



Exploring the Potential Impact of Buckwheat (*Fagopyrum esculentum*) on PI3K/AKT/mTOR Signaling Pathways in Rat Ovarian Tissue

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Abstract

This study examined the effects of adding buckwheat to rat diets on the antioxidant status of ovarian tissue, histopathological parameters, gene expression levels, and serum Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) concentrations. Four groups of female Sprague Dawley rats were used. Groups 1, 2, and 3 were fed diets containing 12%, 24%, and 36% buckwheat, respectively, while the control group was given a basal diet. From roughly 50 to 106 days of age, the animals were fed the experimental diets. Six animals from each group were sacrificed at the conclusion of the study, and samples of serum and ovarian tissue were taken. The control group had the most corpora lutea, while the buckwheat-fed groups had significantly fewer ($p < 0.001$). Phosphoinositide 3-Kinase (PI3K), Mechanistic Target of Rapamycin (mTOR), Protein Kinase B (AKT), Caspase-3, B-cell Lymphoma 2 (Bcl-2), and NF- κ B-p65 (Nuclear Factor kappa-light-chain-enhancer of activated B cells) signaling pathways in ovarian tissue were significantly impacted by buckwheat supplementation ($p < 0.01$). Catalase (CAT) level varied significantly ($p < 0.01$), while Superoxide Dismutase (SOD), Glutathione (GSH), and Malondialdehyde (MDA) levels were similar across groups ($p > 0.05$) among antioxidant parameters. According to serum hormone analyses, Group 3 had the lowest FSH level, while Groups 2 and 3 had the lowest LH levels ($p < 0.01$). In summary, buckwheat supplementation had a negative impact on several signaling pathways in ovarian tissue, as well as on serum FSH and LH concentrations, cystic follicle counts, and corpus luteum counts. Additionally, as the amount of buckwheat in the diet increased, so did the severity of these negative effects.

Keywords Antioxidant · Buckwheat · Gene expression · Histopathology · Hormone · Ovary

1 Introduction

The crude nutrient composition, nutrient profile, chemical components, and bioactive contents of food sources are some of the factors that affect healthy growth and development. One of the most widely used ingredients in animal nutrition and human nutrition is wheat. Wheat is a significant dietary component because of its protein, lipid, vitamin, and mineral content, even though it is mainly thought of as an energy source [1]. However, some conditions are known to be brought on by the gluten present in wheat. For those who are sensitive to gluten, gluten-free foods are therefore advised. Buckwheat, which is categorized as a pseudo-cereal, is one of the most popular substitutes for gluten. Despite having a similar crude nutrient composition to wheat [2, 3], buckwheat has different chemical and bioactive components [3, 4]. Huda et al. (2021) have defined buckwheat as a gluten-free plant and an important plant in terms of phytochemicals [5]. They also reported that buckwheat has high nutritional

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value due to its bioactive compounds, including flavonoids (rutin, quercetin, orientin, isoorientin, vitexin, and isovitexin), fatty acids, polysaccharides, proteins and amino acids, iminosugars, dietary fiber, fagopyrins, resistant starch, vitamins, and minerals. Furthermore, buckwheat has become increasingly important for health in recent years as a highly nutritious, gluten-free grain full of different bioactive compounds [3, 6]. In addition to the well-known illnesses linked to gluten, there have also been reports of negative effects on the reproductive system, including infertility, delayed menarche, irregular menstruation, recurrent miscarriages, and a propensity for early menopause [7]. Male and female fertility are adversely affected by celiac disease, which is caused by gluten intolerance [7]. Raguindin et al. (2021) compared buckwheat and oats in their study, identifying 113 phytochemical compounds in oats and 178 in buckwheat [8]. They reported that buckwheat had higher levels of phytosterols, tocopherols, and flavonoids, while oats had higher levels of avenanthramides, saponins, and β -glucan content. They also noted that further research is needed on the bioavailability of phytochemicals in oats and buckwheat.

Reproductive performance is known to be impacted by nutrition. Analyzing tissue antioxidant levels, histological structure, signaling pathways, and hormone concentrations is usually used to explain how dietary components affect the organism. This topic has been the subject of numerous studies. According to Erbaş et al. [9], feeding rats buckwheat reduced the activity of the Bcl-2, P38-MAPK, STAT3, JAK2, and PI3K signaling pathways while increasing the levels of caspase-3 in testicular tissue. According to Can and Imik [10], SOD, CAT, GSH, GPx, and LPO levels in kidney, lung, and heart tissues were considerably impacted by wheat gluten, corn gluten, and soybean meal. According to Imik et al. [11], ovarian LH and serum FSH levels varied significantly, but the histopathological (primordial, primary, secondary, Graafian follicles, and corpus luteum) and immunohistochemical (transglutaminase 2, gliadin, Immunoglobulin A (IgA), Immunoglobulin M (IgM), Cluster of Differentiation (CD4), Cluster of Differentiation (CD8) structure of ovarian tissue was not significantly affected. Taha et al. [12] reported that supplementing the diets of rats with ovarian cancer with plant- or animal-based protein sources led to reduced tumor cell growth and suggested that plant proteins improved prognosis by preventing activation of the IGF/Akt/mTOR pathway.

Gluten has been shown to affect intestinal tissue CD3 and CD8 receptor expression, as well as serum levels of interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) [13]. This study aims to determine the effects of administering buckwheat, whose bioactive components have been identified, at different levels on hormone levels, histopathological parameters, ovarian antioxidant status, and

signaling pathways in rats taken into the study before the onset of the estrus cycle.

