International Scholars Journals

International Journal of Medical Sociology and Anthropology Vol. 9 (4), pp. 001-007, April, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Genetic and histopathology studies on mice: effect of fenugreek oil on the efficiency of ovarian and liver tissues

Aziza M. Hassan[1], Wagdy K. B. Khalil[1*], Kawkab A. Ahmed[2]

[1]Cell Biology Department, National Research Center, 12622 Dokki, Giza, Egypt.

[2]Pathology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

Accepted 05 January, 2019

There is a growing interest in understanding the biological effect of medicinal plants. In the present investigation, the effects of fenugreek oil administration on the liver and ovarian activity genetically (i.e., meiotic progression in collected oocytes as well as changes in DNA and RNA content in the liver and ovarian tissues) and histopathologically (i.e., alterations in the liver and ovarian tissues) were examined in mice. Swiss albino female mice were orally administrated with different doses of fenugreek oil for 10 days. The mode and magnitude of effect were found to be depending on the dose of fenugreek oil and type of tissue. Administration with fenugreek oil at 0.1 and 0.15 ml/mouse increased the total number of cumulus-oocyte complexes as well as improved their quality. Cytogenetically, fenugreek oil was able to stimulate the oocytes collected from treated mice at all doses to progress in meiosis. Levels of nucleic acids content in all groups did not significantly change neither in the DNA nor RNA in ovarian - or liver-tissues. Histopathological examination of the ovaries collected from untreated mice as well as from mice treated with 0.05 ml/mouse of fenugreek oil showed no histopathological alterations. However, ovaries of mice treated with 0.1 or 0.15 ml/mouse of fenugreek oil showed improvement in several tissues. To our knowledge, this is the first study that suggests significant stimulating effects of fenugreek oil on the ovarian activity in mice.

Key words: Fenugreek, mice, ovaries, oocytes, meiosis, DNA, RNA, histopathology.

INTRODUCTION

Fenugreek (*Trigonella foenum graecum*) is an annual herb belonging to the family Leguminosea, widely grown in India, Egypt, and Middle Eastern countries (Alarcon-Aguilara et al., 1998). Its seeds are commonly used for flavoring and as a spice in curries due to their strong flavor and aroma. Fenugreek contains 4,5-dimethyl-3-hydroxy-2 [5H]-furanone, known as sotolon, which is frequently used as a flavoring agent for artificial maple syrup. Fenugreek natural extractives, oleoresins, and essential oils are generally recognized as safe (GRAS) approved (21 CFR 182.20), and included by the Council of Europe in the list of substances granted approval (COE No.460), as well as by the Flavor and Extract Manufacturer's Association (FEMA No.2485) (Flammang et al., 2004). *T. f. graecum* is one such plant whose seeds and leaves are used not only as food but also as an ingredient in traditional medicine. Its leaves are consumed widely in India as a green leafy vegetable and are a rich source of calcium, iron, β-carotene and other vitamins (Sharma et al., 1996). *T. f. graecum* seeds contain lysine and L-tryptophan rich proteins, mucilaginous fibre and other rare chemical constituents such as saponins, coumarin, fenugreekine, nicotinic acid, sapogenins, phytic acid, scopoletin and trigonelline, which are thought to account for many of its presumed therapeutic effects (Billaud, 2001). Various components of its seeds have varying activities such as fenugreekine, a steroidal sapogenin peptide ester has hypoglycemic

*Corresponding authors E-mail: wagdykh@yahoo.com. Tel: +2-02-3371433. Fax: +2-02-337 0931.

properties (Jellin et al., 1999). It is shown to lower blood glucose level and partially restore the activities of key enzymes of carbohydrate and lipid metabolism close to normal values in various animal model systems (Amin et al., 2005). It can increase the erythrocyte insulin receptors and peripheral glucose utilization, thus showing improved pancreatic function (Raghuram et al., 1994). Therefore, fenugreek seeds are used as a traditional remedy for the treatment of diabetes and hypercholesterolemia in Indian and Chinese medicine (Basch et al., 2003; Miraldi et al., 2001). In Saudi Arabia, fenugreek was found to be among the most common herbs used among people with diabetes (Al-Rowais, 2002). Fenugreek have also been reported to exhibit pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflamatory, hypotensive and antioxidant activity (Cowan, 1999; Shetty, 1997). Fenugreek powder failed to induce any signs of toxicity or mortality in mice and rats who received acute and subchronic regimens (Muralidhara et al., 1999). Moreover, there were no significant histopathological changes in weanling rats fed fenugreek seed for 90 days (Rao et al., 1996).

