



Original Article

Activity of *Corylus avellana* seed oil in letrozole-induced polycystic ovary syndrome model in rats



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ABSTRACT

The aim of the present study was to assess the activity of the hazelnut oil in the treatment of polycystic ovary syndrome in rats. Serum follicle-stimulating hormone, luteinizing hormone, estradiol, progesterone, testosterone, serum lipid parameters, leptin and glucose levels were evaluated. Moreover, antioxidant activity was tested using superoxide dismutase, malondialdehyde, catalase, glutathione peroxidase levels. The phytosterol content of the oil was determined by HPLC. The plasma high density lipoprotein-cholesterol level was found to be significantly high and leptin and glucose concentrations were found to be significantly low in the treatment group. According to the phytochemical analysis, the main components of the oil were detected as α -tocopherol, γ -tocopherol, squalene, β -sitosterol, campesterol and stigmasterol. *Corylus avellana* oil was found to be effective in the treatment of polycystic ovary syndrome *via* regulating gonadotropins, steroids and serum lipid parameters and possesses antioxidant activity.

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Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder which causes anovulation in animal and women of reproductive age (Dehghan et al., 2012; Zhai et al., 2012; Arikawe et al., 2013). Its hyperandrogenic manifestations include menstrual irregularity, acne, hirsutism and oligo-ovulation/anovulation. Metabolic abnormalities such as dyslipidemia, insulin resistance, therefore, diseases including diabetes, obesity, cancer and infertility as well as coronary heart diseases could be seen along with PCOS (Maharjan et al., 2010; Zhai et al., 2012; Ghasemzadeh et al., 2013).

PCOS is characterized by small arrested antral follicle formation in the development process. In PCOS estrogen level decreases, however, the progesterone level increases and LH/FSH ratio becomes three times of the normal level. Androgens are synthesized by the theca cells (Walters et al., 2012).

Corylus avellana L., Betulaceae, is growing wild in Europa, Western Asia and Northern Africa as large shrubs or small trees about 3.5–4.5 m high. The leaves are deciduous, rounded, 6–12 cm long, softly hairy on both surfaces, with a double-serrate margin. The flowers are monoecious, with single-sex catkins, the male pale

yellow and 5–12 cm long. The edible part of the hazelnut is the roughly spherical seed, which is covered by a dark brown perisperm and protected by a hard, woody shell. The ripening nut is enclosed in a green fringed tube (Könemann, 1999; Contini et al., 2011). This plant was reported to contain vitamins including vitamin E, B6 and B9, unsaturated fatty acids, plant sterols and polyphenols (Jakopic et al., 2011). One of the most important features of *C. avellana* is to have the highest ratio of unsaturated/saturated fatty acids. Moreover, *C. avellana* was shown to reduce plasma total and LDL cholesterol concentrations, by its polyunsaturated fatty acid, phytosterol and soluble dietary fiber content (Weststrate and Meijer, 1998; Brown et al., 1999; Feldman, 2002). A high fiber diet can be beneficial for the health of heart and digestive system and can help to regulate the blood glucose level (Anderson et al., 2009). *C. avellana* is a poor source of isoflavones, indeed, seed oil contains only trace amounts of phytoestrogens (Mazur, 2000).

According to the data reported in ethnobotanical studies, *C. avellana* has been used for the treatment of varicose veins, hemorrhoids, diabetes mellitus and gynecological disorders (Sezik et al., 1997; Ramalhosai et al., 2011; Abeer and Amr, 2013). The aim of the present study was to evaluate the activity potential of *C. avellana* oil in the treatment of PCOS and analyze the phytochemical constituents by using chromatographic methods in order to find out the compounds responsible from the efficacy.

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Materials and methods

Plant material

Seeds of *Corylus avellana* L., Betulaceae, were collected from Traşon, Akçabat village, Turkey in August 2013. A voucher specimen was deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Preparation of the oil

The plant material (50 g) was air dried and grinded. Ground hazelnut was weighed (10 g) and the oil was extracted with hexane in a Soxhlet apparatus during 6 h (James, 1995). The extract was evaporated at 40 °C. The oil content of the hazelnut was determined to be 51.23%. The oil was kept in a dark bottle in a freezer at –25 °C until the time of analysis.

