregnant rats and the neonatal mortality rates among their offspring. An interesting fact was observed among newborns exposed daily to the aqueous extract of fenugreek, it is a case of hydrocephalus characterized by a large head and an abundant amount of cerebrospinal fluid. This is the first time such a birth defect is reported by animal testing, in conjunction with the ingestion of fenugreek seeds. Thus, our results highlight the consumption risk of fenugreek seeds during pregnancy and the importance of advocacy for the benefit of young mothers into the excessive use of the seeds of this plant in the Moroccan traditional medicines.

Citation:

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1. Introduction

Fenugreek, Trigonella foenum-graecum L, the Arabic name Helba, is among the oldest medicinal and culinary plants, its seeds are a rich source of proteins, unsaturated fatty acids and phytosterols, carbohydrates, saponins, alkaloids as trigonelline, vitamins (A, B1, B2, C), potassium, phosphorus, calcium and magnesium (Meghwal and Goswami, 2012).

In Morocco, fenugreek seeds fall into the preparation of many traditional dishes, are given to convalescents, pregnant and lactating women. They are widely used in folk medicine against asthenia, diabetes, anorexia, lack of appetite and as lactagogue (Ghedira et al., 2010; Wani, and Kumar, 2016).

However, other studies have shown that fenugreek seeds are not recommended during pregnancy, because their saponins can induce uterine contractions and abortions (Dasch et al., 2003; Skali, 2006; Srinivasan, 2006). In addition, fenugreek seeds suspected to be responsible for birth defects. Between March and August 2006, the Moroccan pharmacovigilance center received eight cases of neonatal malformations whose mothers drank during their pregnancies, fenugreek seeds (Skali, 2006). Furthermore, Kassem et al. observed a significant reduction in fetal weight and placental gestation to 20 days, suggesting a toxic effect embryofetal (Kassem et al., 2006).
2. Materials and Methods

2.1. Animals
In this study, a total of 30 female adult Wistar rats were used. Animals were housed individually in cages and raised on 12 hours of light / 12 h dark cycle, 50% to 60% moisture and at a standard ambient temperature of 21°C ± 1°C. The animals were given water and commercial food enriched with barley and maize ad libitum.

The animals were distributed into 2 groups of 15 rats: a group of females in proestrus phase were caged overnight with sexually mature males. Rats exhibiting vaginal plug on the following morning were separated, and that day was considered as the first day of gestation. These rats are treated during the entire gestation period. The second group of females were treated for 30 days before pregnancy and during the gestation period. Each group is randomized into three sub-groups of five animals. The first subgroup used as a control received tap water, while the second and third batches were treated daily oral administration with aqueous extracts of fenugreek at doses of 450 and 900 mg/kg/day, respectively.

All rats (control and treated) were kept individually in cages until parturition. Each female is regularly checked and weighed every 3 days until parturition to verify the success of pregnancy and detect any changes in attitude or behavior. Then, we determined number and weight of neonates, duration of pregnancy and percent of abnormal or dead fetuses.

2.2. Preparation of aqueous extracts
Trigonella seeds were collected in the Chaouia-Ouardigha region of Morocco. The fenugreek aqueous extract was prepared in the traditional way by decoction process. The fenugreek seeds soaked in drinking water and allowed to heat for 5 minutes. Then, recovering the aqueous extract by filtration. The extract was conserved at 4°C until required for use. Decoction, very simple method compared to other methods used (infusion and powder), collects most of the active ingredients while minimizing or even nullifying the toxic effect (Salhi et al., 2010).

2.3. Macroscopic study
After farrowing, all newborns were scrutinized to identify any physical abnormality, weighed and their size was measured. Individual organs (Liver, kidney and brain) were immediately removed and weighed after dissection.

2.4. histological study
After macroscopic examination, liver and kidney samples were fixed in Bouin–Hollande solution for 24 hours, while the brain was immersed in Karnowsky's fixative for 96 hours. Then, organs were dehydrated in successive ethanol baths, embedded into blocks of paraffin wax and cut into 5-µm sections. The slides were rehydrated in successive ethanol baths and stained with hematoxylin-eosin by a standard procedure. Histological studies were done using LEICA microscope and LAS EZ software.

2.5. Statistical analysis
The values were compared using the one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. The P values less than 0.05 were considered to be statistically significant. All results are presented as mean ± SEM.

