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Evaluation of The Effects of Carob (*Ceratonia siliqua* L.) Fruits on the Puberty of Rats

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What is already known on this topic?

Natural and organic nutrition, which parents prefer to support their children's immunity and development, can sometimes act as endocrine disruptors due to the constituents of the food and the frequency of consumption.

What this study adds?

This is the first study showing that the use of carob in the prepubertal period causes early puberty and tissue damage by increasing doses. *C. siliqua,* preferred by parents for organic nutrition, induces early puberty and increases spermiogenesis and folliculogenesis. Furthermore, antioxidant mechanisms can come into effect and cause tissue damage at high doses.

Abstract

Objective: This study was planned to determine the effects of carob use on puberty because of the observation of early puberty or pubertal variants due to the long-term use of carob in our clinic.

Methods: Forty-eight Wistar albino rats, on postnatal day 21, were assigned into two groups female (n = 24) and male (n = 24). Groups were divided into four groups Control, and Carob-150, Carob-300, and Carob-600. *Ceratonia siliqua* L. extract was given to rats in a 0.5% carboxymethylcellulose (CMC) solution. CMC (0.5%) was given to the control, *Ceratonia siliqua* L. extract was given 150 mg/kg/day to the Carob-150, 300 mg/kg/day to the Carob-300, 600 mg/kg/day to the Carob-600 by oral gavage. The treatments were performed once daily until the first sign of puberty. Serum follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, total testosterone, leptin, glutathione, glutathione peroxidase (GPx), and malondialdehyde were measured by commercial rat-specific ELISA kits. Testis, uterus and ovarian tissue were examined histologically.

Results: The median time of preputial separation in male rats was 38th, 31st, 31st, and 31st days in the Control, Carob-150, Carob-300, and Carob-600 groups, respectively (p = 0.004). The median day of vaginal opening day was the 39th, 31st, 34th, and 31st days in the Control, Carob-150, Carob-300, and Carob-600 groups, respectively (p = 0.059). FSH, LH, testosterone (male), estradiol (female) and leptin levels of the groups were similar. However, GPx levels were higher in male and female animals given *C. siliqua* extract compared to the Control (male p = 0.001 and female p = 0.008). Testicular and ovarian tissues were concordant with the pubertal period in all groups. As the dose

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of *Ceratonia siliqua* extract increased, it induced spermatogenesis and spermiogenesis, causing abnormal changes, such as ondulation in the basement membrane, capillary dilatation, and increased congestion in males. In females, edema in the medulla gradually increased with increased dosage, and granulosa cell connections were separated in Carob-300 and Carob-600 groups.

Conclusion: This study demonstrated that *C. siliqua* caused early puberty and increased spermiogenesis and folliculogenesis. Antioxidant mechanisms were impaired with increasing dose, possibly leading to tissue damage at high doses.

Keywords: *Ceratonia siliqua* L., carob, puberty, antioxidant

Introduction

In recent years, chemical substances such as sweeteners, flavoring, and preservatives have been increasingly used in foods. These foods are the primary source of chemicals in our daily lives. Moreover, natural and organic nutrition can sometimes act as endocrine disruptors due to the content of the food and the frequency of consumption (1,2,3,4).

After weaning, parents tend to prefer organic foods, such as carob, for their children. Carob (Harnup-*Ceratonia siliqua* L.) is a plant species belonging to the legumes (*Fabaceae*) family and grows naturally in a Mediterranean climate (5). It produces a pod-like fruit, consisting of two parts, fruit (pod), and seeds. Carob is a sweet fruit considered a healthy food by families and may be consumed as raw fruit, flour or syrup. These forms are obtained from the fruity pod of the carob (6). Carob is also a natural sweetener and a source of vegetable carbohydrates. *C. siliqua* is rich in polyphenols and flavonoids in addition to its carbohydrate, protein, and fat content (7,8). Due to its rich polyphenol and mineral content, it is used especially for enhancing immune function. Several studies have demonstrated the antioxidant, anti-inflammatory, analgesic, and lipid-lowering effects of *C. siliqua* and there is also evidence of blood sugar regulation (9,10,11,12).

When the medical histories of children attending our clinic because of early puberty and puberty variants, anecdotal evidence emerged of long-term and regular use of *C. siliqua* in some cases. This animal study was planned to experimentally investigate the effects of long-term use of *C. siliqua* on puberty. To the best of our knowledge, this is the first study to examine the effect of *C. siliqua* on puberty.