Saponification of sterol, tocopherol and squalene for HPLC analysis

The oil (30 mg) was mixed thoroughly with 50% KOH (w/v) (200 µl) and 1% ethanolic pyrogallol (w/v) (1.5 ml). The tubes were kept at 70 °C in a water bath for 40 min. Water (0.5 ml) and hexane (3 ml) were added to the tubes after cooling on ice. The tubes were shaken and centrifuged at 400 × g for 10 min. The hexane phase was removed and the extraction repeated with hexane (1 ml). The combined hexane extracts were dried and was redissolved in ethanol (150 µl) and transferred into an HPLC vial.

Analyses of phytosterols, squalene and tocopherols by HPLC

HPLC conditions on Varian a ProStar HPLC system include Varian ProStar 240 Pump, Varian ProStar 410 AutoSampler, Varian ProStar 510 Column Oven, Varian ProStar 335 UV/VIS Detector. Sample (10 µl) was injected onto a Phenomenex luna C8 5u Silica 100A (150 mm × 4.6 mm LC Column) for phytosterol analysis. The chromatographic separation of the sample was performed using a mobile phase consisting of 80% acetonitrile and 20% water at a flow rate of 1.5 ml/min. For the analyses of tocopherol and squalene, Supelcosil LC-18 Column, 3 µm, (4.6 mm × 150 mm i.d.; Supelco, Bellefonte, PA, USA) and mobile phase consisting of 99% methanol and 1% water was used at a flow rate of 1.3 ml/min. Peak areas were recorded using Galaxie Chromatography Workstation software version 1.9.3.2 (Varian, Inc. Marketing Department 2700 Mitchell Drive Walnut Creek, CA 94598). Chromatograms were extracted at 205 nm, 292 nm and 215 nm for phytosterol, tocopherol and squalene analysis respectively (Maguire et al., 2004).

Animals

Eleven-week-old female, non-pregnant, eighteen mature Sprague Dawley rats (200–250 g) with 4–5 days regular estrus cycles were used in the study. All rats were purchased from Kobay Experimental Animals Laboratory, Ankara, Turkey and quarantined for at least two weeks. The animals were housed in polysulfone cages at 21–24 °C, 40–45% humidity, and light and dark cycles of 12 h conditions at Laboratory Animals Breeding and Experimental Researches Center, Faculty of Pharmacy, Gazi University, Ankara, Turkey. The experiments were conducted in accordance with the directions of Guide for the Care and Use of Laboratory Animals. The experiment was approved by the Experimental Animal Ethics Committee of Gazi University (G.Ü. E.T-14-022).

Experimental design

Letrozole (Letrasan[®], DevaHolding A.S., Tekirdag, Turkey) (concentration of 1 mg/kg) was dissolved in 0.5% carboxymethylcellulose (CMC) was administered to the rats by gavage once daily throughout 21 days. This dose was chosen according to the previous studies in which the cystic follicle formation was induced (Rezvanfar et al., 2012a). During the experiment, the estrus cycle was microscopically evaluated by the analyses of relative proportion of leukocytes, epithelial and cornified cells. Body weight change due to the administration of letrozole was weekly observed. All rats were randomly divided into following three groups consisting of six rats in each group: (i) control group (CMC; 2 ml/rat/day, *p.o.*), (ii) reference group (buserelin acetate; 20 mg/rat/week, *s.c.*) and (iii) treatment group (*C. avellana*; 2 ml/rat/day, *p.o.*). The test materials were administered throughout 45 days.

Termination of the procedure

The animals were euthanized 24 h after the last dose of the treatment. Blood samples were collected by cardiac puncture. Serum was separated and kept in a freezer at –20 °C for determination FSH, LH, estradiol, progesterone, testosterone, TC, HDL-C, LDL-C, TGs, leptin and glucose, SOD, MDA, catalase, GPx levels. Uteri and ovaries were dissected, and weighed for the evaluation of the endocrine function.

Measurement of circulating levels of serum gonadotropins and steroids

Serum gonadotropin levels were determined using radioimmunoassay (RIA). All RIA kits were obtained according to the manufacturer's instruction from Beckman Coulter Co., Marseille, France. Serum FSH was assayed by sandwich RIA using a commercially available RIA kit. Serum LH, estradiol, progesterone, and testosterone were measured by competition radioimmunoassay with commercially available RIA kits.