3. Results

3.1. Evolution of body weight in pregnant rats
We observed no mortality or symptoms of toxicity in the treated mothers and control groups during the entire gestation period. Analysis of the data in Figure 1 shows a steady body weight gain among all pregnant rats treated with different concentrations of fenugreek compared to control rats (Figures 1A, 1B).

However, we observed that animals who consumed high doses of fenugreek had a delay in weight gain compared to those treated with a dose of 450mg/kg/day. This very marked difference in early treatment diminishes towards the end of the gestation period.

Moreover, in rats treated over a long period (1 month before pregnancy + during gestation) we noted a decline in the fair weight gain in early pregnancy, probably of a temporary decrease in food intake. This weight loss disappears very quickly and there is a more pronounced increase in body weight during the gestation period (Figures 1A, 1B).

Meanwhile, we noted that the gestation period takes more than 22 days (up to 25 days) in 55% of cases in females treated with fenugreek, whereas this rate does not exceed 10% in controls. It therefore appears that the aqueous extract of fenugreek treatment lengthens the period of gestation and farrowing delays of 3 to 6 days.
Figure 1: Evolution of weight gain in both groups of rats treated with different concentrations of fenugreek, compared to controls. Lot A: rats treated during gestation. Lot B: rats treated over a long period (1 month before pregnancy + gestation).

Thus, measurement of body weight in rats after parturition showed a significant body weight gain in females treated with fenugreek, particularly in low doses (Figure 2). However, no significant weight changes were observed in newborns to rats treated with fenugreek compared to those of control (Figure 3).

Figure 2: weight gain in both groups rats treated with different concentrations of fenugreek compared to controls. Lot A: rats treated during gestation. Lot B: rats treated over a long period (1 month before pregnancy + gestation).
Figure 3: Influence of the aqueous extract of fenugreek seeds on body weight of newborns whose mothers were treated with different concentrations. Lot A: Newborn rats treated during gestation. Lot B: Newborn rats treated over a long period (1 month before pregnancy + gestation).

3.2. Body length
Treatment of pregnant rats with aqueous extract of fenugreek seeds caused no significant change in body length of newborns exposed to concentrations of 450 or 900 mg/kg/day, compared to controls (Figure 4).

Figure 4: Effect of the aqueous extract of fenugreek seeds on body length among newborns whose mothers were treated with different concentrations of fenugreek and those of control mothers. Lot A: rats treated during gestation. Lot B: rats treated over a long period (1 month before pregnancy + gestation).

3.3. Stillbirth
In general, the pregnancy in all rats (case-control and treated by fenugreek) was normal, except for one rat treated by the aqueous extract of the fenugreek with a dose of 450 mg/kg/day. She had vaginal bleeding after only 13 days of gestation, and this is an obvious symptom of a miscarriage.

In addition, several cases of neonatal mortality were observed in both groups (A and B) of rats treated with aqueous extract of the fenugreek seeds and for the two doses administered (Table 1). In total, we identified a stillbirth in 20% of cases. However, we did not find any stillborn with the cases-control rats.

Table 1: Number of the female rats that gave birth to stillborn after treatment with various concentrations of aqueous extract of the fenugreek seeds.
Lot A: rats treated during gestation. Lot B: rats treated over a long period (1 month before pregnancy + gestation).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Lot A</th>
<th>Lot B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>450 mg/kg/day</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Number of stillbirths</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>900 mg/kg/day</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Number of stillbirths</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
3.4. Morpho-histological study of organs

No visible abnormalities were found during the morphological analysis of the organs (liver, kidney and brain) of the offspring who were exposed to the aqueous extract of the fenugreek. However, we noticed that the liver of the treated newborn, unlike the case-control rats, had dark red hue.

3.4.1. Weight

An analysis of the results showed no significant differences (P > 0.05) with the relative weights of the liver, kidneys and the brain of the newborn rats treated with fenugreek compared to those of the case-control rats (Figure 5).

Figure 5: Effects of the aqueous extract of the fenugreek seeds on the relative weight of the brain (A), liver (B) and kidneys (C) in newborns from pregnant rats treated with different concentration of fenugreek compared to the case-control.
Lot A: rats treated during gestation. Lot B: rats treated over a long period (1 month before pregnancy + gestation).

