

Flavonoids Fraction of *Mespilus Germanica* Alleviates Insulin Resistance in Metabolic Syndrome Model of Ovariectomized Rats via Reduction in Tumor Necrosis Factor- α

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Objectives: The rate of metabolic syndrome (MetS) in women diagnosed as they age is one of the main concerns of health cares. Recently new strategies used to prevent progressions of MetS toward the diagnosis of diabetes have focused on plant flavonoids. This study was aimed to investigate the beneficial effects of flavonoids fraction of *Mespilus germanica* leaves (MGL) on MetS in ovariectomized (OVX) rats.

Methods: Twenty-four adult female Wistar rats, weighing 200 to 250 g, were divided into 3 groups: Sham surgery, OVX + Salin, or OVX + Flavonoid. Three weeks after ovariectomy, animals displayed MetS criteria received flavonoid injection (10 mg/kg, intraperitoneally) for 21 days. Then the body weight, body mass index, waist circumference, visceral fat, fasting blood glucose, serum insulin, lipid profiles and tumor necrosis factor- α (TNF- α) were measured.

Results: Treatment with flavonoids fraction of MGL significantly decreased serum level of insulin ($P = 0.011$), glucose ($P = 0.024$), TNF- α ($P = 0.010$), also MetS Z score ($P = 0.020$) and homeostasis model assessment of insulin resistance ($P = 0.007$). Lipid profiles and visceral fat showed insignificant reduction.

Conclusions: Flavonoids of MGL attenuates some of the MetS components possibly via reduction in TNF- α inflammatory cytokine. (J Menopausal Med 2018;24:169-175)

Key Words: Menopause · Metabolic syndrome · Ovariectomy · Polyphenols

Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common chronic diseases with increasingly higher prevalence worldwide due to epidemic of obesity.¹ Metabolic syndrome (MetS) is a pre diabetes stage with multiple risk factors consisting of abdominal obesity, hypertension, hyperglycemia, and dyslipidemia.² In women, the prevalence of the MetS

increases in menopause parallel with estrogen withdrawal, and is associated with visceral adiposity, dislipidemia and impaired glucose tolerance.^{3,4} Also ovariectomy in rodents as a model of menopause has been associated with weight gain, visceral fat increase and disrupted lipid profiles.⁵

Insulin resistance (IR) is assumed to be related to the certain inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6. These cytokines interfere

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with peripheral insulin receptors and impair glucose utilization to induce IR and further pathological consequences.⁶⁻⁸

Therefore, finding new and safe treatments for preventing the progress of MetS to diabetes is of great importance. Over the last decade, growing body of evidences on plant-based dietary nutrients, especially flavonoids have been found different beneficial effects including antioxidant activity with potency of scavenging free radicals.⁹⁻¹¹ Previous studies reported that flavonoids such as quercetin and naringenin are capable to lower serum lipid,¹² and glucose.¹³ Also anti-hyperglycemic effects have been reported before from some of the plants extract such as *Croton lobatus*¹⁴ and *Eugenia punicifolia*.¹⁵

Mespilus germanica leaves (*MGL*; general name, Medlar) is a species belonging to the *Rosaceae* family and is mainly distributed in the north of Iran and Turkey,^{16,17} and traditionally has been used for gastrointestinal tract infection.¹⁷ Hydro-alcoholic extracts of *MGL*. Leaves¹⁶ and fruit¹⁸ are rich sources of bioactive compounds such as polyphenol and flavonoids. Here we hypothesized that isolated flavonoid fraction of *MGL* may have capability to alleviate MetS indices. To approach this, ovariectomized (OVX) rats were used as an acceptable model of menopause-induced MetS.^{19,20}

Materials and Methods

1. Extraction of flavonoid of *MGL*

The *MGL* were collected from Guilan province of Iran and was identified by specialist from the herbarium center of the Guilan University (Herbarium code, 6157). Then 5 g of the dried leaves was dissolved in 104 mL of 70% ethanol and kept on 40°C heater. Then 2 molar of hydrochloric acid and ethyl acetate were added, and mixed solution was transferred to a rotary evaporator to get pure flavonoids. In addition, 2-dimensional paper chromatography (2-DPC) and thin layer chromatography (TLC) were used for detecting the types of flavonoids.