Methods

Ceratonia siliqua L. Extract Preparation

Ceratonia siliqua L. fruits were provided from Doğal Kurucu Gıda Sanayi ve Ticaret Limited Şirketi, Malatya, Turkey as collected fruit material from Tarsus district of Mersin province, Turkey, in 2021. After the fruits (500 g) were dried and separated from their seeds and crushed, a 50% aqueous-alcoholic extract was prepared. The extract was concentrated in a Rotavapor® R-100 (Buchi, Switzerland) under reduced pressure and at a temperature not exceeding 40 °C. The resulting dry extract was prepared to be given to rats in a 0.5% carboxymethylcellulose (CMC) aqueous solution.

When previous *in vivo* studies on carob was reviewed, aqueous-alcoholic fruit extracts were administered to animals by oral gavage at doses of 50 mg/kg to 2000 mg/kg. In studies on the reproductive system, it has been reported that the extracts have been studied at dose ranges of 150 mg/kg and 600 mg/kg (13,14). In light of this, the dosing groups for experimanetal animals were planned to be 0 mg/kg, 150 mg/kg, 300 mg/kg, and 600 mg/kg and designated control, Carob-150, Carob-300, and Carob-600, respectively. According to the guideline, the tested extract doses in rats (150, 300, 600 mg/kg) can be converted to a human dose based on body surface area as 0.72 mg, 1.44 mg, 2.88 mg per day for a child weighing 30 kg (15). The extracts were prepared and administered to the animals in a 0.5% CMC aqueous solution. The same volume of the vehicle without extract (0.5% CMC) was administered orally to the control.

Animals and Study Design

Forty-eight Wistar albino rats weaned on postnatal day 21, were assigned into two groups female (n=24) and male (n=24). Animals were kept in a 12-hour light and 12-hour dark cycle and fed standard rodent chow (Korkuteli Food Industry, Turkey). Female and male groups were divided into control, Carob-150, Carob-300, and Carob-600 with six animals in each group for the male and female sub-groups. CMC (0.5%) was given to the control, and *Ceratonia siliqua* L. extract was given at 150 mg/kg/day to the Carob-150, 300 mg/kg/day to the Carob-300, and 600 mg/kg/day to the Carob-600 by oral gavage. The treatments were performed once daily (6 days/week), at the same time (between 8:00 and 10:00 AM), until the first sign of puberty. The first sign of puberty in male rats is preputial separation and for female rats is the first oestrus stage following vaginal opening. Body weights were recorded, and weight gain was calculated by the formula weight gain (%) = (Last day-First day)/First day. Vaginal cytology was performed to determine the estrus stage (cornified epithelial cells) after vaginal opening. Vaginal secretion was collected with a plastic pipette filled

with 10 IU of normal saline (NaCl 0.9%) by inserting the tip into the vagina. The vaginal fluid was dripped onto glass slides and was evaluated under the light microscope (Leica CME Microscope, 1349522X, NY, USA, 40x objective lenses) according to Cora et al. (16). Female rats in the first estrus stage and preputial separated male rats were euthanized by taking intracardiac blood under ketamine 45 mg/kg and xylazine 5 mg/kg anesthesia.

This study was performed in Gazi University Laboratory Animal Breeding and Experimental Research Center and approved by the Ethical Animal Research Committee of Gazi University (protocol no: G.Ü.ET-21.053, date: 09.07.2021). The experimental procedures and animal care are conducted per the EU Directive 2010/63/EU.

Biochemical Methods

Collected blood was centrifuged at 3000 rpm for 10 minutes at 4 °C and stored at -80 °C.

EA0015Ra rat follicle-stimulating hormone (FSH), EA0013Ra rat luteinizing hormone (LH), E0259Ra rat testosterone, E0174Ra rat estradiol, E0561Ra rat leptin, E1101Ra rat glutathione, E1759Ra rat glutathione peroxidase (GPx) (antioxidative markers), and E0156Ra rat malondialchehyche (MDA) as an oxidative marker were measured (Bioassay Technology Laboratory, Shangai, China).

Histopathological Methods

In the female rat groups, after euthanasia, ovaries and uterus were dissected, and ovarian (right and left) and uterus weights were measured. After euthanasia, testes (right and left separately) were dissected in male rat groups, and their lengths were measured. Uterus, ovarian (single), and testis (single) were fixed in Bouin's fixative, paraffin blocks were obtained, and 4-5 micron-thick sections were taken from the paraffin blocks. Sections taken were stained with hematoxylin-eosin. Ten sections from each rat tissue were evaluated, and data were obtained by examining ten independent fields in each section. The histomorphological changes in the obtained samples were examined with light microscopy using the Leica DM4000 (Leica, Wetzlar, Germany) computer-assisted imaging system. Captured images were evaluated using the Leica-Qwin program.