3.4.2. Histology

Treatment with fenugreek resulted in no change in the tissue architecture and reveals no damage in hepatocytes and kidney cells. Histologic examination of the liver and kidneys showed a normal structure in the newborns previously exposed to the aqueous extract of the fenugreek (6A-C, 6D-F). Hepatocytes are arranged in plates of cells separated by an anastomosing system of sinusoids. Nuclei are distinctly round with dispersed chromatin and prominent nucleoli. Renal histology essentially has proximal and distal convoluted tubules and appear as dense, rounded structures, the glomeruli, surrounded by narrow spaces.

These results showed that fenugreek in doses of 450 and 900 mg/kg/day would not have toxic effect and do not interfere with the normal functioning of the vital organs of the newborns. Furthermore, no cyto-architectural abnormalities were observed in the histological sections of neonates exposed to the fenugreek brains compared to case-control rats (Figure 6G-I), the cerebral cortex showing the cell bodies of neurons and glial cells normal granular appearance.
Figure 6: micrographs of histological sections of the liver levels (A, B, C), kidney (D, E, F) and the brain (G, H, I) of newborns previously exposed to 450 mg/kg/day of fenugreek (B, E, H) or 900 mg/kg/day (C, F, I) compared to the case-control (A, D, G). (G X 400)

3.5. Congenital anomalies
A careful scrutiny was applied to all the newborns of the case-control rats and those previously exposed to the aqueous extract of the fenugreek. No external congenital malformations were observed in newborn in the case-control group. However, the newborn rats treated with fenugreek have, after morphological examination, bruising, located mainly at the ends of the legs, around the head, on the back and sometimes near of the nose (Figure 7). Their size vary and their color is purplish red, sometimes pink.
Furthermore, a case of congenital malformation was noted in neonates of the treated rats that have been exposed over a long period (1 month before pregnancy and during the entire period of gestation) in low dose fenugreek (450 mg/kg/day). This is a case of hydrocephalus (Figure 8) characterized by a large head and an abundant amount of cerebrospinal fluid.

Figure 8: A: Photographs of the hydrocephalus case (A) characterized by a head size higher than normal. B: Brains bathed in an unusually high amount of cerebrospinal fluid.

A detailed study of the case of hydrocephalus was performed. Data on the size and body weights and organ weights (liver, kidney and brain) are noted in the table below (Table 2).

Table 2: Data on the various parameters measured in the baby rat suffering from hydrocephalus

<table>
<thead>
<tr>
<th></th>
<th>Weight (g)</th>
<th>Body length (cm)</th>
<th>IMC (g/cm²)</th>
<th>Kidney weight (g/100g of bw)</th>
<th>Liver weight (g/100g of bw)</th>
<th>Brain weight (g/100g of bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocephalus</td>
<td>6.367</td>
<td>6.866</td>
<td>0.135</td>
<td>0.918</td>
<td>5.99</td>
<td>4.044</td>
</tr>
<tr>
<td>Control</td>
<td>5.850</td>
<td>6.52</td>
<td>0.137</td>
<td>1.017</td>
<td>5.274</td>
<td>3.509</td>
</tr>
</tbody>
</table>

According to these results, we note that this newborn suffering from hydrocephalus has a length and a body weight above normal. The brain weight is relatively higher than case-control rats. Meanwhile, histologic examination of vital organs (liver, kidney) of this baby rats (Figure 9A/B) showed no significant differences with the normal tissue architecture in the case-control rats. As for the brain, no particular neural damage has been spotted at this stage (Figure 9C/D).

Figure 9: micrographs of histological sections of the liver levels (A), kidney (B) and brain (C) of the baby rat suffering from hydrocephalus (G X400).
4. Discussion

In this study, we have tested the teratogenic effects of the aqueous extract of the fenugreek seeds used in traditional Moroccan pharmacopoeia at doses of 450 and 900 mg/kg/day in pregnant rats.

Daily oral administration of the aqueous extract of the fenugreek seeds caused no observable clinical sign in pregnant rats. Similarly, no deaths occurred among the different treated group. These results are similar to those obtained by Muralidhara and al. (1999). These authors have shown that even a dose of 5 g/kg body weight of the powder of the fenugreek seeds administered intragastrically to mice and albino rats of both sexes did not induce any signs of toxicity or mortality.