As already known, ovary of the mammals produces steroidal hormones. One of the steroidal hormones in the ovary is estrogens (α- estradiol, β-estradiol, estriol, and estrone), which play an important role in the estrous cycle (Ying and Zhang, 1999). During the mammalian estrous cycle, 17 β-estradiol induces a sudden increase in gonadotropin-releasing hormone (GnRH) secretion from the neurosecretory cells of the hypothalamus, which in turn brings about a surge in luteinizing hormone (LH) secretion from the pituitary gland. This LH surge initiates final maturation of oocytes and the ovulatory process (Espey, 1999). Seeds of *T. f. graecum* contain steroidal saponin such as gitogenin and traces of trigogenin and vitamin A (Jayaweera, 1981; Petit et al., 1995). This report evaluate the effect of *T. f. graecum* oil at different doses on kinetics of ovarian activity (oocyte quality and meiotic progression), changes in DNA and RNA content in liver and ovarian tissues as well as the histopathological alterations to determine the toxicity probabilities of fenugreek.

MATERIALS AND METHODS

Chemicals

Fenugreek oil was purchased from Elcaptain Company (CAP. PHARM., Egypt).

Animals and maintenance

Seventy two Swiss albino adult female mice (8 weeks old) were obtained from the Animal House of the National Research Center, 12622 Dokki, Giza, Egypt. The animals were housed in several groups in rectangular polypropylene cages with dust-free paddy husk as bedding material. Prior to the experiments, they were acclimatized for one week by feeding on commercial pellet diet and water *ad libitum*.

Experimental design

The experiment was conducted to investigate the effect of fenugreek on kinetics of ovarian and liver tissues. Seventy two animals were used for meiotic progression test and determination of nucleic acid content as well as histopathological analysis. Animals in each assay were divided into 4 groups and treated with fenugreek as follows: First group was served as control group (untreated); the three other groups were treated orally with fenugreek oil for 10 days successively at 0.05, 0.1 and 0.15 ml/ mouse, respectively.

Cytogenetical analysis

Collection of oocytes

After the treatment period female mice were killed by cervical dislocation. Mice ovaries were collected from slaughtered mice and transported in a warm (32–35ºC) saline solution in small Petri dishes (35 x 10 mm). Ovaries were washed three to four times using phosphate buffered saline (PBS). Cumulus-oocyte complexes (COCs) were retrieved from the ovaries by slicing. In this method, ovaries were kept in a sterile Petri dish containing PBS. Ovaries were sliced with a sterile surgical scalpel blade into small pieces. The Petri dishes containing the ovarian pieces were screened under a stereo zoom microscope (WILD Heerbrugg, Switzerland) for oocytes. COCs recovered from ovaries were examined under a stereo microscope at x 35 to x 45 and classified into one of three categories based upon the appearance of the surrounding cumulus cells as follows:

Good: Oocytes with more than three layers of compact cumulus cell masses and a homogenous ooplasm;
Fair: Oocytes with a homogenous evenly granulated ooplasm surrounded by fewer than three layers of granulosa cells;
Poor: Oocytes with a homogenous evenly granulated cytoplasm surrounded by less than one layer of granulosa cells or loosely attached granulosa cells (partially denuded or naked oocytes) (Leibfried and First, 1979; Kolbe, 1998).

Staining and assessment of nuclear maturation

Cumulus cells were removed by incubating COC's in mDPBS (supplemented with 1 mg/ml polyvinyl alcohol, 0.1 % {w/v} porcine trypsin and 0.2 % EDTA {w/v}) for 20 min at 37°C. Oocytes were denuded mechanically by repeatedly pipetting them with a fine Pasteur pipette. Denuded oocytes of good and fair categories were subject to cytogenetical analysis to determine the nuclear maturation state. Immediately after removal of cumulus cells, the denuded oocytes were fixed in Carnoy's solution (25% {v/v} acetic acid in ethanol) for at least 48 h at 4°C and stained with 1% (w/v) orcein in 45% (v/v) acetic acid. For classification of different meiotic stages, the system described by Pola ski and Kubiak (1999) was adopted.