2 Materials and Methods

2.1 Animals and Diet

Forty female Sprague Dawley rats, with an average age of fifty days, were used in this investigation. Ten animals were assigned to each of the four groups used in the experiment: three treatment groups and one control group. The ratios of wheat and buckwheat given to the groups in the study are shown in Table 1. Table 2 shows the proportions and chemical makeup of the diet used to feed the rats. The groups were given wheat and buckwheat in the following arrangement. Additionally, buckwheat and its hulls (whole buckwheat) were part of the ration.

From roughly 50 days of age until 106 days of age, the animals in the study were fed the diets listed in Table 2 for 56 days. Every day at 9:00 a.m., the animals received the experimental diets and were given free access to water in specially made water bottles. Six animals from each group were sacrificed under anesthesia at the conclusion of the experiment, and tissue and blood samples were taken for the required analyses. Serum was obtained by centrifuging blood samples at 3000 rpm for 10 min at +4 °C. The serum was then stored at –80 °C until analysis. Furthermore, ovarian tissues taken from the animals that were sacrificed were prepared for Western blot analysis, antioxidant parameter evaluations, and histopathological analyses.

2.2 Determination of Phenolic Compound Ratios

2.2.1 UHPLC-ESI-MS/MS Conditions

Phenolic compounds were analyzed using an Agilent 1290 Infinity UHPLC system coupled to an Agilent 6460 triple quadrupole mass spectrometer (Palo Alto, USA), following the method described by Can et al. [14].

2.2.2 Method Validation

The developed UHPLC-ESI-MS/MS method was validated in terms of linearity, precision, trueness, sensitivity, and

Table 1 Wheat and buckwheat ratios (%) given to the test groups

Groups	Wheat	Buckwheat
Control	36	0
Group 1	24	12
Group 2	12	24
Group 3	0	36

Table 2 Dietary ingredient ratios and chemical composition for the rats

Ingredients (%)	Control	Group 1	Group 2	Group 3
Wheat	36	24	12	0
Buckwheat	0	12	24	36
Sunflower seed Meal (34–36 g)	24,01	22,05	22,33	22,44
Soybean meal (44–46 g)	23,52	24,51	23,9	23,83
Corn	6,97	8,09	8,37	8,32
Soybean oil	2,5	2,29	2,32	2,34
Molasses	2,5	2,5	2,5	2,5
DCP	2,14	2,21	2,22	2,24
Marble powder	0,9	0,86	0,85	0,85
Salt	0,83	0,83	0,83	0,82
Sodium Bicarbonate	0,36	0,37	0,37	0,37
D-L methionine	0,19	0,2	0,2	0,2
Vit-Min premix	0,1	0,1	0,1	0,1
<i>Chemical composition (%)</i>				
Crude protein	24,31	24,16	24	24
Crude fat	3,76	3,53	3,53	3,50
Crude fiber	7,26	7	7	7
Ash	7,67	7,68	7,71	7,74
Methionine	0,6	0,6	0,6	0,6
Lysine	1,08	1,08	1,07	1,07
Cystine	0,4	0,4	0,4	0,41
Calcium	1	1	1	1
Phosphorus	0,9	0,9	0,9	0,9
Salt	1	1	1	1
Sodium	0,5	0,5	0,5	0,5

selectivity, as outlined by Can et al. [10]. Calibration curves for each phenolic compound were constructed using seven concentration levels ranging from 100 to 2000 µg/L. The method showed excellent linearity with correlation coefficients (R^2) ≥ 0.9986 .

Recovery tests were performed by spiking plant extracts with known standard concentrations. The mean recovery ranged between 93.06 and 106.12%, and matrix effects were below $\pm 20\%$, demonstrating high method reliability and minimal interference from the sample matrix [15].

2.2.3 Histopathological Analysis and Evaluation

At the conclusion of the experiment, ovarian tissues from anesthetized rats were submerged in a 10% neutral formaldehyde solution, which was changed every 24 h. This solution was used to fix the ovarian tissues for a full day. Following graded alcohol series (70%, 80%, 96%, and 100%) and xylene processing, the fixed tissues were embedded in paraffin blocks. A microtome (Leica RM2125 RTS) was used to cut 5 µm-thick sections from these paraffin blocks

Table 3 Primary antibodies were used in the Western blot analysis

Antibody	Manufacturer	Dilution
Caspase-3	Santa Cruz, sc- 56,053	1/1000
Bcl-2	Santa Cruz, sc-7382	1/1000
PI3K	Affinity Biotech, AF3242	1/1000
mTOR	Affinity Biotech, AF6308	1/1000
AKT	Affinity Biotech, AF0836	1/1000
NF-κB-p65	Santa Cruz, sc-109	1/1000
Beta-actin	Santa Cruz, sc-47778	1/2000
Secondary antibody (Goat anti-rabbit IgG-HRP)	Santa Cruz, sc-2004	1/2000
Secondary antibody (Mouse anti-rabbit IgG-HRP)	Santa Cruz, sc-2357	1/2000

for histopathological analysis. The sections were examined under a light microscope after being stained using Crossman's adaptation of Mallory's Triple Staining technique. For microscopic analysis, a light microscope with a computer and camera system (Nikon Eclipse E600) was utilized. Three sections from each ovary were examined to determine the number of corpora lutea, follicular cysts, and ovarian histomorphology [16].