2. Animals and treatment

Twenty-four adult female Wistar rats (3 months age and 200-250 g weight), were housed 4 per cage and fed standard-pellet rat chow and tap water ad libitum. Room

temperature was maintained at $22 \pm 2^\circ\text{C}$ with a 12/12 hour light/dark cycle (light on 7:00 A.M.). All experiments were performed in accordance with National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) modified by ethical committee of Guilan University of medical sciences, Rasht, Iran.

The animals were randomly divided into 3 groups of: Sham surgery, OVX + Salin (SAL), OVX + Flavonoid (FLA). Sham control rats had all surgical procedure except removal of ovaries. Animals were injected intraperitoneally combination of 75 mg/kg ketamine (50 mg/mL; Rotexmedica GmbH, Trittau, Germany) and 5 mg/kg of xylazine (20 mg/mL; ScienceLab, Houston, TX, USA). After complete anesthesia, ovaries were accurately removed through midline incision.²¹

Three weeks after ovariectomy, animals showing 3 criteria of MetS (significant high serum triglyceride [TG], cholesterol low-density lipoproteins [LDL], fasting glucose and large waist circumference [Wc]) received daily injection of isolated fraction of flavonoid (10 mg/kg/21 days).²²

3. Morphometric and chemical analysis

Body weight, Wc and visceral fats were measured after 14 hours fasting. Wc was measured on the largest zone of the rat abdomen.²¹ Visceral fats were isolated from surrounding tissues of mesenteric, urogenital and retroperitoneal regions and weighed using a weighing-scale (Doulton).²¹ Body mass index (BMI) was calculated using the formula: $BMI (g/cm^2) = \text{body weight (g)} / \text{height}^2 (cm^2)$.

Then blood was collected from the inferior vena cava, and centrifuged at 3,000 g/15 minutes and serum was kept at -80°C .²¹

Measurement of food intake was carried out according to the previous work,²³ briefly an equal amount of food (20 g/day) was given to each cage in the morning, and consumed food was measured by subtracting the weight of the uneaten food from the total given one in the evening.

Serum glucose, TG and total cholesterol (TC) (Pars Azmoun kit, Tehran, Iran) and high-density lipoprotein (HDL) (Pishtaz Teb kit, Tehran, Iran) were measured by Enzymatic-Photometric-Endpoint method using an automated analyzer (Alfa-Classic; Tajhizat-Sanjesh Co., Ltd., Isfahan, Iran). LDL levels were estimated based on the Friedewald formula:²⁴ $LDL [mg/dL] = TC [mg/dL] - HDL$

[mg/dL] – TG [mg/dL] / 5)

The level of insulin was assayed using Rat insulin enzyme-linked immunosorbent assay (ELISA) kit (East-biopharm; Hangzhou Eastbiopharm Co., Ltd., Hangzhou, China). According to the manufacturer's instructions, the sensitivity was 0.05 mIU/L and intra-assay and inter-assay coefficients of variation (CV) were $CV < 10\%$ and $CV < 12\%$, respectively. IR index (IRI) was assessed by homeostasis model assessment of IR (HOMA-IR) by formula of (Fasting insulin [μ IU/mL] \times Fasting glucose [mmol/L] / 22.5).⁵

Metabolic Z score was calculated using the formula of: $([40-HDL] / 5.49) + ([TG-150] / 149.53) + ([Fasting\ glucose-110] / 22.69) + ([Wc-102] / 11.13)$.²⁵ Finally serum TNF- α was determined using Rat TNF- α ELISA kit (Diaclone SAS, Besançon, France).

4. Statistical analysis

Normality of variables was estimated by Kolmogorov-Smirnov and Shapiro-Wilk test, then variables were analyzed using one-way analysis of variance (ANOVA) followed by post hoc least significant difference (LSD) test. Results are expressed as mean \pm standard error (SE) and values of $P < 0.05$ were considered statistically significant (SPSS Version 22; SPSS Inc., Chicago, IL, USA).

Results

Results of TLC and 2-DPC revealed that extract provided from the leaves of MGL consisted of: Chrysin, Kaempferol, Luteolin, Myricetin, Naringenin, Quercetin, and Rutin.