Determination of nucleic acids

Contents of the nucleic acids (DNA and RNA) in the tissues of liver and ovary were determined according to the method of Schneider (1957) and Dische (1955). The tissues were homogenized using standard procedures (Peares, 1985) and were initially precipitated

Table 1. Effect of fenugreek oil treatment on the body and ovary weights.

Fenugreek oil treatment (ml/mouse)	Body weight		Weight of ovary
	Initial	Final	
Control (0)	15.8 ± 0.94	19.1 ±0.44	0.17 ± 0.01
0.05	15.0 ± 0.86	19.3 ± 1.4	0.18 ± 0.01
0.1	15.8 ± 0.68	25.3 ±1.6*	0.19 ± 0.01
0.15	16.0 ± 0.57	21.6 ± 1.2	0.19 ± 0.01

Each value represents mean ± SEM obtained from 72 animals.
*Significant change (P <0.05) from controls.

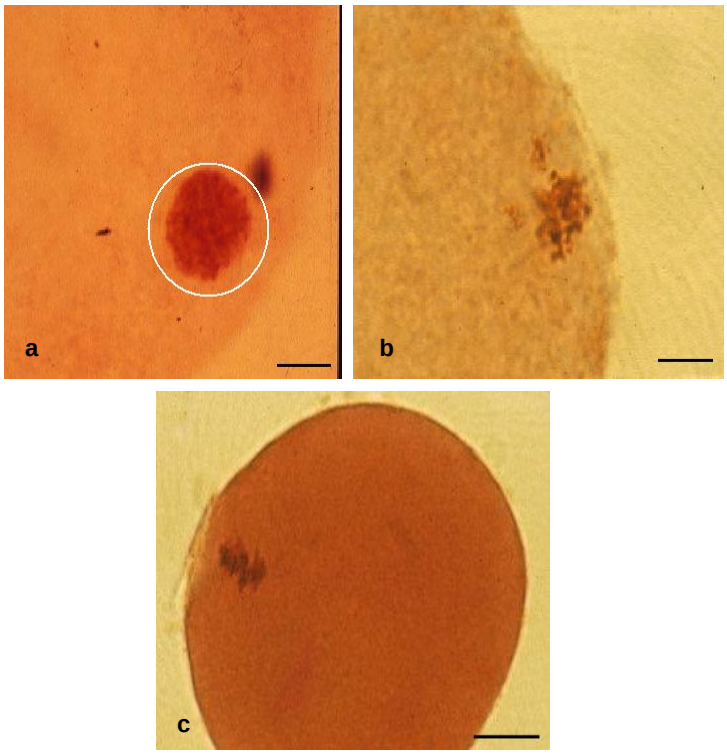

Figure 1. Photomicrographs of stained whole mounted mouse oocytes representing various nuclear stages during maturation. (a) Germinal vesicle stage (GV (bar = 20 µm). (b) Germinal vesicle breakdown stage (bar = 20 µm). (c) Metaphase I (bar = 15 µm)

in 10% cold trichloroacetic acid (TCA) followed by centrifugation for 10 min at 2500 rpm. The pellet was washed once with cold TCA and twice with 95% ethanol then digested in boiled mixture of 95% ethyl alcohol and diethyl ether (3:1). The pellet was centrifuged for 10 min at 2500 rpm and resuspended in 5% TCA. The supernatant was used for assessment of DNA and RNA content. DNA content was determined using diphenylamine method (Dische, 1955). The optical density of the resulting colour was read at wave length 600 nm. RNA contents were determined using orcinol method according to Schneider (1957). Optical density of the resulting color was read at 660 nm.

Body weight and histopathological studies

After the acclimatization period and prior to fenugreek treatment, animals were weighed. After oral administration of fenugreek oil for a continuous period of 10 days at different doses, all animals were weighed again then killed. The ovaries of each mouse were excised, blotted, weighed and processed for routine microscopic examination. Samples from mouse ovaries in all experimental groups were collected and fixed in neutral buffered formalin 10%, washed in tap water overnight and exposed to ascending concentrations of ethanol (70, 80, 90 and 100%), cleared in xylene and embedded in paraffin. Sections of the tissues (4-5 µ thick) were prepared and stained with Hematoxylin and Eosin for subsequent histopathological examination (Bancroft et al., 1996).

