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Original Article

Effects of *Vitex agnus-castus* fruit on sex hormones and antioxidant indices in a D-galactose-induced aging female mouse model

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Abstract

Background: Aging is associated with the loss of endocrine function. In this study, *Vitex agnus-castus* (Vitex), which has antioxidant effects and high levels of phytoestrogen, was investigated with regard to the hypothalamic-pituitary-gonadal axis and antioxidant indices in natural aging and in a D-galactose induced aging model in female mice.

Methods: The mice were subcutaneously injected with D-galactose (500 mg/kg/d for 45 days). Extract of Vitex (600 mg/kg/bid for 7 days by gavage) was used to treat D-galactose-induced aging and natural aging in mice. Seventy-two female NMRI mice (48 3-month-old normal mice and 24 18-24-month-old mice), weighing 30–35 g were randomly divided into six groups: control, Vitex, D-galactose, Vitex + D-galactose, Aging, and Vitex + Aging. The antioxidant indices and sex hormone levels were subsequently measured by enzyme-linked immunosorbent assay kits.

Results: Body weight and the levels of malondialdehyde (MDA), follicle-stimulating hormone, and luteinizing hormone levels were significantly increased in the D-galactose aging and natural aging groups, whereas catalase and superoxide dismutase (SOD) activity and estrogen level were significantly decreased in these same groups. D-Galactose can also disrupt the estrous cycle and damage the uterus and ovarian tissues. Vitex could effectively attenuate these alterations.

Conclusion: Vitex improved some aging events in the reproductive system of female mice. Therefore, because of its apparent antiaging effects, Vitex can be suitable for some aging problems such as oxidative stress, female sex hormone deficiency, and an atrophic endometrium.

Keywords: aging; antioxidant index; D-galactose; estrogen; *Vitex agnus-castus*

1. Introduction

Aging is a biological process that leads to oxidative stress in cells and tissues. This process can enhance human vulnerability to cognitive dysfunction and impairment in physical, mental and social activities.[1,2] Continuous changes in neuroendocrine responses occur because of aging. These changes cause a "vicious cycle of endocrinosenescence and immuno-senescence".[3] In fact, aging is correlated with immune system dysregulation; a deficiency in sex hormones; an increase in oxidative stress and the level of inflammatory cytokines; and the development of chronic diseases such as cancer, type 2 diabetes mellitus, and neurological dysfunction.[4,5] Oxidative stress is generated by an imbalance between free radicals and antioxidants. Oxidative imbalance leads to aging because of the

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impairment of various biomolecular processes and the accumulation of damage over time.[6] Some reproductive diseases such as endometriosis, polycystic ovary syndrome (PCOS), and unexplained infertility are generated because of dysfunction in the antioxidant system caused by the production of reactive oxygen species (ROS).[7] D-Galactose is a reduced sugar that can be changed into glucose at normal concentrations and oxidized into aldehydes and hydrogen peroxide at high concentrations.[8] D-Galactose accelerates aging in rodents.[9] In addition, D-galactose induces oxidative stress by increasing the malondialdehyde (MDA) level and decreasing catalase and superoxide dismutase (SOD) activity.[10] D-Galactose results in the development of aging in the brain, kidney, liver, and blood cells, and it is associated with diabetes, arteriosclerosis, nephropathy, Alzheimer's disease, metabolic abnormalities, and the formation of extra ROS and neuronal damage.[10,11]

Vitex agnus-castus L. (Vitex) is a plant that belongs to the Verbenaceae family and is native to the middle Asian, southern European, and Mediterranean countries.[12] It is used as a treatment for premenstrual syndrome (PMS), abnormal menstrual cycles, amenorrhea, mastodynia, hyperprolactinemia, premenstrual dysphoric disorder, lactation difficulties, and low fertility.[13,14] This plant is composed of iridoid glycosides, flavonoids, diterpenes, and volatile oil.[14] Water extracts and ethanolic extracts of Vitex have antioxidant activity because of its flavonoid, diterpenoid, and ecdysteroid content.[15,16]

The number of aged individuals in societies is increasing and few research documents exist on aging models and on the effects of medicinal plants such as Vitex in aging models. This study therefore investigated the antiaging effects of Vitex on ovarian and uterine tissues, sex hormones, and antioxidant indices in female mice that underwent natural aging and D-galactose-induced aging.