2.2.4 Western Blot Analysis

Western blot analysis was performed as follows: Ovarian tissues were collected, washed with cold PBS, and lysed with RIPA buffer (Ecotech Biotech, Turkey) containing protease/phosphatase inhibitors. Lysates were centrifuged at $14,000\times g$ for 15 min at 4 °C to obtain the supernatant. Protein amounts were determined using the BCA kit (Pierce BCA, Thermo Scientific, USA). Equal amounts of protein (30 µg) were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked with 5% skim milk in TBS-T and incubated with primary antibodies (Table 3) at 4 °C overnight. Subsequently, incubation was performed with HRP-conjugated secondary antibodies for 1 h at room temperature. Bands were developed using an ECL substrate (3405, Thermo Fisher Scientific, USA) and analyzed with Image Lab software (Bio-Rad, USA). All samples were analyzed in triplicate.

2.3 Biochemical Analyses

2.3.1 Blood and Ovarian Tissue Sampling

Under sevoflurane anesthesia, approximately 7–8 mL of blood was collected directly from the heart into blood tubes (Becton Dickinson Co., USA), and the rats were sacrificed by cervical dislocation. Blood samples were

allowed to clot at room temperature and then centrifuged at 3500 rpm for 10 min at 4 °C (Hettich 38R, Germany). The resulting serum was aliquoted into sterile tubes and stored at – 80 °C until analysis.

2.3.2 Determination of FSH, LH, and PG Levels in Blood Serum

FSH and LH analyses in serum were measured by using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) method. ELISA kits were used for Rat FSH (Cat. No. ELK1315, ELK Biotechnology) ve Rat LH (Cat. No. ELK2367, ELK Biotechnology). The analyses were performed according to the manufacturer's instructions. The absorbances were measured at 450 nm using spectrophotometry (ELISA microplate reader, BioTek, µQuant, USA).

2.3.3 Determination of Oxidative and Antioxidative Levels in the Ovary

Malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) levels in ovarian tissue were determined by ELISA. Tissues were frozen in liquid nitrogen and lysed with a tissue lyser (Tissue Lyser II, Qiagen) at 30 Hz for 3 min. PBS (pH 7.4) was added, the mixture was homogenized at 30 Hz for 20 s, and then centrifuged at 3000 rpm to obtain the supernatant. These phases were analyzed using MDA (YLA0029RA), GSH (YLA0121RA), SOD (YLA0115RA), and CAT (YLA0123RA) ELISA kits (YLBiont, China), as well as Bradford reagent for tissue protein (BR05, EchoTech Biotechnology, Turkey). Measurements were performed spectrophotometrically (BioTek µQuant, USA) at 450 nm for MDA, GSH, SOD, and CAT, and at 595 nm for protein, according to the manufacturer's instructions.

2.3.4 Statistical Analyses

Since the highest effect size (D coefficient) was detected in the MDA parameter, sample size calculations were performed based on the FSH, LH, MDA, SOD, and CAT parameters. Analyses for these parameters were planned to test predefined hypotheses [17, 18]. GSH, follicular cyst and corpus luteum counts, and NF-κB, AKT, mTOR, PI3K, Bcl-2, and Caspase-3 parameters were evaluated within the scope of exploratory analysis; therefore, no sample size calculations were performed for these variables. The SPSS software program was used to statistically analyze the data collected for this study. One-way ANOVA was used to evaluate differences in ovarian tissue and serum parameters among the groups. Tukey's post hoc test was used to assess the significance of group differences.

3 Results

Flavonoids, anthocyanidins, and phenolic acids and polyphenolic derivatives of buckwheat were identified in the study along with their ratios (Fig. 1). Gallic acid, chlorogenic acid, ellagic acid, 4-OH-benzoic acid, and rosmarinic acid were identified as phenolic acids in buckwheat; catechin, epicatechin, hesperidin, vitexin, luteolin, and quercetin were identified as flavonols; cyanidin-3-o-glucoside and keracyanin chloride were identified as anthocyanins; and quinic acid and fumaric acid were identified as polyphenols/phenolic derivatives (Table 4).

The results of the histopathological analysis of ovarian tissue, including the number of cystic follicles and corpora lutea, are presented in Fig. 2, while the statistical analysis results are shown in Fig. 3. The lowest number of cystic follicles was observed in the control group, whereas the highest was found in Group 2. The control group had a significantly lower number of cystic follicles compared to all other groups, while the findings for Group 2 and Group 3 were similar ($p > 0.05$) but significantly higher than those of

Fig. 1 LC–MS/MS fingerprint analysis of buckwheat (*Fagopyrum esculentum*) fruit extract analyzed in negative ionization mode

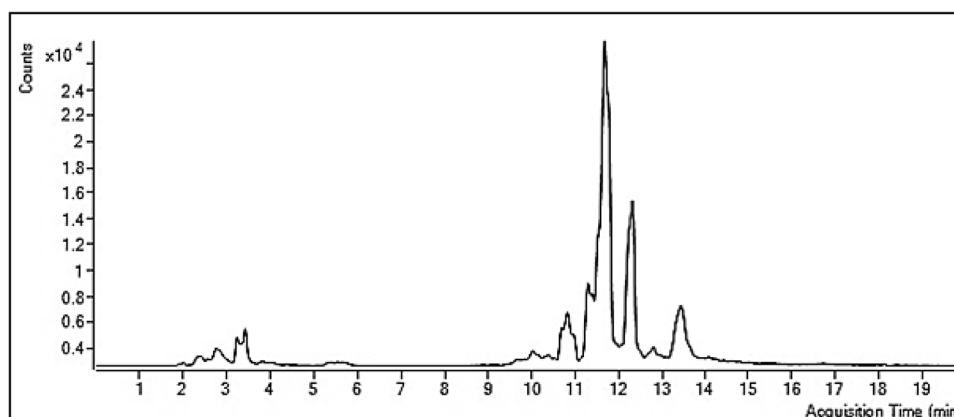


Table 4 LC-MS/MS mass spectral profile of buckwheat phenolic acids, flavonoids, anthocyanins and other polyphenols/phenolic derivatives

