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ORIGINAL ARTICLE

Efficacy of *Trigonella foenum-graecum* Seed Extract on Ovariectomy-Induced Hyperlipidemia, Oxidative Stress, and Histopathological Changes in Rats

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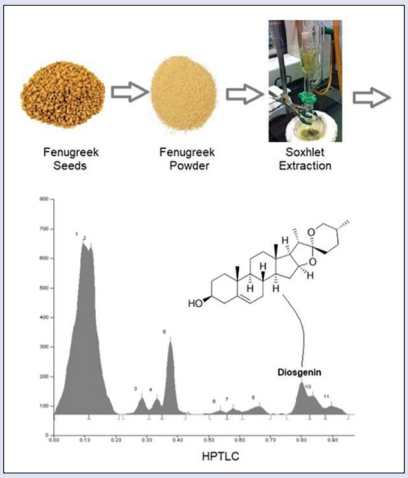
ABSTRACT

Objectives: This study was to standardize *Trigonella foenum-graecum* (fenugreek, TFG) seed extract by high-performance thin-layer chromatography (HPTLC) and to test its efficacy in ovariectomy-induced hyperlipidemia in rats. **Materials and Methods:** Hydroalcoholic extract of TFG-seed was standardized by HPTLC using diosgenin as standard. Female rats were selected and bilateral-ovariectomy was performed. Rats in Group-1 and Group-2 were healthy control and Ovariectomized (OVX) respectively. After 14-days of ovariectomy, Group 3 and Group 4 rats were administered TFG-extract (200 mg/kg/day) and 17β-estradiol (100 µg/kg/day), respectively, for 30 days. Serum lipid profile and liver oxidative markers were determined to compare between the groups. **Results:** The phytoestrogen, diosgenin, was found to be 0.83% w/w in the TFG-seed extract. The OVX rats exhibited a significant increase ($P < 0.001$) in serum cholesterol, triglycerides, low-density lipoprotein, and decreased high-density lipoprotein compared to control rats. The significant increase in thiobarbituric acid reactive substances and decreased glutathione was the indication of oxidative stress in the liver of OVX rats. Treatment with TFG extract significantly reverted these parameters in OVX rats which was comparable to that of standard 17β-estradiol. **Conclusion:** The favorable effect of TFG extract on ovariectomy-induced hyperlipidemia could be because of diosgenin along with other flavonoids and phenols in it. Thus, TFG has the benefits in menopause-induced hyperlipidemia and oxidative stress.

Key words: Histopathology, lipid profile, menopause, oxidative stress, phytoestrogens, *Trigonella foenum-graecum*

SUMMARY

- In this study, the seed extract of *Trigonella foenum-graecum* revealed the presence of phytoestrogen as diosgenin along with other flavonoids and phenols in it. From the biochemical estimations and histopathological analysis, the *T. foenum graecum* has positive effect on menopause-induced hyperlipidemia and oxidative stress.



Abbreviations used: TFG: *Trigonella foenum-graecum*; HPTLC: High-performance thin-layer chromatography; OVX: Ovariectomized; ERT: Estrogen replacement therapy; CVD: Cardiovascular disease; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; VLDL: Very low-density lipoprotein; HRT: Hormone replacement therapy; SERMs: Selective estrogen receptor modulators; CPCSEA: Control and Supervision of Experiments on Animals; TC: Serum total cholesterol; TG: Triglyceride; CRI: Coronary Risk Index; AI: Atherogenic Index; H and E: Hematoxylin and eosin; ANOVA: Analysis of variance.

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INTRODUCTION

Menopause is a physiological phenomenon that marks the termination of women's reproductive capacity and generally occurs during middle age. However, the early onset of menopause is noted in the case of ovariectomy or in certain ovarian diseases. It is characterized by a deficiency of ovarian hormones, mainly estrogen. The declined estrogen is associated with physiological and psychological consequences such as hyperlipidemia, obesity, cardiovascular diseases (CVDs), osteoporosis, anxiety, depression, and changes in cognitive performance.[1] The WHO has predicted that by the year 2030, the expected number of perimenopausal

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or postmenopausal females would be about 1.2 billion.[2] Studies reported that postmenopausal estrogen replacement therapy regulates the lipid profile and in turn reduce the risk of CVD.[3,4] Estrogen regulates low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels, those are correlated with CVD.[5] Contrary to this, the Heart and Estrogen/Progestin Replacement Study showed no beneficial effect of hormone replacement therapy (HRT) on CVD risk despite modulating LDL and HDL levels.[6] Studies have also reported that HRT in women can lead to invasive breast cancer, stroke, and coronary artery disease.[7] Disadvantages associated with chronic treatment with estrogen or delayed treatment several years after menopause have a higher risk of breast, ovarian, and endometrial cancers.[8,9] Hence, the attention was drawn to naturally occurring selective estrogen receptor modulators, including the phytoestrogens.[10,11] One such phytoestrogen containing hypolipidemic medicinal product is seeds of *Trigonella foenum-graecum* (TFG) commonly known as fenugreek seeds.