2. Methods

2.1. Plant material

The fruits of Vitex were collected from Qom, Iran, and stored at the Department of Botany of Ahvaz Jundishapur University (Ahvaz, Iran) under voucher specimen number A14311001P. They were dried in the dark and then powdered by an electric mill. Two hundred fifty grams of the Vitex powder was mixed with 1 L of 70% ethanol, and then soaked for 72 hours in ethanol. The content was then filtered through a paper filter and funnel glass. The filtrate was transferred to a balloon and the solvent (purity of 10%) was removed in an environment with an ambient temperature under 70°C. The yield ratio of the extract was 9.8%. A dose of 600 mg/kg body weight was administered, based on the description of an earlier study by Ibrahim et al.[17]

2.2. Animals and experimental design

In this study, 72 female NMRI mice (48 young mice, 3 months old; 24 aged mice, 18–24 months old), weighing 30–35 g, were obtained from the Ahvaz Jundishapur University of Medical Sciences (AJUMS) Animal Facility (Ahvaz, Iran). All procedures involving animals were approved by the Animals Committee of AJUMS, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The mice were maintained in cages with free access to water and food with a 12/12 hour light-dark cycle and controlled temperature (22°C ± 2°C). The mice were randomly divided into six groups (12 animals per each group), as follows:

(1) Control group: Three-month-old mice were injected subcutaneously with normal saline for 45 days and concomitantly administered normal saline by gavage twice daily for the last 7 days.
(2) Vitex group: Three-month-old mice were injected subcutaneously with normal saline for 45 days and concomitantly administered 70% ethanolic extract of Vitex (600 mg/kg/bid; by gavage for the last 7 days).[17]
(3) D-Galactose group: Three-month-old mice were injected subcutaneously with D-galactose (Merck, Germany; 500 mg/kg/d for 45 days) and concomitantly administered normal saline by gavage twice daily for the last 7 days.[18]
(4) Vitex + D-galactose group: Three-month-old mice were injected subcutaneously with D-galactose (500 mg/kg/d for 45 days) and concomitantly administered Vitex (600 mg/kg/bid; by gavage for the last 7 days).
(5) Aging group: Mice 18–24 months old were injected subcutaneously with normal saline for 45 days and concomitantly administered normal saline by gavage twice daily for the last 7 days.
(6) Vitex + Aging group: Mice 18–24 months old were injected subcutaneously with normal saline for 45 days and concomitantly administered Vitex (600 mg/kg/bid; by gavage for the last 7 days).

2.3. Estrous cycle

In some respects, the process for correctly matching the estrous cycle in the mice was an experimental method. In the beginning, 100 μg of estradiol valerate was dissolved in 0.2 mL olive oil and injected intramuscularly. After 42 hours, 50 μg of progesterone was injected intramuscularly. All animals were smeared after 6 hours,[19] and the mice were also smeared in the last 4 days of the experiment. The smears were fixed on slides and stained with 1% aqueous methylene blue, after which they were examined microscopically.[17]

2.4. Analysis of tissue and serum

After the experiment, the mice were anesthetized with ketamine/xylazine. Thereafter, blood samples were collected from the heart and centrifuged. The serum was isolated and placed in a freezer at -20°C until hormonal assays were performed. Uterine and ovarian tissues were dissected and dried, and their collective weight relative to body weight was

registered. At the end of the experiment, the left piece of the uterus and the left ovary were homogenized, and supernatants were used for the antioxidant indices. The right piece of the uterus and the right ovary were also fixed in 10% formalin for histological analyses.

2.5. Hormonal assessment

Serum levels of estrogen and progesterone were measured by using enzyme-linked immunosorbent assay (ELISA) kits (Diagnostics Test Canada, Inc., Ontario, Canada). The sensitivity of hormone detection per assay tube was 10 pg/mL for estrogen and 0.1 ng/mL for progesterone. The serum levels of mouse FSH and LH were evaluated by ELISA assay kits (Cusabio China Inc., Wuhan, China). This method has high sensitivity and excellent specificity for detecting mouse FSH and LH, with a minimum detectable dose of less than 0.35 mlU/mL and 0.5 mlU/mL, respectively. No significant cross-reactivity or interference between mouse FSH and LH analogs was observed.

2.6. Antioxidant enzyme activities

Catalase and SOD activity in the ovarian and uterine tissues were assessed by ELISA assay kits (Biocore Diagnostik; Ulm GmbH, Veltlinerweg, Germany) in accordance with the manufacturer's instructions. Biocore catalase and SOD activity assays can be used to determine the catalase and SOD activity within a range of 1–100 U/mg of protein and 5–100 U/mg of protein, respectively, with a sensitivity of 0.5 U/mg of protein and 1 U/mg of protein, respectively.