Specification	Compound	RT (min)	Response	Conc (µg/L)
Phenolic acids	Gallic acid	5,577	4932	154,0354
	Chlorogenic Acid	10,700	37,949	1409,2274
	4-OH-Benzoic Acid	11,305	1102	0,7353
	Ellagic Acid	12,108	684	162,7005
	Rosmarinic Acid	12,526	151	49,3132
Flavonoids	Catechin	10,978	8466	933,9255
	Epicatechin	11,312	20,475	1778,9207
	Hesperidin	11,625	120,160	10,581,9843
	Vitexin	11,699	202,795	2820,6834
	Luteolin	13,414	20,668	123,2436
Anthocyanins	Quercetin	13,485	26,173	576,3423
	Cyanidin-3-o-glucoside	10,394	263	50,9893
	Keracyanin Chloride	10,422	17	10,9099
Other polyphenols/phenolic derivatives	Quinic Acid	2,417	6827	2574,8328
	Fumaric Acid	4,015	2141	1255,9812

LC-MS/MS liquid chromatography-tandem mass spectrometry, RT retention time

Group 1 ($p < 0.01$). Regarding the number of corpora lutea, significant differences were observed between the control group and all other groups ($p < 0.01$). However, no significant differences were detected among Group 1, Group 2, and Group 3 ($p > 0.05$).

The live weights of Sprague–Dawley rats fed diets containing different levels of buckwheat during the trial period were compared between groups. No statistically significant difference was found between groups in terms of live weight values at baseline ($p = 0.661$), day 28 ($p = 0.348$), and day 56 ($p = 0.187$). These results indicate that the addition of buckwheat to the diet has no significant effect on live weight development (Table 5).

In the histopathological evaluation of ovarian tissue from all experimental groups, ovarian tissues exhibited a normal corpus luteum, developing follicular structures within a healthy histological cortex, and a medulla with a structure consistent with normal structure in the control group (Fig. 2). Vascular congestion, atretic follicles, and cystic follicles were identified in the ovarian tissues of Groups 1, 2, and 3. Notably, cystic follicles were more prevalent in Group 2. The number of corpora lutea in the control group was significantly higher than in the other groups ($p < 0.01$).

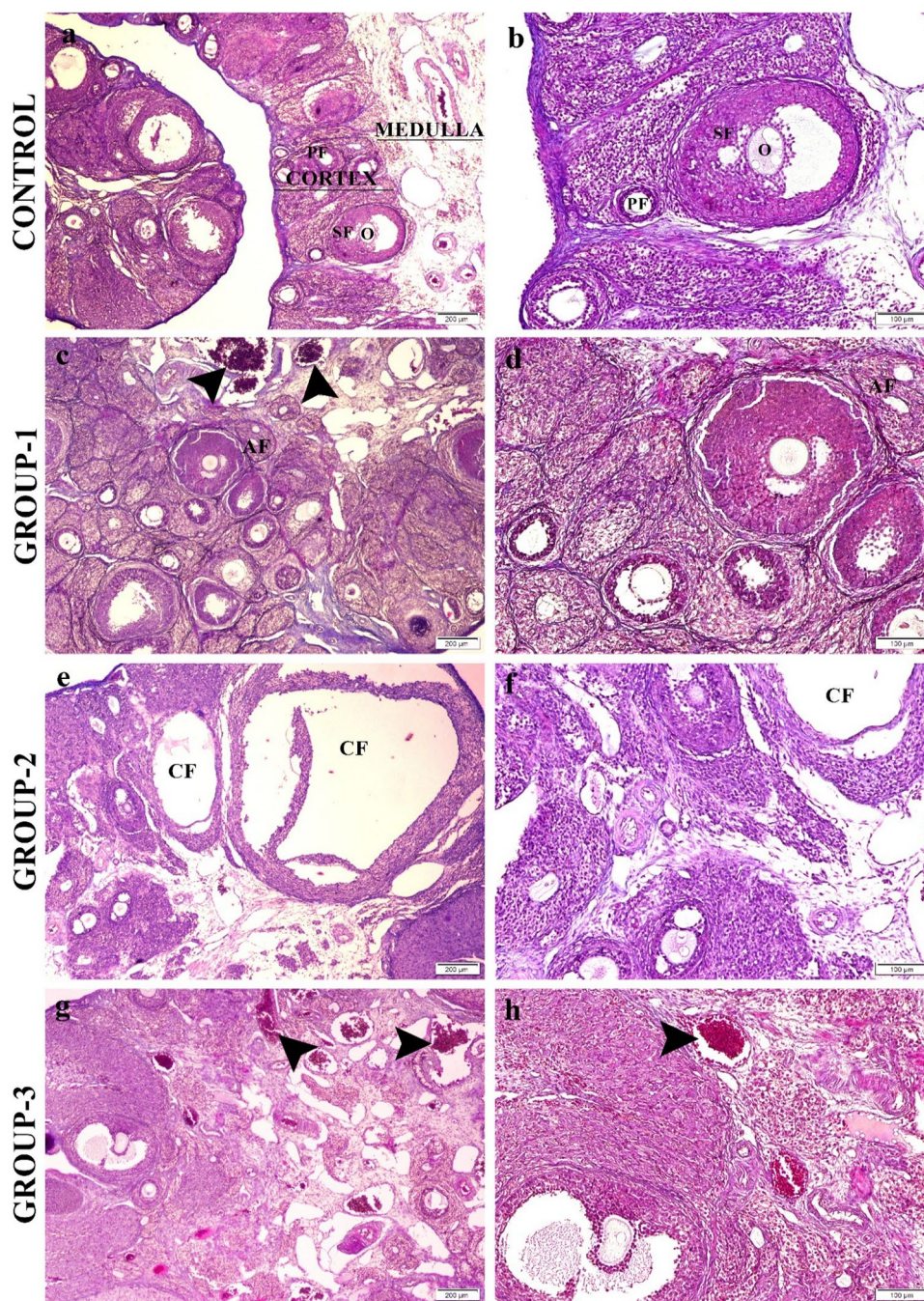
The antioxidant parameter values of ovarian tissue are presented in Fig. 4. The effects of the experimental groups on MDA, SOD, and GSH levels in ovarian tissue were found to be insignificant ($p > 0.05$), whereas CAT level showed a significant change ($p < 0.01$). The CAT level in Group 1 and Group 2 was significantly higher than in Group 2 and Group 3 ($p < 0.01$).