Analysis of ANOVA followed by LSD test showed significant elevation in body weight ($F [2,21] = 18.962; P = 0.001$), BMI ($F [2,21] = 12.597; P = 0.001$), Wc ($F [2,21] = 13.886; P = 0.001$) and visceral fat ($F [2,21] = 16.069; P = 0.001$) in OVX compared with Sham group.

As Table 1 shows BMI ($P = 0.366$), visceral fat ($P = 0.652$), body weight ($P = 0.990$), food intake ($P = 0.130$) and Wc ($P = 0.672$) didn't change significantly in OVX + FLA group compared with OVX + SAL.

On the other hand, significant elevation was found in serum TG ($P = 0.033$), TC ($P = 0.047$) and LDL ($P = 0.029$) levels, but reduction in HDL ($P = 0.018$) in OVX compared

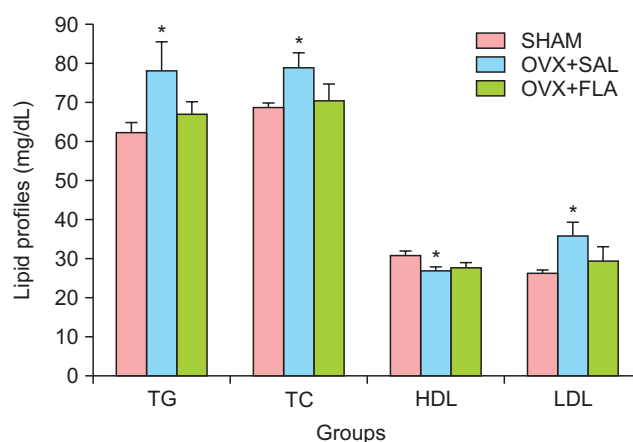


Fig. 1. The effect of treatment with flavonoid of Mespilus germanica leaves (10 mg/kg) on triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), and low density lipoprotein (LDL). The data is presented as the mean \pm standard error for 8 animals per group. TG $*P = 0.033$, TC $*P = 0.047$, HDL $*P = 0.018$, and non-LDL $*P = 0.047$, ovariectomized (OVX) + saline (SAL) group compared with sham-operated (SHAM) group. FLA: flavonoid.

Table 1. Effect of flavonoid of Mespilus germanica leaves on body weight, body mass index, waist circumference, and visceral fat in ovariectomized rats

Variables	SHAM	OVX + SAL	OVX + FLA	P value
Body weight (g)	214.50 \pm 4.73	264.88 \pm 5.09	265.00 \pm 9.26	0.001*
Body mass index (g/cm ²)	0.523 \pm 0.005	0.568 \pm 0.006	0.559 \pm 0.007	0.001*
Waist circumference (cm)	15.35 \pm 0.39	17.88 \pm 0.39	18.13 \pm 0.44	0.001*
Visceral fat (g)	5.50 \pm 0.50	12.50 \pm 0.66	11.88 \pm 1.46	0.001*