2.7. Malondialdehyde assessment in uterine and ovarian tissues

The MDA levels of tissues were assessed by ELISA assay kit (Biocore Diagnostik). The assay detects the MDA level colorimetrically in a range of 0.78–50 μM with 0.1 μM sensitivity.

2.8. Histology analysis

The formalin fixed samples were embedded in paraffin. They were then sectioned (5-μm thick) and stained with hematoxylin and eosin for histopathology. Six microscopy stained slides per animal were examined for histopathological features such as atrophy of the endometrium, and follicle degeneration was examined by microscopy.[20]

2.9. Statistical analysis

The data were analyzed using one-way analysis of variance, followed by *post hoc* least-significant difference test (SPSS version 15, Chicago, IL, USA) to evaluate differences between groups. The results are expressed as the mean ± standard error of the mean. A value of $p < 0.05$ was considered statistically significant in each group.

3. Results

3.1. Effect of Vitex on the serum levels of sex hormones

In this study, the LH level was significantly increased in the D-galactose group and the Aging group, compared to the control animals ($p < 0.01$ and $p < 0.001$, respectively). Vitex significantly decreased the LH level in the Vitex + D-galactose–treated animals, compared to the D-galactose-treated animals ($p < 0.05$). The LH level was also significantly decreased ($p < 0.05$) in the Vitex + Aging group, compared to the Aging group (Fig. 1A).

The FSH level was significantly increased in the D-galactose and Aging groups, compared to the control group ($p < 0.01$ and $p < 0.001$, respectively). Vitex did not change the FSH level in any experimental group (Fig. 1B).

The estrogen level was significantly decreased in the D-galactose and Aging groups, compared to the control group ($p < 0.001$). Vitex significantly increased the estrogen level in the Vitex + D-galactose group, compared to the D-galactose group ($p < 0.05$). Vitex also significantly increased the estrogen level in the Vitex + Aging group, compared to the Aging group ($p < 0.05$; Fig. 1C).

D-Galactose significantly decreased the progesterone level, compared to the control group ($p < 0.01$). Progesterone was also significantly decreased in the Aging group in comparison to the control group ($p < 0.001$). The progesterone level was significantly increased in Vitex + Aging group, compared to the Aging group ($p < 0.05$). The progesterone level was not significantly changed ($p > 0.05$) in the Vitex + D-galactose group, compared to the D-galactose group (Fig. 1D).

3.2. Effect of Vitex on the histology of the ovarian and uterine tissues

In the control group, the ovaries contained the graafian follicle with a clear zona pellucida. The histological architecture of the Vitex-treated ovaries was similar to that of the control animal ovaries. In D-galactose-treated ovaries, degenerative changes occurred in the follicles. The graafian follicle and zona pellucida of the ovaries were not evident in the D-galactose group, and the nucleus of the oocytes was not defined. In the Aging group, follicular degeneration in different stages was evident. Vitex effectively reversed the histological changes in the ovaries in the D-galactose and Aging groups (Fig. 2).

The uterus of the control and Vitex groups had normal histological architecture. Atrophy of the endometrium and swelling of the glands were observed in the D-galactose and Aging groups. Vitex effectively improved these alterations (Fig. 3).

3.3. Effect of Vitex on body weight and on the weight of the ovaries and uterus

There was a significant increase in body weight, whereas the ovary and uterus weight/body weight percentage decreased