Evaluation of the blood lipid, leptin and glucose levels

Plasma leptin (Cat. EZRL-83K) concentration was determined by ELISA using a rat kit (Linco Research, Inc., St. Charles, USA). Plasma triacylglyceride, total cholesterol, HDL-C LDL-C values were measured with commercially available assay kits (Human Diagnostica, GmbH, Germany). Blood glucose level was determined using Glucose Roche Diagnostic glucometer strip.

Determination of antioxidant activity

SOD, GPx, MDA, catalase levels were analyzed for the determination of the antioxidant activity potential. SOD and GPx activities were measured as described in Rezvanfar et al. (2012b). SOD assay was evaluated by the rate of increase at 560 nm. GPx activity was read by the determination of the decrease in absorbance at 365 nm. MDA level and catalase activity were studied biochemically in erythrocyte lysate samples. Catalase activity was measured and calculated according to Aebi's method by recording the hydrogen peroxide degradation spectrophotometrically at 240 nm (Aebi, 1984). MDA was measured by the modified Yagi (1984).

Histology

The specimens were fixed in 10% buffered formalin, processed, and embedded in paraffin and sectioned 5 µm thicknesses. These sections were stained with hematoxylin-eosin (HE). Pathological-physiological structures in ovaries were classified on the HE stained

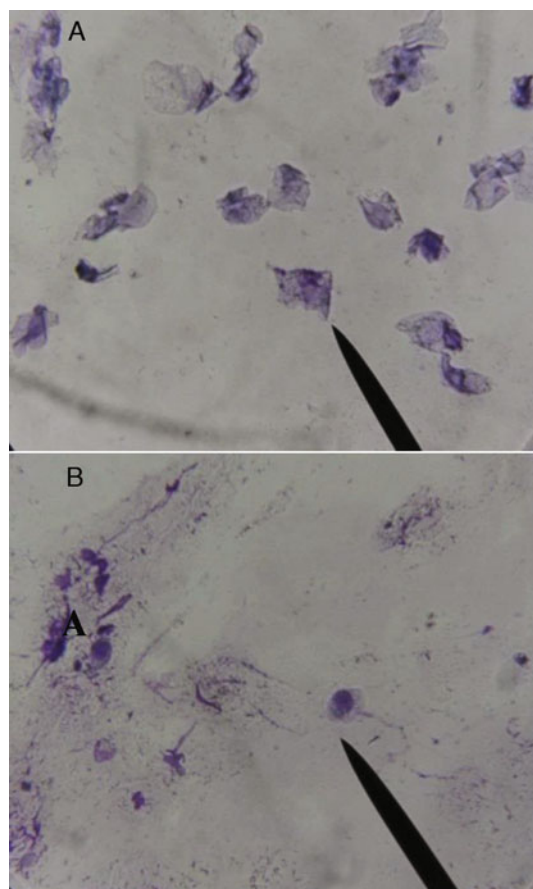


Fig. 1. View of the vaginal cytology in control (A) and treatment (B) groups.

sections with a light microscope and Kameram[®] image analysis system (Microsystem, Istanbul, Turkey).

Statistical analysis

Statistical analysis was performed using SPSS version 16. The results were expressed as the Mean \pm S.D. Dunnett's test was used to determine the significance of differences between groups. In order to compare two groups Student's *t* test was used. A probability value <0.05 was considered statistically significant.

Results

The levels of total α -tocopherol and gamma-tocopherol was calculated as 301.5 $\mu\text{g/g}$ and 52.8 $\mu\text{g/g}$, respectively. 173.7 $\mu\text{g/g}$ squalene was detected in the nut. The levels of phytosterols were detected as follows; β -sitosterol 875.4 $\mu\text{g/g}$; campesterol 75.4 $\mu\text{g/g}$ and stigmasterol 44.9 $\mu\text{g/g}$.

After letrozole administration to the rats, reproductive cycle was found to be irregular. Following the administration of test materials, the control group displayed irregular estrous cycles and exhibited constant estrus (Fig. 1A), whereas the treatment group and the reference group exhibited regular estrous cycles (Fig. 1B).

Twenty one days after the letrozole administration, all rats exhibited significant increase in the body weight (control group: 384.5 ± 12.5 g, treatment group: 384.7 ± 13.0 g and reference group: 386.8 ± 12.7 g). After the treatment with hazelnut oil and the reference drug busserelin, a remarkable decrease was observed in the body weight which was detected as 304.7 ± 11.0 g and 308.2 ± 12.1 g, respectively. However, the body weight of the control group animals increased to 424.6 ± 10.6 g.