Statistical Analysis

Statistical Package for the Social Sciences, version 26 was used for statistical analysis (IBM Inc., Armonk, NY, USA). The Kruskal-Wallis test was used when comparing the medians of four independent groups in the data that did not fit the normal distribution, and the Mann-Whitney U test and the Spearman's correlation test were used when comparing the medians of two independent groups. Bonferroni correction was used in *post-hoc* tests. Statistically, $p < 0.05$ was considered significant. A power analysis was performed using GPower version 3.1.9.7 to determine the minimum sample size required of male and female rat groups to test the study hypothesis. Results indicated that a sample size of $n = 18$ is required to achieve 80% power for detecting a large effect at a significance of $\alpha = 0.05$.

Results

Male Results

At the beginning of the study, the mean weight of the male rat groups control, Carob-150, Carob-300, and Carob-600 were 45±2.9, 47±11, 49±10, 48±8.8 g, respectively. The median time of preputial separation in male rats was 38th (37-39th), 31st, (30-35th) 31st (30-34th), and 31st (30-34th) days in control, Carob-150, Carob-300, and Carob-600. The day of the beginning of puberty was statistically significantly earlier in all groups given *C. siliqua* extract than in the control group ($p = 0.04$). The median (minimum-maximum) of % weight gain was 126% (83-133), 72.5% (53-121), 60% (52-82), and 60.1% (45-89) in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. Percentage weight gain (%) was higher in the control group than in all groups given *C. siliqua* extract ($p = 0.006$). A positive correlation was found between weight gain and the day of the beginning of puberty ($p = 0.001$ $r = 0.636$). There was no statistical difference between the groups in terms of FSH, LH, testosterone and leptin (Table 1).

Table 1. The mean ± SD hormone levels of the male groups

	FSH (mIU/mL)	LH (mIU/mL)	Testosteron (ng/L)	Leptin (ng/mL)
Control	14.5 ± 7.3	70 ± 45	263 ± 52	3.3 ± 0.3
Carob-150	11.6 ± 5	92.2 ± 29.5	232.6 ± 25.5	2.8 ± 0.3
Carob-300	6.1 ± 2.8	50.4 ± 38.3	227.2 ± 73.3	3.2 ± 0.6
Carob-600	7.6 ± 2.5	68.6 ± 24.5	285.2 ± 21.5	3.2 ± 0.7
p value	0.051	0.273	0.136	0.319

FSH: follicle stimulating hormone, LH: luteinizing hormone, SD: standard deviation

The mean ± standard deviation (SD) glutathione level was 166.8 ± 28.8 mg/L, 164.5 ± 27.4 mg/L, 158.9 ± 26.8 mg/L and 178.2 ± 15.8 mg/L in the control, Carob-150, Carob-300, and Carob-600 groups, respectively, while the MDA levels were 1 ± 0.3 nmol/mL, 0.9 ± 0.2 nmol/mL, 1 ± 0.2 nmol/mL and 1.2 ± 0.1 nmol/mL in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. There was no difference between the groups regarding glutathione and MDA levels (p = 0.612, p = 0.144). The mean ± SD GPx level was 85.9 ± 11.4 U/mL, 123.4 ± 12.4 U/mL, 128 ± 20.9 U/mL and 144.0 ± 21.7 U/mL in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. There was a significantly higher level of GPx in Carob-300 (p = 0.042) and Carob-600 (p = 0.001) compared to the control group (p = 0.002) (Figure 1).

Testicular lengths in animals from the control group were longer than the groups given *C. siliqua* extract. Although % weight gain was higher in control animals, a positive correlation was found between % weight gain and both right and left testicular length (right testis p = 0.038, r = 0.425 and left testis p = 0.019, r = 0.474). Left testis length was shorter in Carob-150 and Carob-300 than in the control group and this was statistically significant (p = 0.011) (Table 2).

On histological evaluation, the seminiferous tubules of the animals from the control group were observed to have the usual histomorphology with regular contoured basement membranes. Secondary spermatocytes and spermatids were observed in some of the tubules, while spermatogonium and primary spermatocytes were observed in most of the tubules. No sperm were found in the lumen of any tubule. These findings indicated that the spermatogenesis process had just started in the tubules, and cells belonging to the later stages of the series did not differentiate. Leydig cells and capillaries in the interstitial area were in normal formation (Figure 2A).

In the Carob-150 group, the basement membranes of seminiferous tubule contours were regular. This group's secondary spermatocytes and spermatids distribution were similar to the control group. Leydig cells and capillaries in the interstitial area were observed to have normal structure and distribution (Figure 2B).