The data is presented as the mean \pm standard error for 8 animals per group

* $P = 0.001$ OVX + SAL group compared with SHAM group

SHAM: sham-operated, OVX: ovariectomized, SAL: saline, FLA: flavonoid

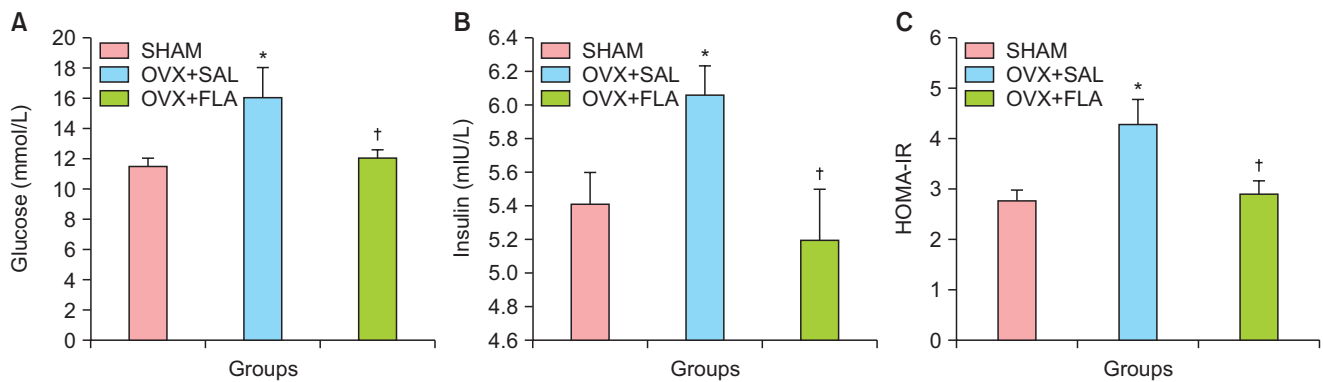


Fig. 2. The effect of treatment with flavonoid of *Mespilus germanica* leaves (10 mg/kg) on: (A) fasting glucose $*P = 0.011$, ovariectomized (OVX) + saline (SAL) group compared with sham-operated (SHAM) group, $^{\dagger}P = 0.024$, OVX + flavonoid (FLA) group compared with OVX+SAL group, (B) fasting insulin $*P = 0.048$, OVX+SAL group compared with SHAM group, $^{\dagger}P = 0.011$, OVX+FLA group compared with OVX+SAL group, (C) homeostasis model assessment estimate of insulin resistance (HOMA-IR) $*P = 0.004$, OVX+SAL group compared with SHAM group, $^{\dagger}P = 0.007$, OVX+FLA group compared with OVX+SAL group. Values are reported as the mean \pm standard error for 8 animals per group.

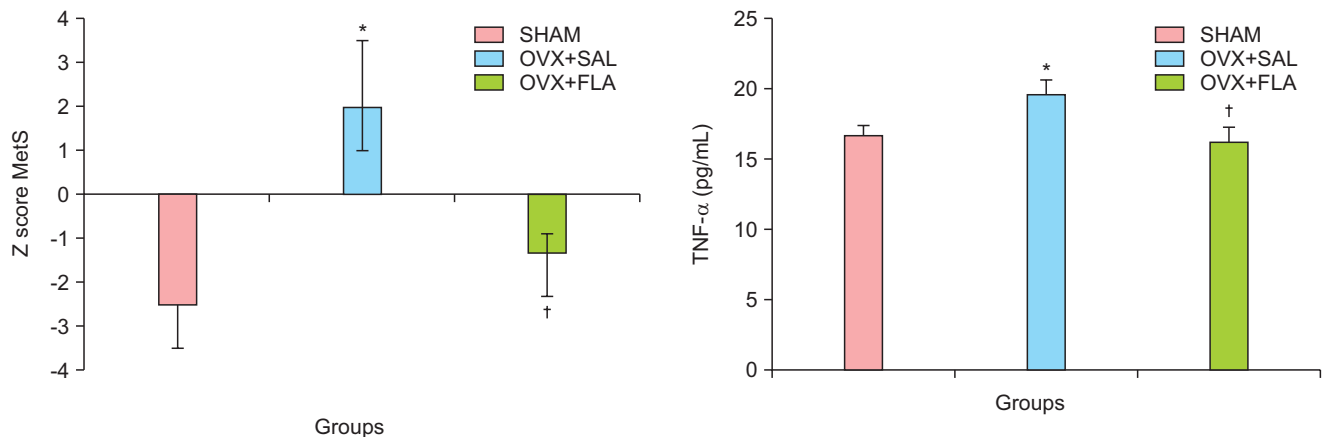


Fig. 3. The effect of treatment with flavonoid of *Mespilus germanica* leaves (10 mg/kg) on metabolic syndrome (MetS) Z score. The data is presented as the mean \pm standard error for 8 animals per group. $*P = 0.003$, ovariectomized (OVX) + saline (SAL) group compared with sham-operated (SHAM) group, $^{\dagger}P = 0.020$, OVX+flavonoid (FLA) group compared with OVX + SAL group.

Fig. 4. The effect of treatment with flavonoid of *Mespilus germanica* leaves (10 mg/kg) on serum tumor necrosis factor- α (TNF- α). The data is presented as the mean \pm standard error for 8 animals per group. $*P = 0.035$, ovariectomized (OVX) + saline (SAL) group compared with sham-operated (SHAM) group, $^{\dagger}P = 0.010$ OVX + flavonoid (FLA) group compared with OVX + SAL group.

with Sham group. Further treatment with flavonoid (Fig. 1) showed no significant change in TG ($P = 0.110$), TC ($P = 0.099$) and LDL ($P = 0.139$), HDL levels ($P = 0.550$) compared with OVX + SAL.