Fenugreek seeds are an important component of Indian spices that are used regularly in Indian food. TFG belongs to the Fabaceae family. It is widely distributed throughout the world and cultivated in Asia, Africa, and Mediterranean countries for the medicinal properties of the seeds.[12] Fenugreek has a wide range of medicinal properties such as antidiabetes, antihyperlipidemic, analgesic, anticancer, and antioxidant activity.[13] Fenugreek seeds contain (25R) spirost-5-en-3β-ol (known as diosgenin) a phytoestrogen mimic estrogen which helps in antihyperlipidemic action. Apart from diosgenin, fenugreek seeds also contain alkaloids such as trigonelline, flavonoids such as quercetin, luteolin, apigenin and isovitexin, and steroidal saponins such as gitogenin and tigogenin.[14,15] The literature search showed few studies on the determination of trigonelline, 4-hydroxyisoleucine, and diosgenin from fenugreek seeds by high-performance thin-layer chromatography (HPTLC).[15,16] In our study, we aimed HPTLC standardization of TFG seed extract using diosgenin as standard phytoconstituent and to see efficacy on ovariectomy-induced hyperlipidemia and oxidative stress in rats.

MATERIALS AND METHODS

Preparation of *Trigonella foenum-graecum* seed extract

The fenugreek seeds were procured from Pro Natural, Bengaluru (assured 100% organic seeds). Seeds were coarsely powdered in a mixer grinder and refluxed at 85°C with 1 L of 70% ethanol in Soxhlet apparatus for complete extraction of the seed powder. Further, the extract was concentrated using Rotavapor (Buchi, India). The final drying was done using a freeze dryer. The extraction was carried out in batches. Further standardization of extract was carried out by phytochemical and HPTLC analysis.

Phytochemical analysis and high-performance thin-layer chromatography standardization

The hydroalcoholic extract was subjected to various qualitative tests for the identification of different phytoconstituents present in it, using standard qualitative methods.[17] The HPTLC analysis was carried out using the standard diosgenin (95% purity, Tokyo Chemical Industry Co., Ltd. Japan). The standard solution was prepared by dissolving 100 µg of diosgenin in 1 ml of methanol. The hydroalcoholic extract of TFG and diosgenin standard was applied on an F_{254} aluminum plate, precoated with silica gel of 10 cm × 10 cm (Merck, India) to a width of 6 mm band using Linomat-5 TLC Applicator (CAMAG with winCATs software). Toluene:ethyl acetate:formic acid (5:4:1) was used as a mobile phase to develop the plate in twin-trough Chamber with lid. Anisaldehyde-sulfuric acid spray was used to derivatization of plate followed by heating at 110°C for 10 min. In the end, the plate was scanned at 620 nm. The CAMAG TLC Scanner was used for chromatogram evaluation. The HPTLC analysis was repeated three times for reproducibility to standardize the extract.

Procurement of rats for experiments

Adult healthy female Wistar albino rats of 10 months' age were allowed to acclimatize for 2 weeks before starting the experiment. Three rats were housed in one polycarbonate cage at Central Animal House Facility, Manipal Academy of Higher Education, Manipal. The experiments on animals were as per the recommendations made by the purpose of control and supervision of experiments on animals, India. The experimental protocol was approved by Institutional Animal ethics committee (IAEC/KMC/16/2016). The rats were maintained under standard environmental condition (12-h day-night cycle with a temperature of 22°C ± 2°C). Rats were fed with standard pellet diet and water *ad libitum* throughout the experiment.

Induction of menopause by ovariectomy (OVX)

During the preoperative period, all rats were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg b.w) and xylazine (5 mg/kg b.w). After checking withdrawal and blinking reflexes, rats were aseptically prepared for abdominal surgery. A lower abdominal cavity exploration was performed by midline incision, followed by bilateral ovariectomy.[18] Proper postoperative care was taken, and rats were placed individually in one polycarbonate cage. After a waiting period of 2 weeks to allow for the development of menopause, the rats were randomly assigned into experimental groups.