In the Carob-300 group, the most striking finding was ondulation in the basement membranes of the all seminiferous tubules. The length of the seminiferous epithelium was elongated in most tubules, and secondary spermatocytes, spermatids and spermatazoa were present in all tubules. Congestion and dilatation were detected in the capillaries. Leydig cells were observed to have normal structure (Figure 2C).

The seminiferous tubule basement membrane ondulation, seen in the Carob-300 group, was much more common and prominent in the Carob-600 group. In all tubules, thickened seminiferous epithelium containing every cell type of

Table 2. Mean ± SD testis lengths of the groups

	Right testicular length (mm)	Left testicular length (mm)
Control	14.6 ± 0.9	15 ± 1.1
Carob-150	12.2 ± 2.2	10 ± 4.8
Carob-300	12.1 ± 1.9	11.8 ± 1.4*
Carob-600	12.3 ± 1.6	12.2 ± 2*
p value	0.059	**0.011**

Values represent mean ± SD. *p < 0.05 vs. control.
SD: standard deviation

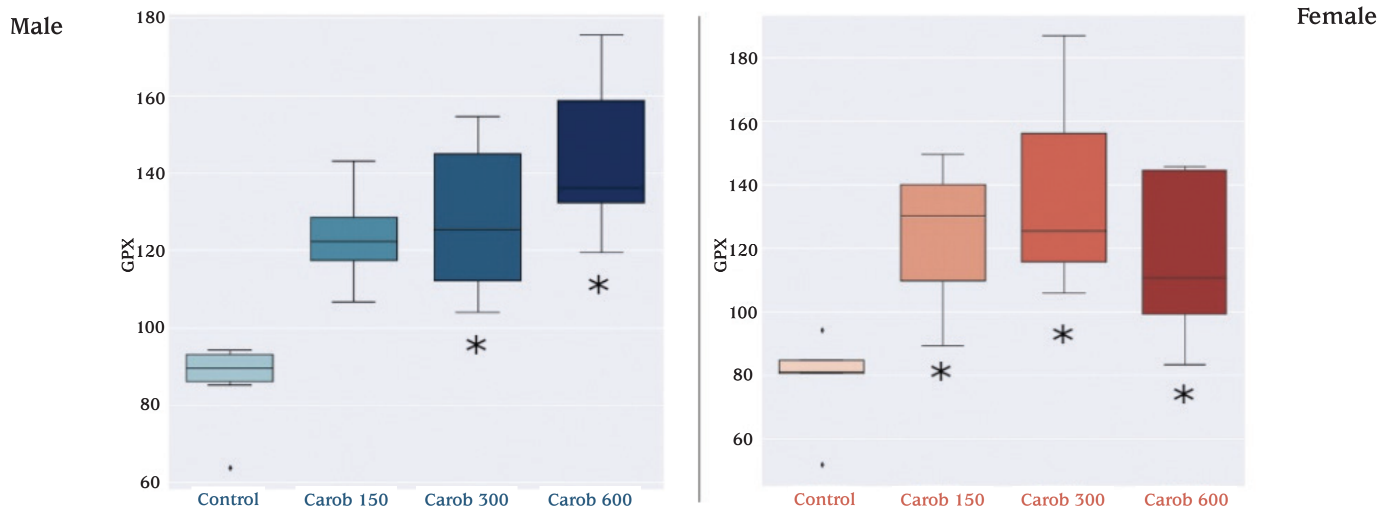

Figure 1. Glutathione peroxidase of male and female rat groups (*p≤0.05)

the spermatogenic series and sperm were evident. The seminiferous tubule walls were significantly thicker. In this group, capillary congestion and dilatation in the interstitial area were more common and prominent. Leydig cells were detected with typical histological structure near these capillaries (Figure 2D).

Testicular tissue was concordant with the pubertal period in all groups. As the dose of carob extract increased, it was observed that spermatogenesis and spermiogenesis became more evident, and although this did not cause structural and numerical changes in Leydig and Sertoli cells there were abnormal changes, such as ondulation in the basement membrane, capillary dilatation, and increased congestion.