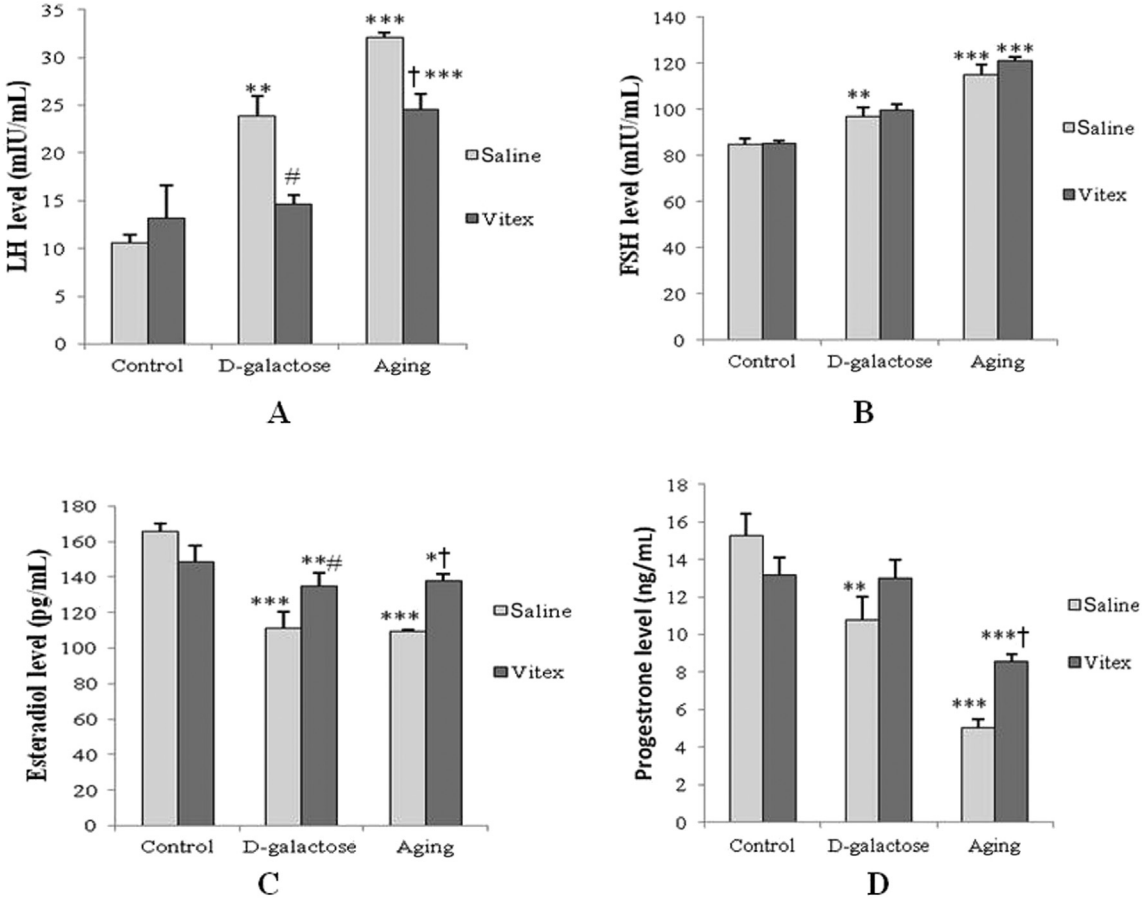

Fig. 1. The effects of *Vitex agnus-castus* (Vitex; 600 mg/kg) on the serum levels of (A) luteinizing hormone (LH), (B) follicle-stimulating hormone (FSH), (C) estrogen, and (D) progesterone in the control, D-galactose-treated, and Aging group mice ($n = 12$). The data are presented as the mean ± the standard error of the mean. Based on one-way analysis of variance and *post hoc* least-significant difference tests, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to the control group; # $p < 0.05$, compared to the D-galactose group, and † $p < 0.05$, compared to the Aging group.

in the D-galactose and Aging groups, compared to the control group ($p < 0.05$). However, the weight of the ovaries and uterus did not decrease significantly ($p > 0.05$). The administration of Vitex significantly reduced the body weight in the Vitex + D-galactose and Vitex + Aging groups, compared to the D-galactose and Aging groups, respectively ($p < 0.05$). Vitex did not significantly change the ovary and uterus weight/body weight percentage in the D-galactose and Aging groups. In addition, Vitex did not significantly increase ($p > 0.05$) the ovarian and uterine weight in these groups (Table 1).

3.4. Effect of Vitex on estrous cycle

D-Galactose may disrupt the estrous cycle, similar to aging, and Vitex may reverse the estrous cycle. The estrous cycle was normal in the control and Vitex group (data were not shown).

3.5. Effect of Vitex on the antioxidant indices of ovarian and uterine tissues

Ovarian and uterine tissue levels of MDA were significantly increased ($p < 0.001$) and SOD and catalase activity was significantly decreased ($p < 0.001$) in the D-galactose group, compared to the control group. The Aging group samples also had a significant increase in the MDA content ($p < 0.001$) and a decline in SOD activity ($p < 0.001$) and catalase activity ($p < 0.01$), compared to the control group (Fig. 4).