Experimental design

Twenty-four rats were selected randomly into four groups of six animals each.

- Group 1 (Normal control): Fed with standard pellet diet
- Group 2 (OVX control): Fed with standard pellet diet without any treatment
- Group 3 (OVX + TFG): The standardized extract of TFG was suspended in 0.5% carboxymethyl cellulose and administered orally (200 mg/kg/day) for 30 days. This dose was selected based on our earlier findings[18]
- Group 4 (OVX + E2): 17β-estradiol was dissolved in sesame oil and injected subcutaneously (100 µg/kg/day) for 30 days.

The dose was decided based on the previous study conducted in our laboratory. At the end of the experiment, rats fasted for 12 h and 2 mL blood was collected from each rat through puncturing retroorbital plexus. To separate serum, blood samples were centrifuged at 3000 rpm for 10 min at room temperature. All the rats were sacrificed, and the liver tissue was isolated for the estimation of oxidative stress markers and histopathological evaluation.

Estimation of serum lipid profile and determination of Atherogenic Index, Coronary Index

Serum total cholesterol (TC), HDL, and triglyceride (TG) were determined enzymatically using standard kits obtained from Aspen Ltd., New Delhi, India. Serum LDL was assessed using the Friedewald formula:

LDL-C = TC − HDL − VLDL (VLDL = TG/5).[19]

The Coronary Risk Index (CRI) and Atherogenic Index (AI) were calculated as TC/HDL and log (TG/HDL), respectively.[20]

Estimation of oxidative stress markers

The oxidative stress marker, TBARS, and glutathione (GSH) were estimated as per the standard literature.[21]

Histopathological analysis

Liver tissue fixed in 10% formalin were further processed for histopathological evaluation by standard histopathological procedures. At the end, 5-μ thickness sections were cut and stained with hematoxylin and eosin. The slides were examined under a light microscope. The histopathology was compared between the groups by expert anatomist at the Department of Anatomy, Kasturba Medical College, Manipal.

Statistical analysis

Data were analyzed using one-way analysis of variance, followed by Bonferroni's *post hoc* test by GraphPad Prism Version 5.0 (San Diego, California), statistics software. Results were expressed as mean ± standard error mean. $P \leq 0.05$ was considered as statistically significant.

RESULTS

Phytochemical constituents in fenugreek and quantification of diosgenin by high-performance thin-layer chromatography

The percentage of yield was 11% w/w. The phytochemical analysis by various qualitative methods revealed the presence of alkaloids, carbohydrates, saponins, tannins, flavonoids, proteins, sterols, fixed oils, and fats in the extract. Analysis of HPTLC confirmed the presence of diosgenin, and the percentage (R_f 0.82) was found to be 0.83% w/w [Figure 1]. The results were reproducible with the different batches of extract.

Trigonella foenum-graecum seed extract could prevent ovariectomy-induced hyperlipidemia in rats

Rat serum TC and TG were significantly increased ($P < 0.001$) in OVX group compared with healthy control rats. However, after the treatment with TFG seed extract, TC and TG were significantly normalized in OVX rats. 17β-estradiol-treated group significantly reduced the TC ($P < 0.05$) and TG ($P < 0.05$) levels compared with OVX-group. Serum HDL was decreased in OVX-group rats compared with the control group, although, the values are not statistically significant. After administration of the TFG seed extract, the serum HDL did not increase. Serum LDL was significantly increased in OVX rats ($P < 0.001$) compared to control rats. This effect was reversed after the supplementation of TFG seed extract [Figure 2].

The AI and CRI were suggestively increased in OVX rats ($P < 0.001$). These values were significantly decreased ($P < 0.05$) after treatment with TFG seed extract [Table 1].

Trigonella foenum-graecum seed extract could prevent ovariectomy-induced oxidative stress in rat liver

The ovariectomy increase in TBARS in the liver significantly compared to the normal control rat livers ($P < 0.001$). The GSH in the liver were decreased upon ovariectomy in rats when compared with control rats. Treatment with TFG seed extract after ovariectomy markedly reverted GSH and TBARS in OVX rats [Figure 3].