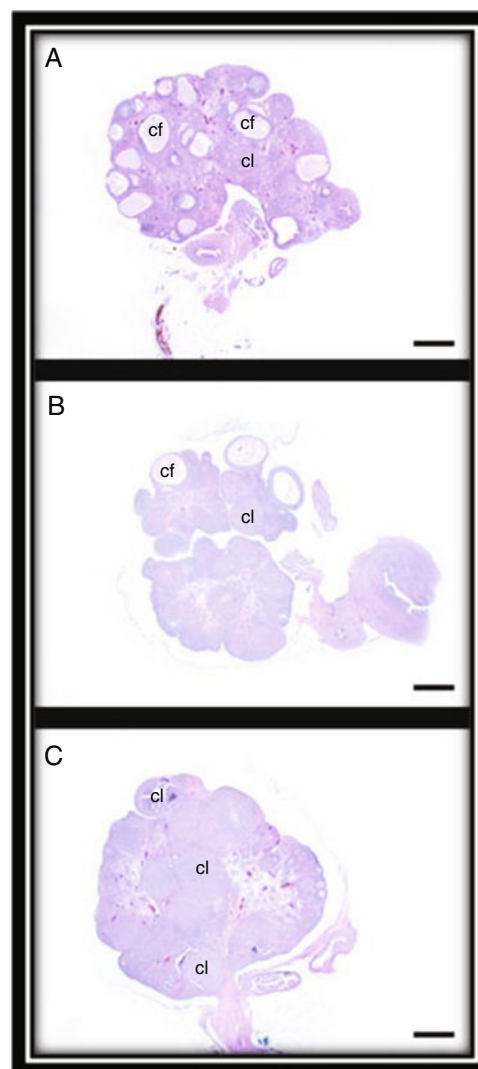


Fig. 2. Histopathological view of the experimental groups. The images are presented were stained with H&E. The original magnification was $1.25\times$ and the scale bars represent 1000 μm . (A) Control group; (B) treatment group; (C) reference group. Abbreviations as shown on the images; cl: corpus luteum, cf: cystic follicle.

In comparison with the control group, hazelnut oil and the reference drug administration caused remission in the ovarian weight at the end of the experiment (25.6 ± 1.2 mg/100 g, 21 ± 1.0 mg/100 g and 22 ± 1.2 mg/100 g, ($p < 0.05$) respectively). However, after the administration of hazelnut oil and the reference drug the uterine weight was increased when compared to control group (88.2 ± 1.2 mg/100 g, 92.0 ± 1.4 mg/100 g and 51 ± 1.1 mg/100 g, ($p < 0.05$) respectively).

Follicular cysts, seconder follicles and atretic follicles were recorded as severe in the control group, moderate in the treatment group, and mild in the reference group. Lumen of follicular cysts was bordered with single or more layered granulosa cells. Degeneration and desquamation of these cells and thickened theca interna were severe in the control group. Compared to the other groups regression of corpus luteum was prominent in the reference group, moderate in the treatment group and mild in the control group (Fig. 2).

High FSH and LH levels were detected in the control group, while the treatment and reference groups' levels were found to be normal. Furthermore, testosterone levels significantly augmented in the control group, indicating the hyperandrogenism. Estradiol and progesteron levels decreased in the control group when compared

Table 1

Serum gonadotropin and steroid levels in control, treatment and reference groups.

Groups	FSH (ng/ml)	LH (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)	Testosteron (ng/dl)
Control	108.3 ± 5.8	8.7 ± 1.6	2.6 ± 0.3	9.85 ± 1.7	267.46 ± 23.5
Treatment	87.6 ± 3.2	2.8 ± 0.2 ^{***}	9.06 ± 1.3 ^{**}	26.8 ± 1.5 ^{***}	92.4 ± 7.3 ^{***}
Reference	75.2 ± 7.5 [*]	3.6 ± 0.4 ^{***}	10.8 ± 1.9 ^{**}	36.4 ± 5.2 ^{**}	72.64 ± 6.2 ^{***}

^{*} $p < 0.05$.^{**} $p < 0.01$.^{***} $p < 0.001$ (treatment and reference groups were compared with the control group).

Data presented as Statistical Mean ± Standard Deviation (SD).