Statistical analysis

Analysis of differences between treatments was carried out according to the chi-square test (Snedecor and Cochran, 1982).

RESULTS

Clinical signs

Oral administration of fenugreek oil did not cause any appreciable alterations in the feed intake (data not shown) during 10 days. Furthermore, there is no significantly different between body weight gain during the observation period and the relative ovaries weights among the treated animals with low and highest doses (0.05 ml and 0.15 ml), comparable to their respective control (Table 1). However, there is a significantly increase in the body weight of mice treated with fenugreek oil (0.1 ml/mouse) compared to control group (Table 1).

Cytogenetical analysis

Numbers of mouse ovaries and oocytes per ovary were counted and classified according to the appearance of cumulus cells. A comparison of the recovery rate of the three different types of COCs is shown in Table 2. Administration with fenugreek oil at different doses increased the total number of COCs as well as improved the quality of COCs (Table 2). At doses 0.1 and 0.15 ml/mouse a significant increase in total number of the COCs (from 170 in control group to 219 and 265 in previous doses respectively) as well as a significantly improving in the percentage of good COCs (from 58.2% in control group to 67, 65 and 80% at doses of 0.05, 0.1 and 0.15 ml/mouse, respectively) was found (Table 2). There is no significantly different in fair COCs between treated groups and control. While, poor mice COCs were significantly decreased at 0.15 ml/mouse in comparison to other groups (Table 2).

Table 3 shows state of meiotic progression of the oocytes collected from albino mice treated with different doses of fenugreek oil. Meiotic progression in most oocytes (75.2 %) collected from untreated female mice

Table 2. Quality of cumulus-oocyte complexes (COCs) recovered from mice treated with fenugreek oil.

Fenugreek oil treatment (ml/ mouse)	Total No of COCs[a]	Quality of COCs								
		Good			Fair			Poor		
		Total No.	%	No/ Animal[b]	Total No.	%	No/ Animal[b]	Total No.	%	No/ Animal[b]
Control (0)	170	99	58	16.5±4.1	30	18	5.0±0.8	41	24	6.8±0.7
0.05	181	122	67	20.3±0.9	34	19	5.6±0.9	25	14	4.2±0.7
0.1	219	142	65	23.6±0.9**	48	22	7.8±0.9	29	13	4.8±0.7
0.15	265	221	83	36.8±4.0**	31	12	5.1±0.7	13	8	2.3±0.7

[a]Total No of COCs collected from 6 animals, [b]Each value represents mean ± SEM.obtained from 6 animals, **Significant change (P <0.01) from controls.

Table (3). Meiotic progression of the albino mouse oocytes after treatment with fenugreek oil.

Fenugreek oil treatment (ml/ mouse)	No. of ovaries	No. of oocytes	State of nucleus									
			GV		GVBD		M I		AI/TI		M II	
			No	%	No	%	No	%	No	%	No	%
Control (0)	12	129	97	75.2	22	17.1	10	7.7	--	--	--	--
0.05	12	156	83	53.2	42	26.9	31	19.9	--	--	--	--
0.1	12	190	98	51.5	54	28.4	34	17.9	2	1.1	2	1.1
0.15	12	252	129	51.2	71	28.2	45	17.8	2	0.8	5	2.0

GV= Germinal vesicle, GVBD= Germinal vesicle breakdown, M I= Metaphase I, A I/T I= Anaphase I/ Telophase I, M II= Metaphase II.

was still in germinal vesicle (GV) stage. While, only half of the oocytes number collected from female mice treated with different doses of fenugreek oil were arrested in GV stage (Table 3). Furthermore, the treatment with fenugreek oil stimulated the nucleus of mouse oocytes to arrive at Germinal vesicle breakdown (GVBD) and M I stages. Where, half of the oocytes number collected from female mice treated with fenugreek oil was arrived at GVBD and M I stages (Table 3). However, a few numbers of the oocytes collected from control mice were arrested in GVBD and M I stages (17.1 and 7.7%, respectively) (Table 3).

Alterations in DNA and RNA content

Changes in DNA and RNA content in the liver and ovarian tissues of the albino female mice treated with fenugreek oil are shown in Table 4. Levels of the DNA and RNA content in all groups did not significantly change neither in the liver nor in the ovary (Table 4).