Hepatic histopathology

The histopathological examination of the control group showed normal cell architecture, whereas the OVX-group showed marked hepatic lipid accumulation compared to control group. However, treatment with TFG seed extract and 17β-estradiol for a period of 30 days lowered hepatic lipid accumulation compared to OVX rats [Figure 4].

DISCUSSION

The diosgenin concentration in TFG seed extract was calculated using the regression analysis was found to be 0.83% w/w. According to the literature data diosgenin concentration in TFG seed varies from 0.42% to 0.98%.[22] The diosgenin concentration in seed was reported in the range of 0.113–0.135% (w/w) by HPTLC method.[23] Report on Indian

Table 1: Effect of *Trigonella foenum-graecum* seed extract on Atherogenic Index and Coronary Risk Index

Groups	AI	CRI
Control	1.57±0.1	0.18±0.01
OVX	2.58±0.3***	0.4±0.02***
OVX + TFG	1.99±0.5$^{\$\$}$	0.27±0.1$^{\$\$}$
OVX + E2	1.98±0.1$^{\#\#}$	0.28±0.05$^{\#\#}$

Values are mean ± SEM; ***$P \leq 0.001$, control versus OVX; $^{\$\$}P \leq 0.01$; OVX versus OVX + TFG; $^{\#\#}P \leq 0.01$, OVX versus OVX+E2.; OVX versus TF $^{\$\$}P \leq 0.01$; OVX versus OVX+E2 $^{\#\#}P \leq 0.01$. AI: Atherogenic Index; CRI: Coronary Risk Index; OVX: Ovariectomized rats; TFG: *Trigonella foenum-graecum* seed extract, 200 mg/kg/day p.o; E2: 17β-estradiol, 100 μg/kg/day p.o; SEM: Standard error mean

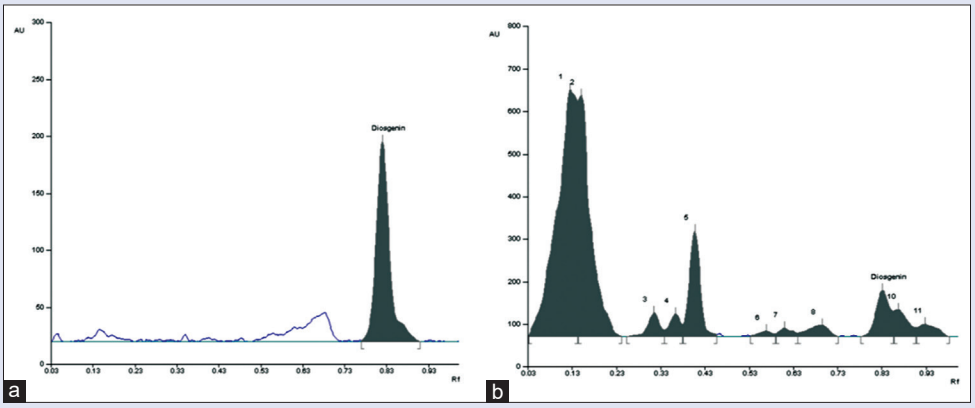

Figure 1: High-performance thin-layer chromatography analysis of hydroalcoholic extract of *Trigonella foenum-graceum* seed extract with diosgenin; high-performance thin-layer chromatography chromatographic profile of (a) standard diosgenin (R_f = 0.82); (b) *Trigonella foenum-graecum* seed extract (R_f = 0.83 indicate the presence of diosgenin)