Vitex significantly decreased the tissue level of MDA ($p < 0.001$) and increased the catalase activity ($p < 0.05$). However, Vitex did not significantly increase the SOD activity in the Vitex + D-galactose group, compared to the D-galactose group. Treating the Aging mice with Vitex (i.e., Vitex + Aging group) significantly decreased the MDA levels ($p < 0.01$) and increased the SOD and catalase activity ($p < 0.05$), compared to the Aging group (Fig. 4).

4. Discussion

The present study demonstrated that Vitex can attenuate D-galactose-induced aging in the female mouse reproductive system. D-Galactose induces aging alterations that are similar to the normal aging processes.[21] D-Galactose can cause a decrease in estrogen, a disruption in the estrous cycle, and an increase the body weight and atrophy of the endometrium. These alterations are similar to what occurs with aging. Features of reproductive aging are associated with the progressive

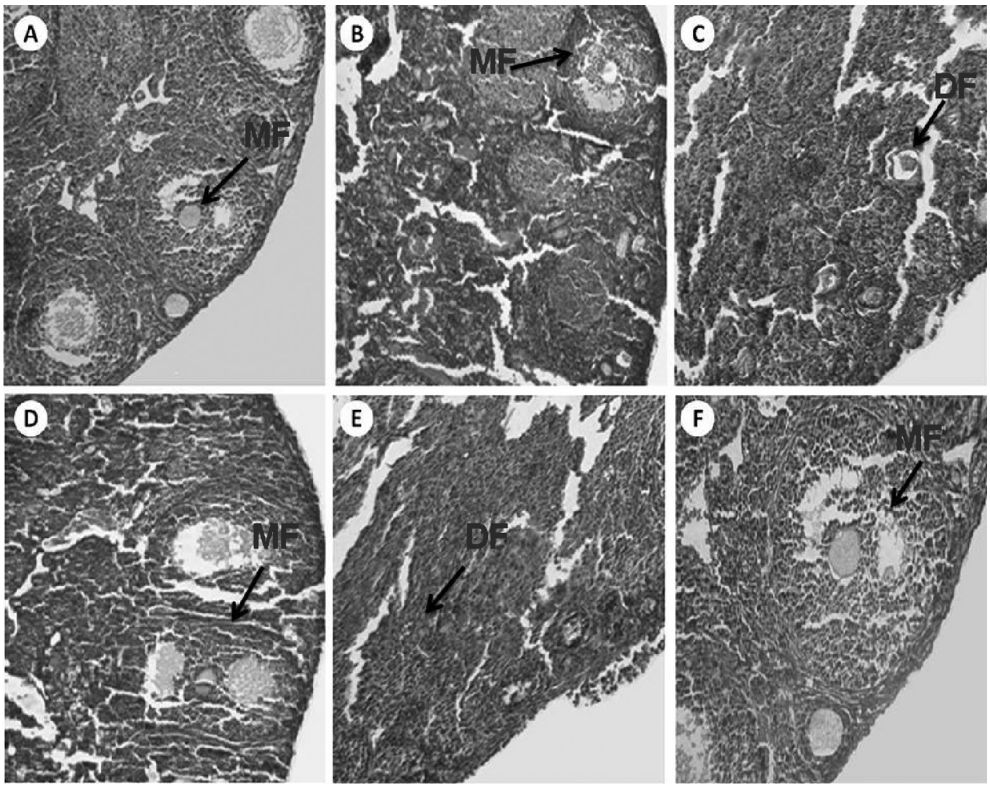

Fig. 2. Light microscopy of cross-sections of hematoxylin- and eosin-stained ovaries from the control and experimental groups: (A) Control, (B) *Vitex agnus-castus* (Vitex), (C) D-galactose, (D) Vitex + D-galactose, (E) Aging, and (F) Vitex + Aging. The magnification of all images is ×400. DF = degenerated follicle; MF = mature follicle.