The serum FSH and LH levels of the experimental groups are presented in Fig. 5. The serum FSH level in

Group 3, fed 36% buckwheat, was significantly lower than in the Group 1 ($p < 0.01$), while the FSH levels in Control, Group 1, and Group 2 were similar. The LH levels in the Control group was significantly higher than in Group 2 ($p < 0.02$), whereas the LH level in Group 3 was similar to those of the Control, Group 1, and Group 2.

The parameters related to signaling pathways representing protein expression in the groups are presented in Fig. 6. The expression level of NFκB -p65 in the Control group was significantly lower than in the other groups ($p < 0.05$). The NFκB -p65 expression in ovarian tissue increased progressively from the Control group to Group 3. The AKT expression was highest in the Control group and lowest in Group 2 ($p < 0.05$). The Control group and Group 1 were statistically similar, while Group 2 was similar to Group 3. The mTOR expression decreased in all experimental groups compared to the Control group, with the lowest level detected in Group 3 ($p < 0.05$). The PI3K expression continuously decreased from the Control group to Group 3. The Bcl-2 expression in the Control group and Group 1 was significantly higher than in Group 2 and Group 3 ($p < 0.05$). The expression of Caspase-3, a marker of apoptosis, was similar between the Control group and Group 1, but significantly lower than in Group 2 and Group 3 ($p < 0.05$). These findings indicate that pro-survival proteins (AKT, mTOR, and PI3K) and the anti-apoptotic protein Bcl-2 were lower in Group 2 and 3. Meanwhile, NFκB and Caspase-3 expression increased in the buckwheat-fed groups, suggesting differential effects on apoptotic pathways. The relative expression levels and corresponding protein bands are presented in Fig. 6.

Fig. 2 Photomicrographs of ovarian tissues of all groups. PF; Primary Follicle, SF; Secondary Follicle, O; Oocyte, Arrowhead; Vascular congestion, AF; Atretic Follicle, CF; Cystic Follicle. Mallory's Triple Stain Modified by Crossman staining. Magnification; a,c,e,g 40X and b,d,f,h 100X



4 Discussion

For living things to grow, reproduce, and live healthy lives, nutrition is known to be essential. For many years, both humans and animals have eaten wheat. Some people may experience a variety of health issues due to the gluten in wheat, which has led to a rise in the number of informed consumers and a shift toward gluten-free foods. One popular source of gluten-free food is buckwheat [5]. To use buckwheat as a food source and to understand its metabolism in organisms, animal experiments are required. Rats are used

in animal experiments for a number of reasons, the most important of which are their accessibility and low cost.

The nutritional value of buckwheat has been the subject of numerous studies. Fatty acids, polysaccharides, proteins and amino acids, iminosaccharides, dietary fibers, fagopyrins, resistant starch, vitamins, minerals, flavonoids (such as rutin, quercetin, orientin, isoorientin, vitexin, and isovitexin), phenolic compounds, stilbenes, triterpenoids, and tannins are among the numerous phytochemicals found in buckwheat [6, 19]. Flavonoids, anthocyanidins, and phenolic acids and their polyphenolic derivatives were found in the

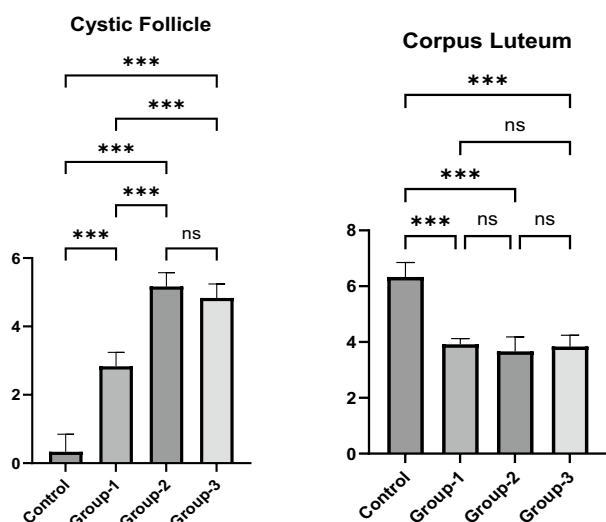


Fig. 3 Histopathological evaluation of follicular cyst and corpus luteum numbers in ovarian tissue for all groups. Values are given as mean $\pm$ SD ($n=6$) and analyzed by one-way ANOVA followed by Tukey's test. ns (not significant, $p > 0.05$), *** ($p < 0.001$)

structure of hulled buckwheat used in this investigation, along with their ratios. A wide and dense profile of bioactive compounds was found through analyses. These results are in line with data from the literature.