Table 2Effects of *Corylus avellana* on plasma concentrations of TC, TG, HDL-C, LDL-C, leptin and glucose.

Groups	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	Leptin (μg/l)	Glucose (mg/dl)
Control	52.01 ± 4.11	114.81 ± 10.24	53.59 ± 5.72	19.61 ± 6.38	5.94 ± 1.79	136.45 ± 10.93
Treatment	41.58 ± 3.18	102.55 ± 11.93	78.36 ± 3.15 ^{**}	15.50 ± 4.19	3.98 ± 1.04 [*]	107.74 ± 3.41 [†]
Reference	31.46 ± 1.09 [*]	84.72 ± 9.08 [*]	85.32 ± 2.10 ^{**}	12.48 ± 3.07 [†]	2.46 ± 1.12 ^{**}	98.06 ± 2.22 ^{**}

^{*} $p < 0.05$.^{**} $p < 0.01$ (treatment and reference groups were compared with the control group).

Data presented as Statistical Mean ± Standard Deviation (SD).

Table 3

Serum levels of MDA, SOD, catalase and GPx levels in all groups.

Groups	MDA (mmol/ml)	SOD (u/mg protein)	Catalase (mmol/ml)	GPx (u/mg protein)
Control	3.41 ± 0.58	846.12 ± 20.03	7.46 ± 2.32	112.42 ± 9.52
Treatment	2.63 ± 0.21 [*]	994.05 ± 17.22	9.24 ± 1.62 [†]	107.25 ± 5.27
Reference	1.24 ± 0.14 ^{**}	1032.26 ± 25.47 [†]	8.43 ± 1.89	104.71 ± 6.13

^{*} $p < 0.05$.^{**} $p < 0.01$ (treatment and reference groups were compared with the control group).

Data presented as Statistical Mean ± Standard Deviation (SD).

to the treatment and reference groups. While estradiol ($p < 0.01$) and progesterone ($p < 0.001$) levels significantly increased, LH and testosterone ($p < 0.001$) levels decreased in the treatment group when compared to the control group (Table 1). However this difference was not significant between the reference group and treatment group.

Serum TC, TG, HDL-C, LDL-C, leptin and glucose levels were respectively 41.58 ± 3.18 mg/dl, 102.55 ± 11.93 mg/dl, 78.36 ± 3.15 mg/dl, 15.50 ± 4.19 mg/dl, 3.98 ± 1.04 μg/l, 107.74 ± 3.41 mg/dl in the treatment group. The plasma HDL-C ($p < 0.01$), leptin and glucose ($p < 0.001$) concentrations were found to be significant in the treatment group when compared to the control group (Table 2).

Serum MDA, SOD, catalase and GPx levels were determined as 2.63 ± 0.21 mmol/ml, 994.05 ± 17.22 u/mg protein, 9.24 ± 1.62 mmol/ml and 107.25 ± 5.27 u/mg protein, respectively in the treatment group. Serum catalase ($p < 0.05$) levels significantly increased in the treatment group when compared to the control group. There was no statistically significant difference between the treatment and reference groups in terms of the antioxidant activity ($p > 0.05$) (Table 3).

For the acute toxicity evaluation, the oil was administered orally to the rats at 1, 2 and 4 ml/rat/day doses. The animals were observed for 48 h and neither toxicity nor mortality was recorded.

Discussion

There are several hypotheses on the etiology of PCOS, which is a common endocrine disorder in women and animals, however, there is limited information regarding the mechanisms of its development (Ball and Peters, 2004; Padmanabhan and Veiga-Lopez, 2013). Treatment of PCOS is highly important for the economically valuable animals such as livestock (Ball and Peters, 2004). The therapeutic approaches to PCOS are mainly symptomatic since

little information is available on the etiopathogenesis of the disease. Some pharmacological agents such as oral contraceptives, antiandrogens, GnRH agonists, steroids, insulin sensitizers such as metformin, troglitazone are preferred both for human and animal in the treatment of PCOS (Dehghan et al., 2012). However, due to the adverse effects caused by synthetic drugs, the plant based remedies are much preferable in the treatment of PCOS.