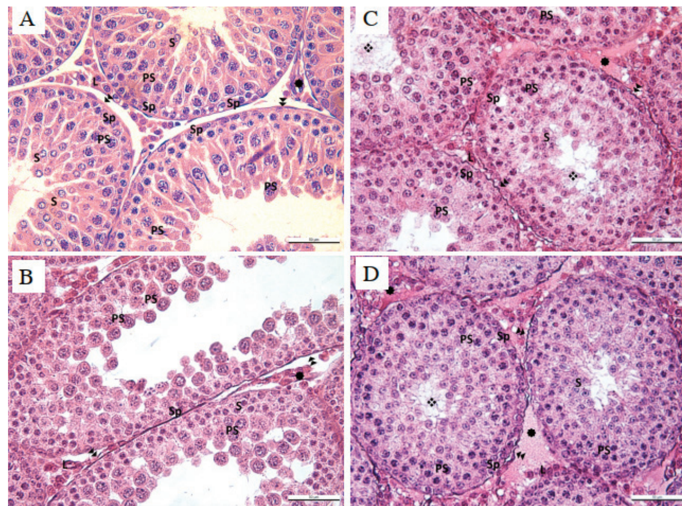

Figure 2. Histological findings of testis. **A)** Control group: Sp: spermatogonia, PS: primary spermatocyte, S: secondary spermatocytes and spermatids, ⏪: basement membrane of the seminiferous tubule in normal configuration, L: Leydig cell, ✹: normal blood vessel (H&E x200). B) Group 1: Sp: spermatogonia, PS: primary spermatocyte, ⏪: basement membrane of seminiferous tubule in normal configuration, L: Leydig cell, ✹: normal blood vessel (H&E x200). **C)** Group 2: Sp: spermatogonia, PS: primary spermatocyte, S: secondary spermatocytes and spermatids, ❖: tails of sperm in the stage of spermiogenesis, ⏪: the corrugated basement membrane of the seminiferous tubule, L: Leydig cell, ✹: congested and dilated blood vessel (H&E x200). **D)** Group 3: Sp: spermatogonia, PS: primary spermatocyte, S: secondary spermatocytes and spermatids, ❖: tails of sperm in the stage of spermiogenesis, ⏪: densely corrugated seminiferous tubule basement membrane, L: Leydig cell, ✹: extensively congested and dilated blood vessel (H&E x200)

The mean seminiferous tubule thickness for the groups (control, Carob-150, Carob-300, and Carob-600) was 234.3±34.5 µm, 258.9±46.4 µm, 301±36.2 µm, 383.8±76.4 µm, respectively. The seminiferous tubule thickness was significantly higher in Carob-300 and Carob-600 than in control and Carob 150 (p=0.001).

Female Results

At the beginning of the study, the mean weight of the female rats was 42.5±4.7, 50±9.5, 49±6.7, 51.1±4.5 g in the control, Carob-150, Carob-300 and Carob-600 groups, respectively. The median (minimum-maximum) day of vaginal opening in female rats was 39th ($37-39^{th}$), 31st ($30-35^{th}$) 31st ($30-34^{th}$), and 31st ($30-34^{th}$) in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. The day of the beginning of puberty was earlier in all groups given *C. siliqua* extract compared to the control group, but this was not significant. The median (minimum-maximum) % weight gain was 165 (127-186) mg/L, 151 (127-197) mg/L, 143 (128-184) mg/L, and 141 (131-156) mg/L in the control group, control, Carob-150, Carob-300, and Carob-600 groups with % weight gain being higher in the control group than in all groups given *C. siliqua* extract, but again this was not significant. A positive correlation was found between weight gain and the time of the beginning of puberty (p=0.001, r=0.682).

There were no statistical differences between the groups in terms of FSH, LH, estradiol, and leptin levels (Table 3).

The mean±SD glutathione levels were 161.7±19.7 mg/L, 162.3±31.1 mg/L, 149.3±18.8 mg/L and 141.5±9.2 mg/L in the control, Carob-150, Carob-300, and Carob-600 groups respectively while the MDA levels were 0.9±0.5 nmol/mL, 1.0±0.0 nmol/mL, 1.0±0.3 nmol/mL and 0.9±0.2 nmol/mL in the same groups. There was no difference between the groups in terms of mean glutathione and MDA levels (p=0.277 and p=0.976). The mean±SD GPx levels were 79.1±14.3 U/mL, 121.4±25.5 U/mL, 123.9±32.1 U/mL and 113.7±25.8 U/mL in the Control, Carob-150, Carob-300, and Carob-600. GPx levels were higher in all groups given

Table 3. The mean±SD hormone levels of the female groups

	FSH (mIU/mL)	LH (mIU/mL)	Estradiol (ng/L)	Leptin (ng/mL)
Control	15.8±4	65.7±23	99.9±37.4	3.2±0.6
Carob-150	12.3±4.3	101.3±45.3	105.9±30	3.3±0.4
Carob-300	6.1±6	56.6±41.8	89.7±40.4	3.2±0.3
Carob-600	11.4±5.9	95.6±33.6	88.6±28	3.1±0.5
p value	0.051	0.167	0.81	0.963

FSH: follicle stimulating hormone, LH: luteinizing hormone, SD: standard deviation

C. siliqua extract compared to the control group (p = 0.008) (Figure 1).

There was no difference between the groups in terms of ovarian length, ovarian weight or uterus weight (p > 0.05) (Table 4).