The first recorded effects of the fenugreek seeds aqueous extract is an increase in body weight gain of pregnant rats. These results are consistent with those observed in non-pregnant rats that consumed the aqueous extract of fenugreek seeds (Xue et al., 2007; Harchane et al., 2012).

However, maternal weight gain is not accompanied by an increase in the fetal weight. The newborn weight of the rats treated with fenugreek was being similar to that of the newborns of the case-control rats. This body weight gain observed is probably related to the increase in the food intake and the motivation to eat in rats. The steroid saponins extracted from fenugreek seeds stimulate the appetite in these animals by altering the circadian rhythm of feeding behavior (Petit et al., 1995). Fenugreek seeds also help to gain muscle while getting rid of excess fat, thanks to its high fiber content. Its positive effects on the development and muscle density have also been demonstrated (Poole et al. 2010).

Furthermore, a very interesting fact was brought forward. We obtained only one case of miscarriage and several cases of stillbirths in pregnant rats treated with fenugreek, regardless of the administered dose (450 mg/kg/day and 900 mg/kg/day). This correlation between stillbirth and consumption of fenugreek has also been reported in traditional medicine. Its positive effects on the development and getting rid of excess fat, thanks to its high fiber content. Its positive effects on the development and muscle density have also been demonstrated (Poole et al. 2010).

Consumption of fenugreek has also been reported in pregnant mice treated with fenugreek, but only at the dose of 1 g/kg/day (Khalki et al., 2012). Thus, our results show that the fenugreek seeds aqueous extract, even at low doses induces inhibitory effects on reproduction, causing in some cases, fetal death and abortion in treated rats.

Unlike the case-control, newborns exposed to fenugreek have localized bruising. These neonatal ecchymosis would be the result of nutritional disorders related to the fenugreek ingestion by pregnant animals or the natural consequence of a difficult parturition in treated rats (O'Brien et al., 2009; Ward et al., 2013).

Moreover, among newborns, only one case of hydrocephalus was found in a neonate exposed over a long period to aqueous extract of fenugreek at low dose (450 mg/kg/day). This is the first time such a congenital deformity is obtained from an animal experiment, in conjunction with the fenugreek seeds ingestion. These results confirm the five cases of human hydrocephalus reported by Skali and that this would be associated with taking fenugreek during pregnancy (Skali, 2006).

Other types of defects have been reported by Khalki and al. in mice pretreated with higher doses of fenugreek seeds, essentially two cases of malformation (cleft lip and palate) (Khalki and al., 2010). Thus, our results combined with all these data confirm the potential teratogenic effects of fenugreek and the risk of using the seeds of this plant in traditional medicine.

In the present study, the other newborns that were exposed to the fenugreek seeds aqueous extract had normal body weight and appearance identical to that of the case-control. The newborns’ liver and kidneys, which are major organs that eliminate toxins from the body, showed no signs of cytotoxicity. They have a relative weight and a histological structure identical to those of the case-control group. Additionally, the dark brown color of the liver observed in the newborns treated with fenugreek may be interpreted as an adaptive metabolic response rather than that liver toxicity.

More than that, the newborn rats’ brains prenatally exposed to fenugreek had a relative weight comparable to untreated newborns. The fenugreek aqueous extract would therefore have no significant effect on the growth and brain development. This result disproves the data reported by Khalki et al.(2012) in mice exposed to a dose of 1g/kg/day, suggesting that exposure to the aqueous extract of fenugreek produce moderate microcephaly which is probably due to an alteration of the neuronal and glial proliferation (Barkovich et al., 2001). Meanwhile, histologic examination of different newborns’ brains exposed to fenugreek showed no visible abnormality. The tissue architecture seemed normal and the general appearance of the sensorimotor cortex has not been altered by prenatal exposure to the fenugreek seeds aqueous extract.

Conclusion

Thus, despite the proven health benefits of fenugreek seeds, all our results combined to other studies reported in the literature, show that the use of this plant is not recommended in pregnant women. Thus, the general population should be
made aware and health professionals must be well informed about the harmful effects of the daily consumption of fenugreek seeds during pregnancy. The seeds aqueous extract, even used at low doses, is a risk factor among both women at a reproductive age and among their offspring.

References


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