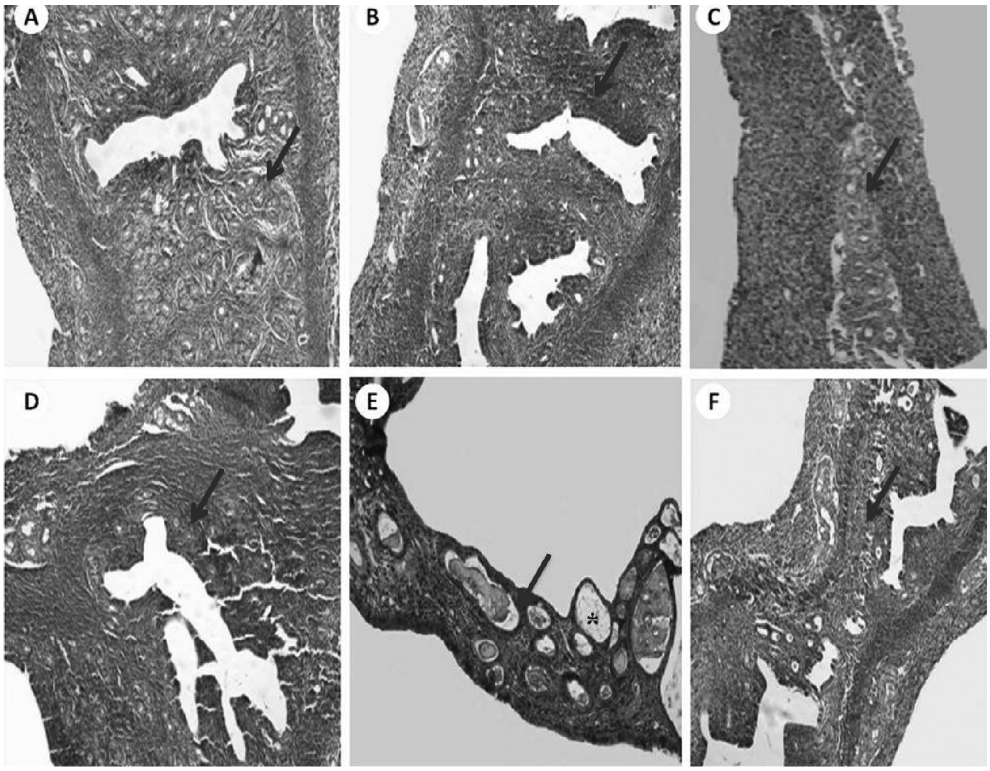

Fig. 3. Light microscopy of cross-sections of hematoxylin- and eosin-stained uterus from the control and experimental groups: (A) control, (B) *Vitex agnus-castus* (Vitex), (C) D-galactose, (D) Vitex + D-galactose, (E) Aging, and (F) Vitex + Aging. The arrow indicates the endometrium and the asterisk (*) indicates swollen glands. The magnification of all images is ×100.

Table 1
Effect of Vitex on body weight, ovary and uterus weight and %ovary and uterus weight/body weight in normal, D-galactose, and Aging group mice

Group	Body weight (g)	Ovary and uterus weight (g)	Ovary and uterus weight/body weight (%)
Control	29.95 ± 0.19	0.155 ± 0.01	0.517 ± 0.05
Vitex	29.82 ± 1.1	0.157 ± 0.02	0.526 ± 0.05
D-galactose	31.98 ± 0.4 *	0.127 ± 0.01	0.399 ± 0.03 *
Vitex + D-galactose	29.58 ± 0.4 **	0.144 ± 0.01	0.490 ± 0.03
Aging	32.78 ± 0.42 *	0.123 ± 0.01	0.376 ± 0.02 *
Vitex + Aging	29.82 ± 0.95 ***	0.149 ± 0.01	0.500 ± 0.04

* $p < 0.05$, compared to the control group.
** $p < 0.05$, compared to the D-galactose group.
*** $p < 0.05$, compared to the Aging group.