On histological evaluation, the ovarian tissue of the control group was observed to exhibit normal histomorphology, compatible with puberty. While all follicles belonging to the developmental stage were seen in the sections, no corpus luteum formation was observed in any section. (Figure 3A).

In the Carob-150 group, vasodilatation and edema were evident in the medulla. Typical follicle structures at the developmental stage were observed in the cortex. However, there was no evidence of corpus luteum in this group (Figure 3B).

In the Carob-300 group, vasodilatation and findings of edema of the medulla edema were increased compared to the Carob-150 group (Figure 3C). In the multilaminar primary follicle structure, separation and pericellular edema were observed in the junctional units between the granulosa cells (Figure 3D in 3C). It is possible that these type of follicles may lead to atresia.

In the Carob-600 group, corpus luteum-like structures was detected in many areas. Degenerative changes were detected in the granulosa cell layer in the multilaminar primary follicle structure. Interstitial edema was observed in regions containing granulosa cells. Edema and congestion in the medulla were found most frequently and markedly in this group. Similarly, corpus luteum was only observed in this group (Figure 3E).

Ovarian tissue was concordant with the pubertal period in all groups and primary, antral and tertiary follicles were observed in all groups but corpus luteum was only seen in the Carob-600 group. Edema and congestion in the medulla gradually increased in all groups starting from the group that received the lowest dose of carob extract. Separation of granulosa cell connections was detected in the two highest dose groups (Carob-300 and Carob-600).

Uterine tissue was observed to exhibit normal structure through all layers in the control and Carob-150 groups (Figure 4A). However, relatively minor edema was observed in the lamina propria in Carob-150 animals (Figure 4B). In Carob-300 vasodilatation and edema in the lamina propria and congestion in the muscle layer were observed, in contrast to the control and Carob-150 groups (Figure 4C). In

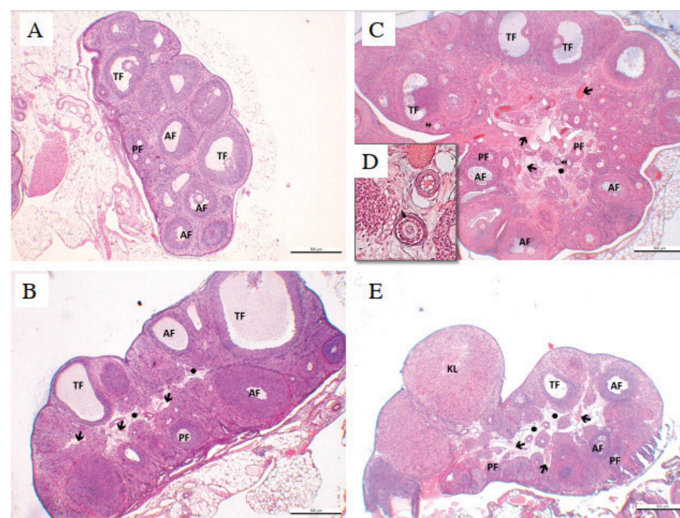

Figure 3. Histological findings of over. **A)** Control: Normal histomorphological ovarian tissue. At various developmental stages, with their normal structures PF: primary follicle, AF: antral follicle, TF: tertiary follicle (H&E x40). **B)** Carob-150: Vasodilatation in all areas, especially in the medulla (←), edema in the medulla (✸). At various developmental stages, with their normal structures PF: primary follicle, AF: antral follicle, TF: tertiary follicle (H&E x40). **C)** Carob-300: Vasodilatation increased in all areas, especially in the medulla (←). Common edema in the medulla (✸). At various developmental stages PF: primary follicle, AF: antral follicle, TF: tertiary follicle, particularly in primary follicles, separations between granulosa cells (◀◀) (H&E x40). D) Separation (←) and pericellular edema in the junctional units between granulosa cells, in the multilaminar primary follicle structure. **E)** Carob-600: corpus luteum (CL), significant vasodilatation and congestion in all areas (←) at the highest level in this group. Progressive medullary edema (✸). At various developmental stages PF: primary follicle, AF: antral follicle, TF: tertiary follicle, particularly in primary follicles, separations between granulosa cells (◀◀) (H&E x40)