The relationship between oxidative stress, apoptosis, and signaling pathways in ovarian tissue has been established in previous studies, and it has been reported that the oxidative/antioxidative balance plays an important role in maintaining ovarian function [20]. It has also been demonstrated that plant-derived bioactive compounds can modulate cellular signaling pathways beyond their antioxidant effects and exert protective effects on the reproductive system [21]. In this context, the rich flavonoid and phytoestrogen content of buckwheat may have contributed to the molecular changes observed in the present study. The mechanistic relationship between oxidative stress and apoptosis is of critical importance for reproductive tissues, and it has been reported that oxidative damage can lead to changes in apoptotic markers [22]. The decrease in Bcl-2 levels and increase in caspase-3 expression in the buckwheat-fed groups in the present study suggest that oxidative and cellular stress responses may be involved in the process. However, since the estrus cycle

phases were not controlled, the findings should be considered as correlational rather than definitive causal effects.

Until they reach sexual maturity, females require a balanced diet to maintain healthy and long-lasting fertility. The groups in this study were fed until sexual maturity (average 106 days of age) and were designed to have similar raw material ratios because nutrition is known to affect fertility. The findings show that adding buckwheat to the diet at different levels has no significant effect on body weight and overall growth performance in rats. Serum FSH and LH levels, as well as the impact of ovarian tissue on cystic follicles and corpus luteum, were measured in order to assess the impact of nutrition on reproduction. The buckwheat-fed groups in this study had significantly higher levels of cystic follicles in their ovarian tissue. It is important to note that the groups supplemented with 24% and 36% buckwheat had the highest number of cystic follicles. Groups 2 and 3, as well as Group 3, which received 36% buckwheat, had the lowest serum FSH levels. It was discovered that eating a lot of buckwheat decreased the secretion of FSH and LH. In comparison to the control group, Rakic et al. [15] found that the groups receiving estradiol valerate and a high-fat diet had significantly more cystic follicles. One possible explanation for the increased number of cystic follicles in the ovaries of the buckwheat groups in this study is that certain compounds found in this food have an impact on hormonal balance and ovarian function. This could cause follicle maturation or cystification by interfering with ovulation. Buckwheat's antioxidants shield cells from harm by scavenging free radicals, but too much antioxidant activity can interfere with certain cellular functions and have an impact on follicle development. It is believed that this will result in more cystic follicles. Flavonoids and lignans, two substances present in buckwheat, may have phytoestrogen qualities. By imitating the natural effects of estrogen, phytoestrogens' effects on ovarian tissue can promote follicle growth and development [23]. Cystic follicle levels may then rise as a result. The effects of phytoestrogens, antioxidants, and other bioactive components are probably the reason buckwheat raises the levels of cystic follicles in the ovaries. Although the control group (36%) fed wheat had the highest corpus luteum counts, the buckwheat groups had much lower counts. Taking into account both factors, we can conclude that buckwheat has an adverse effect on ovarian

Table 5 Body weight changes of Sprague–Dawley rats fed diets containing different levels of buckwheat during the experimental period

	Control	Group 1	Group 2	Group 3	P-value
Live weight (Day 1)	172,14 $\pm$ 25,67	171,68 $\pm$ 26,66	173,67 $\pm$ 22,09	172,86 $\pm$ 18,62	0,661
Live weight (Day 28)	232,80 $\pm$ 20,51	226,53 $\pm$ 22,70	225,60 $\pm$ 18,52	235,49 $\pm$ 20,95	0,348
Live weight (Day 56)	272,46 $\pm$ 21,81	264,11 $\pm$ 24,20	267,54 $\pm$ 19,40	277,91 $\pm$ 19,84	0,187

The values are expressed as mean $\pm$ standard deviation. No statistically significant difference was observed among the groups ($p > 0.05$)