Previous studies on the family Betulaceae revealed that the plants are rich in the phenolic constituents including flavonoids, tannins, caffeic acid derivatives, diarylheptanoid-type compounds (Jin et al., 2007; Martineau et al., 2010). Diarylheptanoids were found to possess various pharmacological effects such as anti-inflammatory, antioxidant, anticancer and antiadipogenic activities (Jin et al., 2007; Martineau et al., 2010). Moreover, diarylheptanoids are the major components, which exhibit high estrogenic activity by ASPP 049 mediated transcriptional activation through a ligand-dependent ERα-estrogen-responsive element-driven pathway and a non-genomic action on vascular relaxation through the ER-Akt-endothelium nitric oxide synthase pathway (Suksamrarn et al., 2008; Winuthayanon et al., 2009a,b; Intapad et al., 2009).

A number of reports have suggested that phytosterols possess estrogenic activity and therefore, have potential effects on the reproductive system. According to the previous published articles especially β-sitosterol was found to act as an estrogenic agent (Malini and Vanithakumari, 1992; Mellanen et al., 1996). Although the estrogenic potential of β-sitosterol was confirmed by several *in vitro* and *in vivo* experiments, a mechanistic study suggested that β-sitosterol is not estrogenic itself but needs to be metabolized to a hormonally active metabolite (Mellanen et al., 1996).

According to the HPLC analysis, campesterol, stigmasterol, β-sitosterol, squalene and tocopherol were determined in hazelnut oil in the present study. These results were found to be in accord with the previous studies (Parcerisa et al., 1998; Baker et al., 1999).

Alteration in the oxidant-antioxidant profile is known to occur in polycystic ovary syndrome (Verit and Erel, 2008). Reactive oxygen species cause the formation of lipid hydroperoxides, conjugated dienes, and MDA, as well oxidize biomolecules leading to extensive lipid peroxidation (LPO) in proteins, membranes, and genes (Kucukkurt et al., 2008). The evaluation of MDA is an indicator of free-radical damage through membrane LPO (Keles et al., 2012). In the present study, blood MDA level decreased in the treatment group when compared to control group which suggest hazelnut include active components against LPO. Antioxidants involve enzymatic systems such as SOD, glutathione peroxidase, and CAT which prevent the oxidative cell injury on tissues (Kyle et al., 1987). SOD protects the tissues from the harmful effects of superoxide radicals and CAT enzyme hydrolyzes H_2O_2 into H_2O and $1/2 O_2$. These enzymes have an important role in the resistance of cellular lipids, proteins, and DNA (Matés and Sánchez-Jiménez, 1999). In the present study, the levels of erythrocyte SOD and CAT activities increased whereas GPx level decreased when compared to control group.

The ethanolic extract of *C. avellana* seed displayed remarkable antioxidant activity in total antioxidant and radical scavenging tests, which could be attributed to the relatively high total phenol content of the extract (Shahidi et al., 2007). Myricetin-3-O-rhamnoside, quercetin-3-O-rhamnoside, gallic acid, protocatechuic acid, phloretin-2'-O-glucoside, (+)-catechin, (-)-epicatechin and procyanidins were reported as the major phenolic compounds of *C. avellana* seed (Weststrate and Meijer, 1998).

Phytosterols such as squalene, campesterol, β -sitosterol, stigmasterol, tocopherol have been determined as the potential antioxidant agents in the previous studies (Smith, 2000; Kmiecik et al., 2011; de França Ferreira et al., 2014). Therefore, it was suggested that phytosterols determined herein, probably promoted the treatment of PCOS by their antioxidant effect.

Insulin plays an important role in the modulation of blood FSH levels (Sudha et al., 2000). The antioxidant activity of *C. avellana* could be responsible from the regulator effect of gonadal hormone levels in rats with high level glucose due to PCOS as a reproductive problem. Therefore, the improvement in the blood glucose and serum insulin levels could be associated with the improvement in the serum FSH and LH hormones.

The main components of the oil were detected as α -tocopherol, γ -tocopherol, squalene, β -sitosterol, campesterol and stigmasterol. *C. avellana* oil was found to be effective in the treatment of PCOS via regulating gonadotropins, steroids and serum lipid parameters and possess antioxidant activity.

Authors' contribution

MAD contributed to the laboratory works, analysis of the data. MI contributed to laboratory work, data analysis and collection of plant samples. IS contributed to manuscript writing, laboratory works and collection of plant samples. HK helped in histopathological studies. EKA designed the study, supervised the laboratory works, manuscript writing, helped in collection of plant samples.

Conflicts of interest

The authors declare no conflicts of interest.

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