Histopathological findings

Mice administrated with fenugreek oil did not develop any clinical signs of toxicity either immediately or during the post-treatment period even at the highest dose (0.15 ml/mouse). Histopathological examination of the ovaries collected from untreated mice as well as from mice treated with 0.05 ml/mouse of fenugreek oil showed no histopathological alterations. The ovary revealed normal developing follicles and corpora lutea (Figures 2a and 2b). However, ovaries of mice treated with 0.1 or 0.15 ml/mouse fenugreek oil showed more or less similar histopathological changes confined as marked congestion of interstitial ovarian blood vessels (Figure 2c) associated with presence of numerous mature ovarian follicles as well as multiple corpora lutea (Figure 2d). Numerous active primordial follicles, primary or secondary follicles were also noticed in the examined sections (Figures 2e and 2f).

Examined liver of untreated mice as well as liver of mice with 0.05 ml/mouse fenugreek revealed no histopathological changes (Figure 3a). Moreover, liver of mice treated with medium dose of fenugreek showed no histopathological changes except slight activation of kupffer cells and slight congestion of central veins and hepatic sinusoids (Figure 3b). Sporadic cell necrosis as well as small focal area of hepatic necrosis replaced by mononuclear leucocytic cells infiltration was the only histopathological findings observed in the liver of mice treated with high dose (0.15 ml/mouse) of fenugreek (Figure 3c).

Table (4). Effect of fenugreek oil on the DNA and RNA content in liver and ovarian tissues of albino mice.

Fenugreek oil treatment (ml/ mouse)	DNA level (mg/g tissues)		RNA level (mg/g tissues)	
	Liver	Ovary	Liver	Ovary
Control (0)	0.45 ± 0.02	0.09 ±0.01	0.18 ± 0.04	0.05 ± 0.00
0.05	0.47 ±0.02	0.10 ± 0.01	0.19 ± 0.03	0.05 ±0.00
0.1	0.51 ±0.02	0.10 ± 0.00	0.20 ±0.01	0.05 ± 0.00
0.15	0.40 ± 0.01	0.09 ± 0.04	0.19 ± 0.01	0.05 ± 0.01

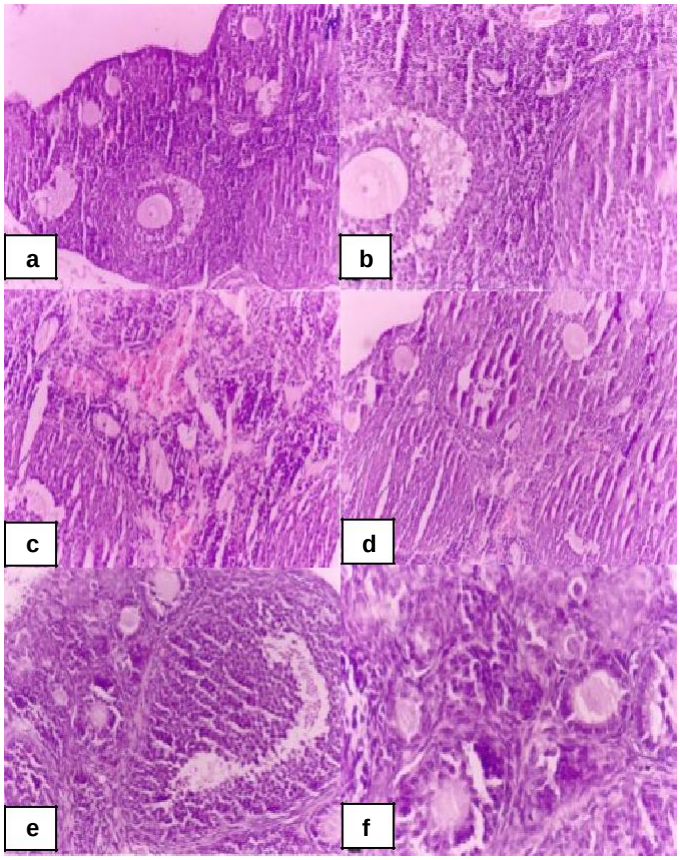

Figure. (2): Photomicrographs of untreated mouse ovaries (a and b) showing normal developing follicles and corpus luteum (H & E X 100 and X 200, respectively). Photomicrographs of mouse ovaries treated with fenugreek (0.1 ml/mouse) showing: (c) Congestion of interstitial ovarian blood vessels (arrows) (H & E X 200); (d) Numerous mature ovarian follicles as well as multiple corpora lutea (H & E X 100). (e) and (f) Photomicrographs of mouse ovaries treated with fenugreek (0.15 ml/ mouse) showing numerous active primordial follicles, primary and secondary follicles (H & E X 200 and X 400, respectively).