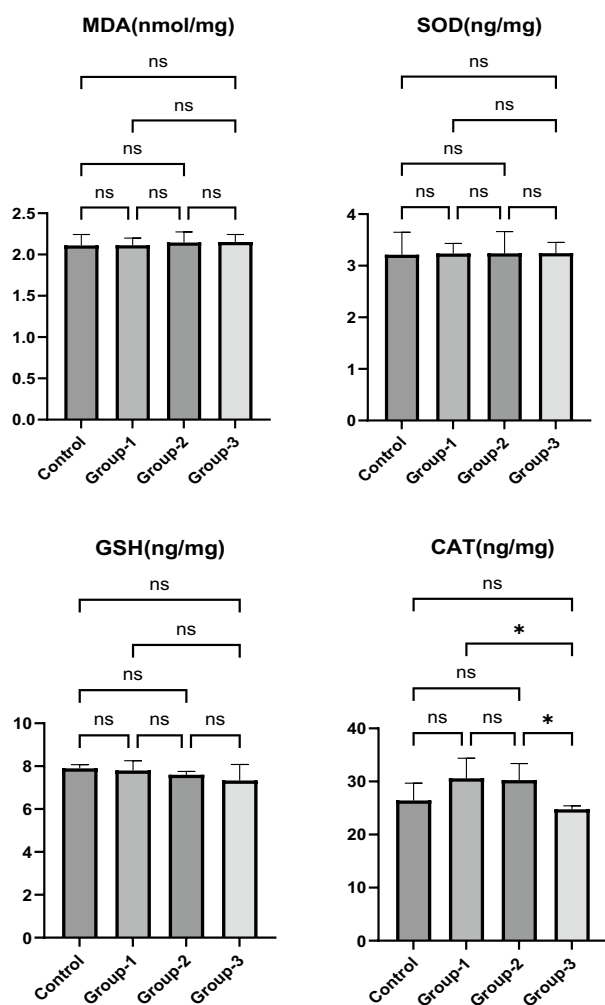


Fig. 4 Antioxidant and oxidant ratios of ovarian tissue. Values are given as mean $\pm$ SD ($n=6$) and analyzed by one-way ANOVA followed by Tukey's test. ns (not significant, $p > 0.05$), * ($p < 0.05$)

tissue histopathology. Because of the presence of follicles, an excess of corpus luteum signifies increased ovulation and folliculogenesis [24]. This is feasible because wheat does not have decreased folliculogenesis. Accordingly, similar findings have been reported [25]. Follicles must mature and ovulation take place in order for the corpus luteum to form [26]. Some follicles may become cystic and fail to mature due to buckwheat's high phytoestrogen content. This may result in insufficient or nonexistent ovulation. The corpus luteum either does not form or becomes fewer in number when ovulation is absent. A low corpus luteum count may result from buckwheat's high phytoestrogen content. Buckwheat's phytoestrogens and other physiologically active substances are known to upset hormonal equilibrium and prevent luteinization following ovulation. The high concentration of bioactive compounds in buckwheat or the fact that the rats' tolerance to these compounds is well above their limits are probably

the causes of the detrimental effects of buckwheat on the histopathological parameters of ovarian tissue. Histological analysis of the ovaries of rats fed a wheat-based diet in the study by Idaguko et al. [26] showed the presence of cortical stroma, loose connective tissue rich in the corpus luteum and inner medulla, and ovarian follicles at different stages of development. The ovaries of the wheat-fed group and the control group in the same study did not differ. In a study by İmİK [27], rats were fed wheat gluten, corn gluten, and soybean meal as a source of protein from 20 to 65 days of age. The results showed that these treatments had a significant impact on primordial follicles, primary follicles, graft follicles, corpus luteum, and atrophic follicles, but had little effect on the formation of secondary follicles. In a study by İmİK et al. [11], ovarian tissue histopathological and immunohistochemical parameters were unaffected when female rats were fed a diet containing wheat gluten, corn gluten, and soybean meal for 165 days. Additionally, they found that the wheat gluten group had the lowest LH levels in ovarian tissue and that the soybean meal group had much lower serum FSH levels than the wheat and corn gluten groups. Despite the fact that the rats in these two literature reviews were fed identical diets, it can be inferred that the animals became accustomed to these diets over longer feeding periods.

One of the body's main defense mechanisms is antioxidants. Antioxidants can be obtained externally through feed or produced internally. Despite the similar raw nutrient ratios of the experimental groups' feeds in this study, it can be observed that their bioactive substances also varied because of the different feed ingredients (the ratios of buckwheat and wheat) [3, 6, 28, 29]. It was established how the addition of buckwheat to the diet affected the ovarian tissue's antioxidant metabolism. Tissue oxidation is represented by the parameter MDA. It is therefore a free radical that needs to be eliminated from the tissues. The most crucial antioxidant defense mechanism against ROS and superoxide anion radicals is SOD. According to Halıcı et al. [30], SOD catalyzes the reduction of another superoxide radical to the less reactive molecule hydrogen peroxide (H_2O_2) and oxidizes one superoxide radical to O_2 .

The body naturally produces glutathione, an antioxidant made up of three amino acids that are produced by the liver. In addition to its role as an antioxidant, glutathione is essential for tissue regeneration and immune system strength. Stress, poor nutrition, and environmental conditions are known to lower glutathione levels. Catalase is essential for scavenging hydrogen peroxide radicals, which can harm cells. The effects of the diets fed to the experimental groups on antioxidant levels in ovarian tissue were investigated in this study. The experimental groups' MDA, SOD, and GSH levels were found to be comparable. Furthermore, CAT activity in the 12% and 24% buckwheat supplemented groups was significantly higher than in the control and 36%

Fig. 5 Effect of buckwheat on serum hormone parameters. Data are expressed as mean $\pm$ SD. Statistical significance among groups was determined using one-way ANOVA followed by Tukey's post hoc test, ns (not significant, $p > 0.05$), ** ($p < 0.01$)