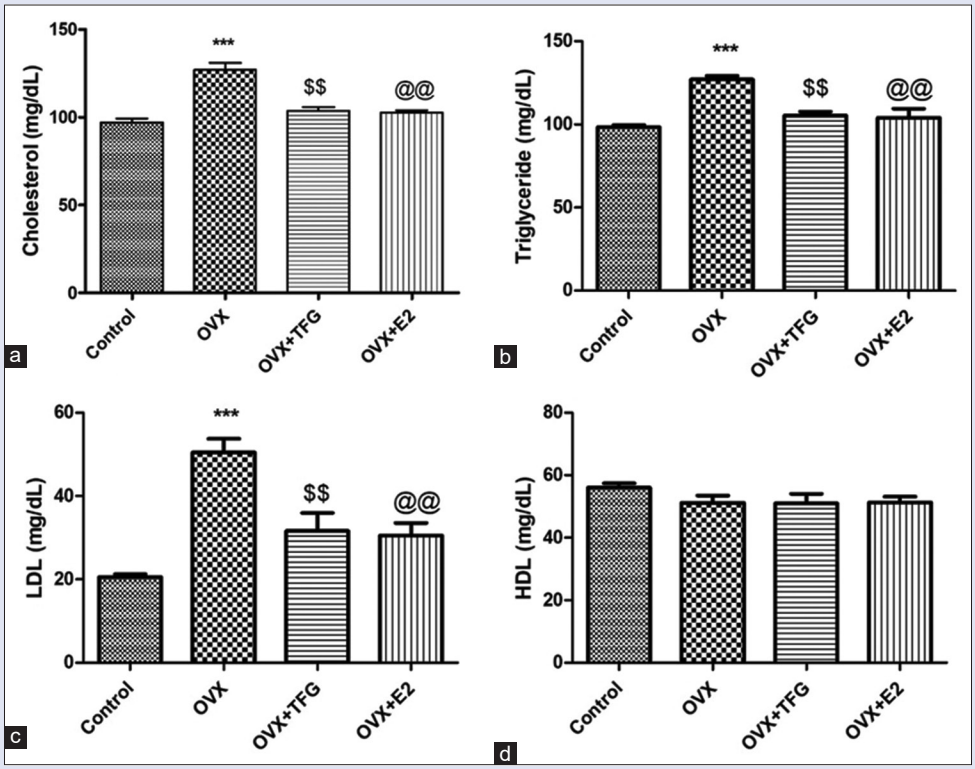

Figure 2: Effect of *Trigonella foenum-graecum* seed extract on lipid profile in OVX rats. (a) Serum cholesterol; (b) Serum triglyceride; (c) Serum LDL; (d) Serum HDL. Value are mean ± SEM; ***$P \leq 0.001$, control versus OVX; $^{\$\$}P \leq 0.01$. OVX versus OVX + TFG; $^{@@}P \leq 0.01$, OVX versus OVX + E2; OVX: Ovariectomized rats; TFG: *Trigonella foenum-graecum* seed extract, 100 µg/kg/day p.o; E2: 17β-estradiol

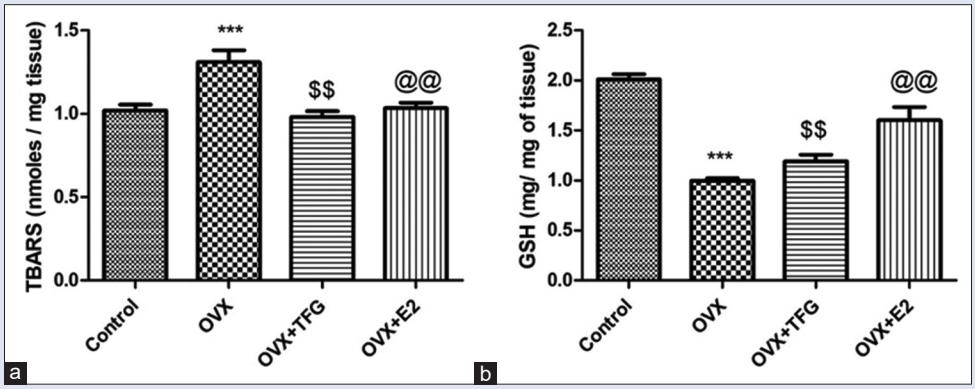

Figure 3: Effect of *Trigonella foenum-graecum* seed extract on oxidative stress markers in liver. (a) Thiobarbituric acid reactive substances (TBARS), (b) Glutathione. Values are mean ± SEM; ***$P \leq 0.001$. control versus versus OVX; $^{\$\$}P \leq 0.01$. OVX versus OVX + TFG; $^{@@}P \leq 0.01$, OVX versus OVX + E2 ; OVX: Ovariectomized rats; TFG: *Trigonella foenum-graecum* seed extract, 200 mg/kg/day p.o.; E2: 17β-estradiol, 100 µg/kg/day p.o.

fenugreek from the market, the diosgenin content in seeds was five-fold higher (0.529%–0.658%) compared to other literature.[24]