Table 4. Mean ± SD ovarian lengths and ovarian and uterus weights of the groups

	Right ovarian (mm)	Left ovarian (mm)	Ovarian weight (mg)	Uterus weight (mg)
Control	4.7 ± 0.7	4.9 ± 0.7	75 ± 33.9	441.7 ± 240.9
Carob-150	5.1 ± 0.7	5.2 ± 0.5	110.8 ± 17.5	451.3 ± 114
Carob-300	4.4 ± 0.9	4.8 ± 1.4	95.1 ± 32.4	353.7 ± 154.4
Carob-600	4.6 ± 0.9	4.4 ± 0.4	97.7 ± 27.7	384 ± 163.8
p value	0.565	0.18	0.22	0.734

p < 0.05 vs. control.
SD: standard deviation

Carob-600, mitosis activation, characteristic of proliferation, and edema in the lamina propria, were more prominent in the juxta epithelial region. Due to this proliferation, the mucosa corrugated towards the lumen. Vasodilatation was very prominent in this group (Figure 4D). These findings suggested that carob extract affected the uterine cycle in a dose-dependent manner, and histological changes mimicked the proliferation phase in the control, Carob-150 and Carob-300 groups and the secretory phase in the Carob-600 group. It appeared that high dose carob extract was especially potent at accelerating the cycle in rat uterus.

Discussion

In this study, the association between ingestion of *C. siliqua* and precocious puberty was investigated in an animal model. *C. siliqua* extract accelerated the time to puberty in male and female rats. Previous studies have investigated the relationship between *C. siliqua* and the fertility of male and female adult rats (13,14,17,18). In these studies, *C. siliqua* extract was given after exposure to gonadotoxic agents such as doxorubicin, cyclophosphamide, lead and monosodium glutamate. Results reported included an increase in gonadotropins, testosterone, and estradiol after different doses of *C. siliqua*. In the present study, gonadotropin and sex steroid levels (total testosterone in males, estradiol in females) of the control group and the groups given varying doses of *C. siliqua* extract were similar. The gonadotropin levels of the control group and *C. siliqua* extract-exposed groups suggest that the onset of puberty was associated with central activity.

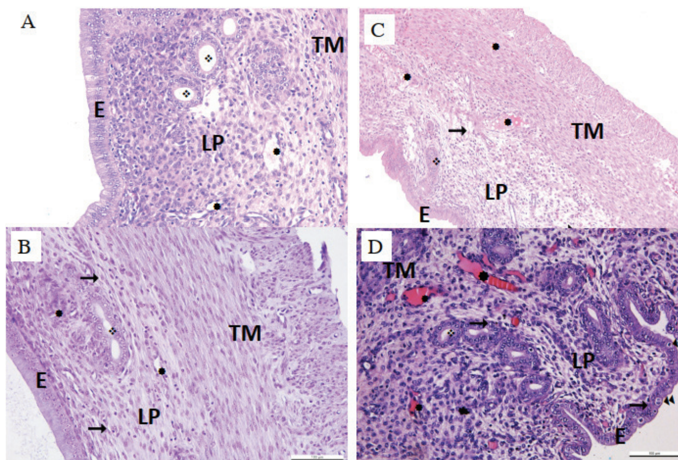

Figure 4. Histological findings of uterus. **A)** Control: Epithelium (E) lamina propria (LP), uterine glands (❖), vascular structures (✹) and muscle layer (Tunica muscularis) (TM) observed with normal histological structures (H&E x100). **B)** Carob-150: Epithelium E, lamina propria (LP), uterine glands (❖),vascular structures (✹) and muscle layer (Tunica muscularis) (TM) were observed with normal histological structures, minimal edema in the lamina propria (→) (H&E x100). **C)** Carob-300: Vasodilatation and congestion in the vessels in the lamina propria and muscle layer (✹). More prominent edema in the lamina propria (→) (❖): normal uterine glands (H&E x100). **D)** Carob-600: Relatively increased mitosis activation in epithelial cells (E) (◂◂). Increased edema in the lamina propria, more prominent in the juxta epithelial region (→). Corrugated mucosa towards the lumen. Vasodilatation is very prominent in this group (✹). (❖): normal uterine glands (H&E x100).

In the male animals exposed to *C. siliqua* extract, the onset of puberty was earlier and the day of start of puberty were the same in all three groups. The histological appearance of the testicular tissue was concordant with the pubertal period in all the male animals given *C. siliqua* extract; increasing doses of carob extract induced spermatogenesis and spermiogenesis and increased seminiferous tubule thickness. Notably, as the dose increased, tissues were more likely to be damaged.