ANOVA analysis followed by LSD test showed significant between group difference in fasting blood sugar (FBS; $F [2,21] = 4.604$; $P = 0.022$), insulin ($F [2,21] = 4.218$; $P = 0.029$) and HOMA-IR ($F [2,21] = 6.358$; $P = 0.007$). As Figure 2 illustrates, FBS was significantly increased in OVX + SAL compared with Sham ($P = 0.011$), then it was de-

creased by 24.83% in OVX + FLA ($P = 0.024$). Serum insulin was significantly increased in OVX + SAL compared with Sham ($P = 0.048$; Fig. 2B), then it was reduced by 14.23% in the OVX + FLA ($P = 0.011$). Moreover HOMA-IR, which showed significant elevation in OVX + SAL ($P = 0.004$), significantly reduced in OVX + FLA group ($P = 0.007$; Fig. 2C). MetS Z score was significantly increased in OVX + SAL ($P = 0.003$), and then decreased in OVX + FLA group compared with counterparts respectively ($P = 0.020$; Fig. 3). Serum levels of TNF- α were significantly changed between groups as determined by ANOVA followed by LSD test ($F [2,19] =$

4.662; $P = 0.023$]. TNF- α was significantly increased in OVX + SAL compared with Sham group ($P = 0.035$) and then, significantly decreased in OVX + FLA group compared with OVX + SAL group ($P = 0.010$; Fig. 4).

Discussion

Our findings in line with the previous investigations,^{5,20,21,26} clearly confirm that ovariectomy leads in MetS, via elevation in body weight, BMI, Wc, serum glucose, insulin, visceral fat and dyslipidemia. Moreover, OVX rats showed elevation in serum levels of TNF- α indicating inflammatory pathway involvement in MetS. Interestingly, 21 days treatment with flavonoids fraction of *MGL* significantly reduced IR and serum glucose by 32%, and 25% respectively. In addition serum TNF- α significantly reduced to almost baseline value. To our knowledge this is the first report investigating the effect of flavonoids fraction of *MGL* on some of the MetS indices. However, similar to our findings, Yang et al.²⁷ and Li et al.²⁸ have previously reported on flavonoids of oxytropis falcate and Tatar buckwheat in diabetic rats.

The mechanisms by which flavonoids could slow down the progress of diabetes are not clear. Based on significant reduction in TNF- α , flavonoids of *MGL* seem to be capable to combat with inflammatory states which involves in pre diabetes stages,²⁹ although other possible mechanisms cannot be rule out. Considering the fact that more than 70% of flavonoids of *MGL* are Kaempferol and Chrysin,²⁹ we can suggest that these 2 components might be responsible for the improvement in MetS indices via reducing serum TNF- α . Kaempferol previously has been known as a potent anti-oxidant flavonoid capable to reduce TNF- α , interleukin-1 β , and lipid peroxidation.^{30,31} It should be mentioned that inflammatory signaling pathways, more notably TNF- α , are the most important initial mechanisms of diabetes and cardiovascular diseases. TNF- α , activates mitogen-activated protein kinases and downregulates peroxisome proliferator-activated receptor γ , and finally induces the transcription of inflammatory genes and impairment of insulin sensitivity.^{32,33} Dislipidemia observed in our MetS model of animals, can be explained by previous finding that TNF- α increases plasma TG concentration by inhibiting LPL activity and apo-

lipoprotein E in hepatocytes, and increases cholesterol level via stimulating the activity of 5-hydroxy-3-methylglutaryl-coenzyme A reductase.³⁴

As Joo and Lee³⁵ stated in their study, the goals of obesity treatment are to improve or prevent complication of metabolic diseases, not weight loss itself. Here we suggest that *MGL* flavonoids are potent insulin sensitizer which alleviates some of the MetS clusters, but not weight gain and visceral fat. However, its side effects remain to be elucidate in future studies.

Therefore, the present study provides new insights into the therapeutic mechanism of *MGL* flavonoids on MetS, which will be a basis for the development of effective and safe preventive natural medicine for treatment of T2DM and cardiovascular diseases.

Conclusion

In conclusion pure flavonoid of *MGL* attenuates IR partially via reducing TNF- α in MetS model of rats.

Acknowledgement

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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