Menopause is associated with hyperlipidemia, which is defined by increased levels of TC, TG, LDL, and decreased levels of HDL. In this study, we have evaluated the TFG for its hypolipidaemic activity in ovariectomized rats, because of its phytoestrogen activity. Ovariectomized rats resulted in a significant increase in serum TC and TG levels. However, after the treatment with TFG seed extract, TC and TG levels were decreased in the OVX group compared with the untreated OVX group. These outcomes can support the use of fenugreek in postmenopausal use other than its proven effects on dyslipidemia and diabetes.[25,26] The reduced TC and TG may be due to increased activity of lecithin cholesterol acyltransferase and also the phytoestrogen content, a large amount of fiber galactose and mannose present in TFG seed extract.[27,28] Estrogen has a role in lowering TC levels through upregulation of the hepatic LDL receptor.[29] This is reflected in our rat model, where 17β-estradiol-treated OVX rats, significantly prevented the increase in serum TC and TG compared to the normal rats. The serum HDL was decreased in the OVX group compared to the control group. One of the studies reports that the treatment with fenugreek seed extract had lower TG, total cholesterol, and higher HDL cholesterol in a dose-dependent manner, compared to diabetic rats.[28,29] In our study, OVX group treated with TFG seed extract, the HDL levels were not increased. These results may be due to the less duration of the treatment.

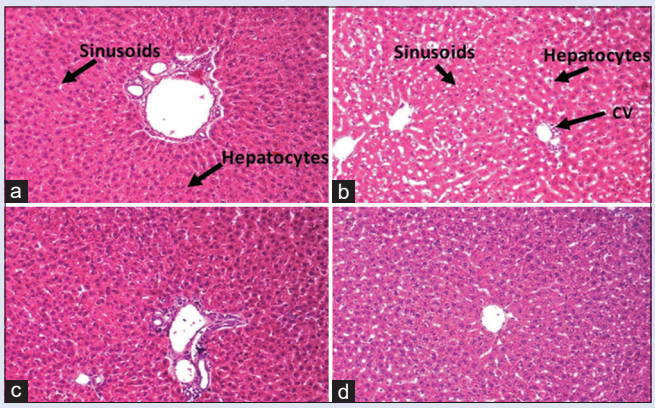

Figure 4: Effect of TFG on histopathological changes in liver (40×). (a) Control rats; (b) Ovariectomized rat; (c) OVX+TFG; d: OVX+ E2. a: Normal architecture of liver; b: Accumulation of lipids in the liver; c: Less hepatic accumulation of lipid; d: lower accumulation of lipids compared to OVX rats in the liver. OVX: Ovariectomized rats; TFG: *Trigonella foenum-graecum* seed extract, 200 mg/kg/day p.o ; E2: 17β-estradiol, 100 µg/kg/day p.o.

The increased serum LDL in OVX rats may be due to the decreased or diminished LDL-receptor clearance and receptor activity resulting in the accumulation of LDL. Endogenous estrogen reduced the circulating very low-density lipoprotein (VLDL), LDL, and Apo-B levels in both male and female.[30] Thus, ovariectomy stimulates the production of VLDL that is destined to become long-lived LDL. The TFG seed extract was reported to revert the estrogen deficiency by upregulation of LDL receptors.[31] The saponins in TFG might be responsible for its phytoestrogenic activity. The TFG seeds contain mainly two steroidal sapogenins, diosgenin and gitogenin in 9:1 ratio.[32] In our study, the AI and CRI were reduced by fenugreek seed extract, indicates that fenugreek seed extract can minimize cardiovascular risk and these results are supported by another study.[33]

Estrogen acts an antioxidant by an electron donor, a free-radical scavenger, and interrupts the lipid peroxidation. GSH peroxidase and manganese superoxide dismutase are up-regulated in females by estrogen.[34,35] Ovariectomy causes metabolic disturbances, which influences the liver (fatty liver) by the generation of reactive oxygen species. Studies have reported that oxidative stress was increased after 8 weeks of ovariectomy in rats.[36] In our study, we observed the elevated levels of TBARS in OVX rats due to lack of estrogen. TBARS levels were reduced and GSH levels were elevated after treating with TFG seed extract. TFG seed extract contains flavonoids and polyphenols; they exhibit antioxidant properties. Decreased lipid peroxidation after fenugreek administration might also be attributed to its estrogenic constituents includes saponins such as diosgenin, trigonelline, and other flavonoids.[37]

CONCLUSION

TFG supplementation showed a favorable effect on OVX-induced hyperlipidemia and oxidative stress. This favorable effect may be due to components such as diosgenin, flavonoids, and phenols. The HPTLC analysis of TFG seed extract confirmed the presence of diosgenin. TFG seed extract can be a therapeutic agent for treating menopause-induced hyperlipidemia or oxidative stress.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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