The onset of puberty was also earlier in all female animals exposed to *C. siliqua* extract. Ovarian tissue was consistent with the pubertal period in all female groups given *C. siliqua* extract, and primary, antral and tertiary follicles were observed in all groups. As the dose increased, the follicle structures of oogenesis tended to be at a more advanced stage. It was observed that increasing the dose of *C. siliqua* extract accelerated the cycle in the rat uterus. It is hypothesized that this may have occurred in response to the increased hormone level due to ovarian-induced folliculogenesis. In the present study *C. siliqua* caused early puberty and increased spermiogenesis and folliculogenesis on histological examination of reporoductive tissues. Similarly, there are studies reporting that *C. siliqua* increases spermiogenesis and folliculogenesis after different doses in adult rats after exposure to gonadotoxic agents (13,14,18). Some studies also report an increase in the number of Sertoli and Leydig cells in the tissue, but this was not observed in the present study (13,14).

One of the main factors leading to puberty in rats is excess weight gain. Increasing leptin levels with the increase of adipose tissue is a trigger to initiate puberty (19,20). Weight gain percentage was calculated to evaluate the effect of weight gain on puberty since increasing doses of extract increased the calories taken. Percentage weight gain in the control groups was higher in both female and male rats compared to groups given the extract. However, the beginning of puberty in the control group was later than the groups exposed to *C. siliqua* extract (21,22). Leptin is known to affect the onset of puberty. In humans, weight gain and an increase in leptin caused delayed puberty in boys and early puberty in girls (23,24). In the present study, leptin levels were similar between the groups.

Fertility markers that were negatively affected at both the hormonal and tissue levels after exposure to gonadotoxic agents showed improvement after *C. siliqua* extract was administered (25). It was suggested that this occured because of the rich polyphenol, vitamin, and mineral content of *C. siliqua* and up-regualtion of antioxidant mechanisms. Arachidonic acid and aspartic acid, present in *C. siliqua*, increase the synthesis of annular adenosine monophosphate and cAMP, stimulating testosterone production (25). In addition, *C. siliqua* may exert an antioxidant effect through polyphenols (gallic acid-tannin) and through iron, manganese, zinc, copper, selenium, and vitamin E, which are cofactors of antioxidant pathways. Antioxidants are critical for protection against oxidative stress created by free radicals. Antioxidants can scavenge free radicals and prevent cell damage (26). GPX, one of the enzymatic antioxidants, breaks down hydrogen peroxide into water in the mitochondria and cytosol. GPX activity is selenium-dependent, and there are eight identified GPXs (27). GPX plays a role in cell differentiation and proliferation in gametes. GPX4 is located primarily in the testis, and its expression pattern in the testis suggests that it may be related to the onset of puberty (28). In the rat study of Roveri et al. (29), it was reported that under stimulation by gonadotropins, GPX increased in the testicular tissue of rats and stimulated spermatogenesis.

In the present study, GPX levels were significantly higher in male and female animals given the extract. Thus, antioxidant processes may also play a role in the progression of puberty, as previously suggested (30). In addition to inducing puberty at high doses, *C. siliqua* also caused histopathological changes, including ondulation in the basement membrane in males and degeneration of intercellular junctions in granulosa cells in females. These findings suggest that high doses may cause damage to gametes. When antioxidants are taken in high doses, they may become pro-oxidants in the tissue and cause tissue damage and death (31,32).

Study Limitations

Limitations of the present study include not analyzing kisspeptin and neurokinin B levels, both of which are mediators involved in the onset of puberty. If these markers had been measured, we hypothesize that there would be results more supportive of the central onset of puberty. Also, we were unable to demonstrate hyperplasia of gonadotroph cells in the rat pituitary histopathologically. This was not studied because the rats were in a small age group, and the tissue was difficult to dissect. Another limitation of our study was *C. siliqua* fruits are rich in flavonoids, phenolic acids, carbohydrates, proteins, vitamins and minerals. It is not known which of these ingredients induces puberty. We did not conduct a content analysis in the extract. But in future studies it will be possible to show the effect of more specific active ingredients.

Conclusion

Extracts of *C. siliqua* appeared to cause early puberty and increased spermiogenesis and folliculogenesis in a rat model. It is suggested that antioxidant mechanisms may also be involved but may cause tissue damage at high doses. We caution that foods consumed for their organic nutrition may become endocrine disruptors when the amount and duration of use increase.

Ethics

Ethics Committee Approval: This study was performed in Gazi University Laboratory Animal Breeding and Experimental Research Center and approved by the Ethical Animal Research Committee of Gazi University (protocol no: G.Ü.ET-21.053, date: 09.07.2021).

Informed Consent: Animal experiment.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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