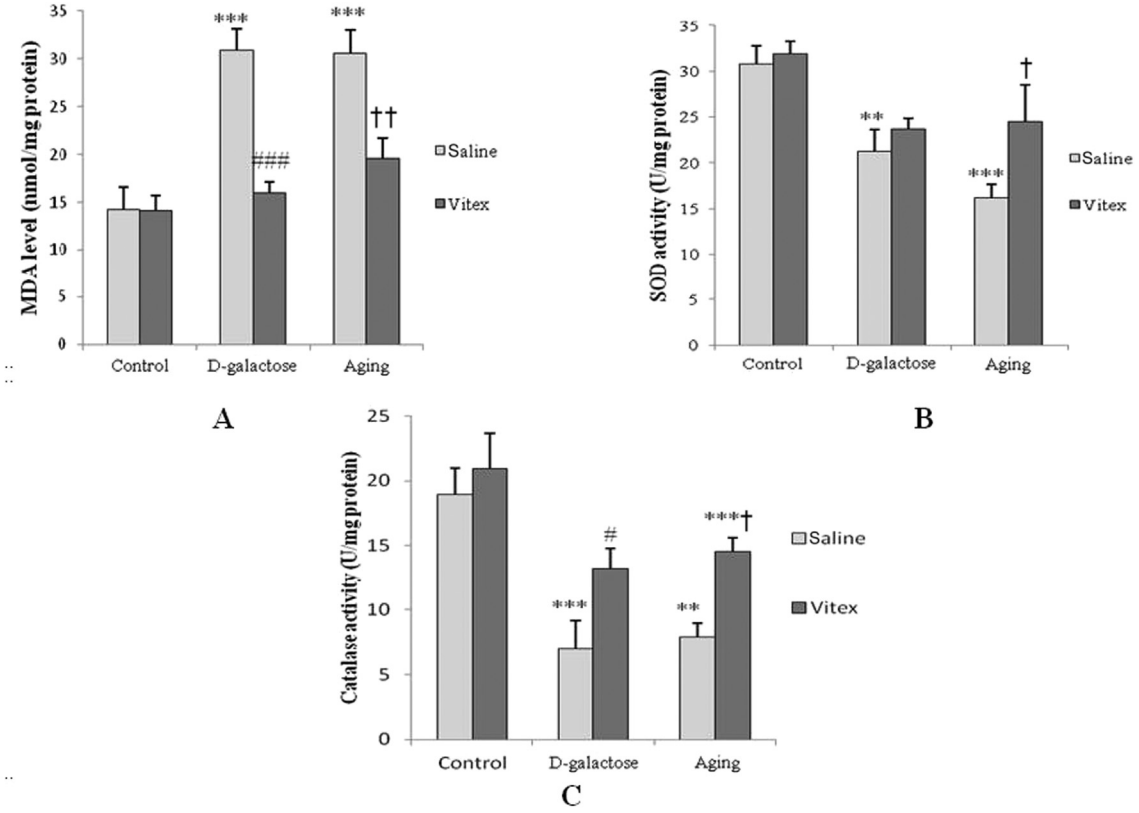

Fig. 4. The effects of *Vitex agnus-castus* (Vitex; 600 mg/kg) on (A) MDA content, (B) SOD activity, and (C) catalase activity in uterine and ovarian tissues of the control, D-galactose-treated, and Aging group mice; ($n = 12$). The data are presented as the mean ± the standard error of the mean. Based on one-way analysis of variance and *post hoc* least-significant difference tests, ** $p < 0.01$ and *** $p < 0.001$, compared to the control group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$, compared to the D-galactose group; and $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$, compared to the Aging group. MDA = malondialdehyde; SOD = superoxide dismutase.

reduction in the oocyte number. In addition, ovarian endocrine function is lost in different animals. For example, the ovaries cannot produce steroid hormones in aged animals.[22]

The findings of the present study also indicated that D-galactose could increase LH and FSH levels. A previous study interestingly reported that D-galactose can increase FSH and LH levels in male mice.[4] The elevated basal FSH (and LH) can induce reproductive and endocrinological signs of aging.[23] In mammals, aging of the reproductive system can reduce the capacity of the hypothalamic-pituitary-gonadal axis.[24]

A study by Jelodar and Askari[25] demonstrated that the Vitex extract can increase the progesterone level and decrease the testosterone level, but Vitex does not change the levels of dehydroepiandrostenedione and estrogen in animals with PCOS. In addition, Ibrahim et al.[17] found that Vitex increased the level of the hormones progesterone and estrogen and decreased LH without affecting FSH in ovariectomized rats. In the current study, the estrogen level was increased after the administration of Vitex to the D-galactose and Aging group mice. Vitex increased the progesterone levels in Aging group mice and correspondingly increased these levels slightly in the D-galactose group mice, while leaving the FSH levels unchanged in all groups. Moreover, Vitex diminished the LH level in the D-galactose and Aging group mice. Through

physiological and pharmacological effects, Vitex modulates the increase or decrease in sex hormones.[26] Vitex secretes substantial amounts of androgen that are converted to estrogen.[17]