DISCUSSION

To date, study of the ovarian activity induced by fenugreek oil has been not studied. Therefore, our present work aims to investigate if the *T. f. graecum* oil has the effectiveness to stimulate the biological roles of the mice ovaries. As previously known, seeds of *T. f. graecum* contain steroidal components (Jayaweera, 1981; Petit et al., 1995), in which they are precursor to form estrogens (Ying and Zhang, 1999). Estrogens, in which they are one of steroidal hormones in the ovary, play an important role in the estrous cycle (Ying and Zhang, 1999). 17 β-estradiol induces a sudden increase in gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus. GnRH is carried to its target cells in the anterior hypophysis (pituitary) where it stimulates secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH induces follicles to grow and increase in size (Espey, 1999) . In the current work, administration with fenugreek oil at different doses increased significantly the total number of the oocytes as well as improved the quality of oocytes compared with oocytes collected from control mice. Dedieu et al. (1998) and Dekel (1999) reported that LH surge initiates final maturation of mammalian oocytes. LH surge induces GV breakdown (GVBD), chromosome condensation, metaphase I spindle formation, extrusion of the first polar body and arrest at metaphase II. We have found that fenugreek oil was relatively effective in stimulating the mouse oocytes to progress in meiosis. Half of the oocytes number collected from female mice treated with fenugreek oil were acquired only at GVBD and M I stages. However, most oocytes collected from untreated female mice were still in GV stage. From these observations, we can suggest that fenugreek may be able to stimulate the pituitary-ovarian axes to secret FSH more than LH, because most oocytes collected from treated mice do not have the efficiency to complete their meiotic progression up to M II stage. Therefore, the mechanism of action of fenugreek on the ovarian activity in mice may be attributed to the endocrine influence and/or the chemicals components of fenugreek. Fenugreek seeds contain lysine and l- tryptophan rich proteins, mucilaginous fiber and other rare chemical constituents which are thought to account for many of its presumed biological effects (Randhir et al., 2004). Lysine is an essential amino acid, which means that it is essential to human health but cannot be manufactured by the body. The deficiency of lysine may cause various problems including fatigue, slow growth and reproductive disorders (Shils et al., 1999). It is not possible to identify the most effective constituent of fenugreek at ovarian kinetics in mice. However, essential amino acids like 4-

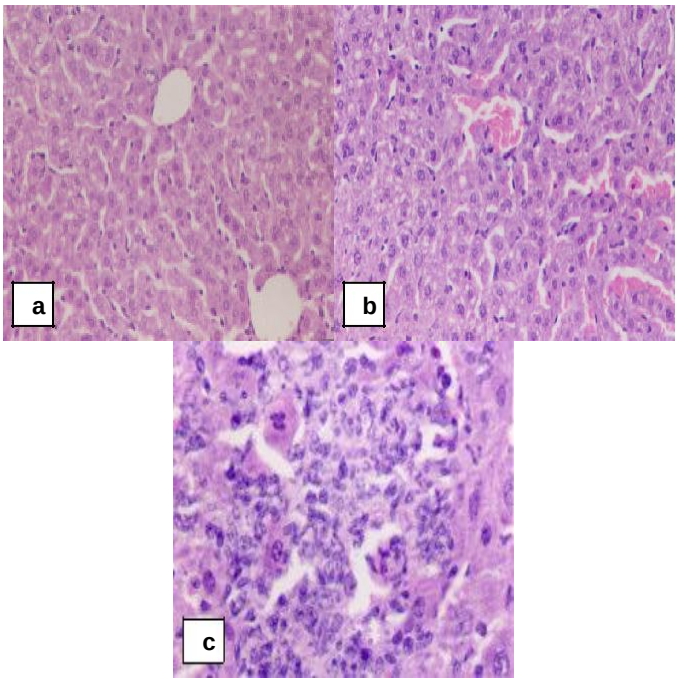

Figure. (3): Photomicrographs of liver of untreated mouse ovaries (a) showing no histopathological changes (H & E X 200). (b) Mice treated with 0.1 ml/mouse of fenugreek oil showing slight activation of kupffer cells and slight congestion of central veins as well as hepatic sinusoids (H & E X 200). (c) Mice treated with 0.15 ml/mouse of fenugreek showing focal area of hepatic necrosis replaced by mononuclear leucocytic cells infiltration (H & E X 400).

hydroxyisoleucine and lysine seem to be eliciting the improving effects.

Nucleic acids and proteins could be considered the most important compounds in the cell, since they are responsible for information, storage and usage (Elser et al., 1996). DNA concentration provides a good estimate of total number of cells. Similarly, RNA concentration also provide useful information about a sample and the ratio of DNA to RNA varies widely between different animals and tissue types because it reflects the metabolic activity of the constituent cells (Bregman, 1990). DNA content of tissues, provide information about genotoxicity by replication or mutation, and DNA content of many tumors provides a good prognosis for the progression of the disease (Silvestrini, 2000). Our present study established that fenugreek has the efficiency to improve the ovarian activity including number and quality of the oocytes without any influence on the total content of DNA or RNA in both liver and ovarian tissues. This is indicated that fenugreek oil have no genotoxic effects on female albino mice, which agrees with the observations of Muralidhara et al. (1999) who concluded that debitterized fenugreek powder does not produce any significant acute and cumulative toxicity at the doses administered (2 and 5 g/kg body weight). Moreover, Amin et al. (2005) suggested that a potential protective effect of fenugreek seeds against 7,12- dimethylbenzanthracene (DMBC) induced breast cancer was shown in rats. At 200 mg/kg body weight, fenugreek seeds extract significantly inhibited the DMBC-induced mammary hyperplasia and decreased its incidence. Furthermore, Basch et al. (2003) postulated that simultaneous administration of aqueous extract of fenugreek seeds with ethanol prevent enzymatic leakage and the rise in lipid peroxidation and enhanced antioxidant potential.

In the current study we have attempt to investigate the histopathological effect of fenugreek oil on liver and ovarian tissues of female mice. We have found that fenugreek oil did not affect significantly on liver and ovary weights in females mice. This is further supported by the lack of any histopathological changes. The histopathological findings in the liver of our present work were agree with those of Abdel-Barry and Al-Hakiem (2000), who described that the main target organ affected among the four different organs studied (liver, kidney, stomach, small and large intestine) was the liver, where early degeneration with infiltration of mononuclear and mild hepatitis was found in some animals treated with toxic doses of glycosidic extract. They concluded that the glycosidic extract of *T. f. graecum* leaves is considered to be safe and have minimal adverse effect. Our histopathological observations indicated that the most vital biological effects of fenugreek were observed only in the ovaries of mice treated with 0.1 and 0.15 ml/mouse of fenugreek oil, which confined as marked congestion of interstitial ovarian blood vessels associated with presence of numerous mature ovarian follicles as well as multiple corpora lutea. Numerous active primordial follicles, primary or secondary follicles were also described. None of the available literatures described the histopathological effects of fenugreek on the rodent ovaries. The histopathological results were confirmed with the results of the cytogenetic study described before. Rao et al. (1996) reported that there were no significant histopathological changes in weanling rats fed fenugreek seeds for 90 days. As well as histopathological examination of various tissues including ovaries in rats revealed no significant changes attributable to the consumption of fenugreek seeds at 5, 10 and 20 g/kg over a 90 day period (Udayasekhara et al., 1996). El-Shayb and Mabrouk (1984) showed also that production of toxinogenic structure of *Aspergillus* was inhibited by 85 – 90% when testing the inhibitory effect of the fenugreek plant extract. Fenugreek has also shown an overall stimulatory effect on the specific as well as non-specific immune functions in mice (Bin-Hafeez et al., 2003).

To understand the mechanism of action how fenugreek can improve the ovarian activity as well as liver tissues genetically and histopathology, further investigations are underway to unravel the molecular mechanism that mediates the improving effects. In addition, we plan to iso

-late and characterize the fenugreekÁs active ingredients that contributes to its effects.

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