Apigenin, which can bind to the estrogen receptor, is the most active phytoestrogen in Vitex.[27] Linoleic acid as an estrogenic compound in Vitex, and can induce certain estrogen-inducible genes by binding to estrogen receptors.[26] Luteal phase dysfunction, which increases as the progesterone level in females increases, is treated by the extract of Vitex; therefore, the chance of conception is improved. In addition, Vitex regulates reproductive function.[28] In the present study, Vitex probably increased the progesterone level and thereby regulated luteal phase dysfunction.[28] Previous studies indicate that the extract of Vitex fruit regulates the imbalance in the levels of sex hormones such as estrogen and progesterone in PMS. In fact, PMS occurs because of the imbalance between estrogen and progesterone hormones.[29]

This study demonstrated that D-galactose disrupts the estrous cycle in mice, as suggested previously by Park et al.[30] Alterations in hormone levels could also explain the disruption of the estrous cycle in the D-galactose and Aging groups. Vitex restored the normal function in the abnormal estrous cycle in D-galactose and Aging group mice, which is consistent with the findings of a study by Xu et al.[31] in phytoestrogen treated-ovariectomized mice.

Estradiol-17β (E2) has an important role in the control of body weight.[32] Estrogen deficiency is associated with the increased probability of weight gain in postmenopausal women.[33] In the current study, body weight was increased, whereas the ovary and uterus weight/body weight percentage was decreased in the D-galactose and Aging group mice. Xu et al.[31] showed that E2 replacement therapy reversed the obesity produced by deficient estrogen levels in ovariectomized mice. In the present study, Vitex reduced the body weight in the D-galactose and Aging group mice. Moreover, Vitex slightly increased the weight of the ovaries and uterus, and the ovary and uterus weight/body weight percentage in the D-galactose and Aging group mice.

Xu et al.[31] also showed that estrogen deficiency caused atrophy in the uterus of ovariectomized mice. Atrophy in the uterus was reversed by estradiol valerate and a phytoestrogen. In the present study, the administration of Vitex effectively reduced the atrophy of the endometrium and the degeneration of the follicles in the D-galactose and Aging group mice. This improvement may be because of the increase in the estrogen hormone level.

As shown in the results, D-galactose increased the MDA levels. The MDA level is a major marker of lipid peroxidation in aging tissues.[34] Malondialdehyde destroys unsaturated fatty acids in the cell membrane. Membrane structure and function are changed by the lipid peroxidation of mitochondrial, lysosomal, and plasma membranes.[35] The human body can be protected from the effects of ROS and the progression of lipid peroxidation can be prevented by antioxidants. Enzymatic and/or nonenzymatic antioxidant defenses provide protection against oxidative injury.[36] D-Galactose can induce aging because of an increase in the MDA content and decrease in the activities of SOD, catalase and glutathione peroxidase, and decrease in the estradiol content in the ovaries.[37] The SOD and catalase tissue activities were reduced in the Aging and D-galactose group mice. In this study, D-galactose likely produced superoxide anions and hydrogen peroxide molecules that led to the accumulation of lipid peroxides in the cell membranes, and thereby increased the MDA levels. Another possibility is a decline in the activity of antioxidant enzymes such as catalase and SOD elevates the peroxidation reaction. Therefore, oxidative stress may result. We suggest that this mouse model may be used as an aging model for the reproductive system. Liu et al.[38] have reported that oxidative stress is implicated in reproductive aging. Oxidative stress damages the oocytes and embryos. Therefore, the use of antioxidants probably suppresses reproductive aging in females.[39] The imbalance caused by ROS is neutralized by antioxidant enzymes in ovarian tissues. Oocytes and embryos are also protected from oxidative stress.[40]

Dietary antioxidants such as certain nutrients and cofactors may affect the oxidative stress that has a principal role in female fertility.[41] Vitex is an antioxidant plant because it contains flavonoids and phenolic compounds. This study showed that Vitex reversed oxidative stress. Phenolics and flavonoids have an important role in the scavenging of the free radicals.[16] Catalase controls the level of peroxidation with the consumption of different phenols.[42] In the present study, Vitex decreased the tissue level of MDA and increased the SOD and catalase activity in aging mice. It also increased catalase activity and reduced the MDA level in the D-galactose group mice.

In conclusion, the present study indicated that, in the female reproductive system, D-galactose induces features of aging such as atrophy of the endometrium, degeneration of follicles, increased serum levels of LH and FSH, and a decline in estrogen through oxidative stress. The administration of Vitex could effectively improve these alterations and reduce their impact. Therefore, Vitex may be useful for the treatment of certain aging problems such as oxidative stress, atrophy of the endometrium, premature-aging female, and postmenopausal syndrome.

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