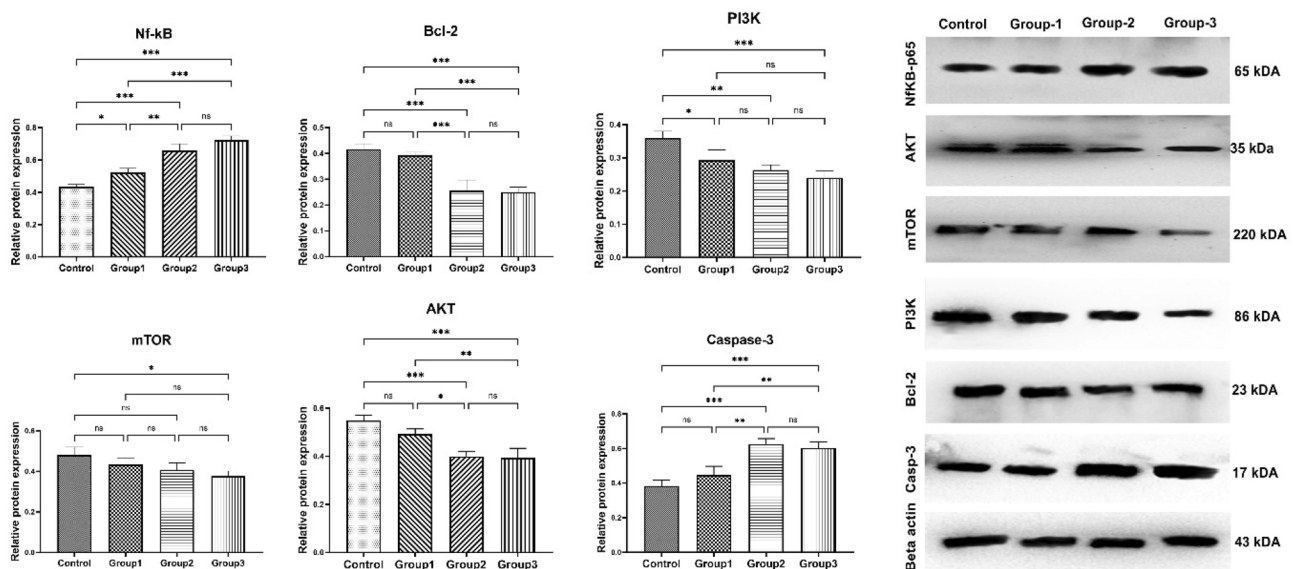
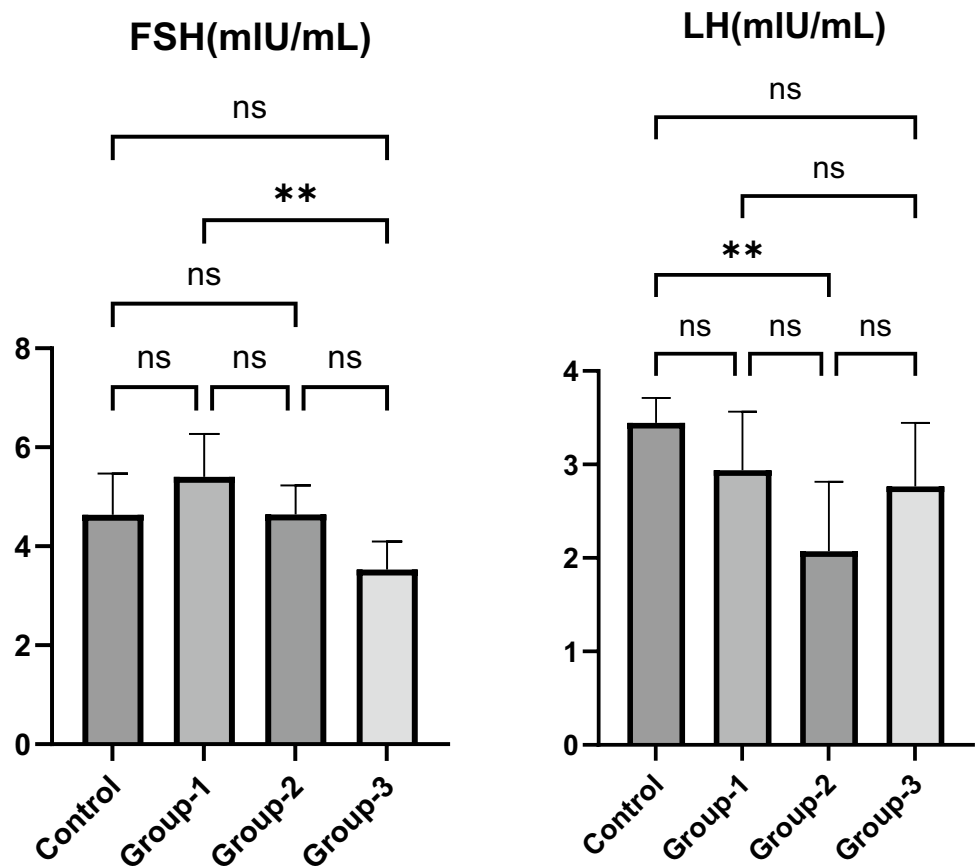


Fig. 6 Relative protein expression levels in ovarian tissues across experimental groups. Representative Western blot images and corresponding quantitative bar graphs illustrate the expression patterns of NF- κ B p65, AKT, mTOR, PI3K, Bcl-2, and Caspase-3 in the Control, Group 1, Group 2, and Group 3. Protein expression levels were normalized to β -actin as the internal loading control. Data from three

independent experiments are expressed as mean $\pm$ SD. Statistical significance among groups was determined using one-way ANOVA followed by Tukey's post hoc test. Differences were considered not significant at $p > 0.05$ (ns), and statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

buckwheat groups. The lower CAT activity in the 36% buckwheat supplemented group suggests that antioxidant metabolism involves a much more complex chain of events. This could be because buckwheat contains a lot of bioactive compounds, or because the ovarian tissue perceives these compounds as free radicals.

The effects of nutrition on tissues and metabolism have been the subject of numerous studies. The study of how nutrients impact signaling pathways or gene expression is still in its early stages. Much work is needed to shed light on this issue. This study sought to elucidate the effects of whole buckwheat on signaling pathways in rat diets. Information about these signaling pathways is also provided in order to fully explain the issue.

A physiological cellular process seen in organisms during normal development is called apoptosis, sometimes referred to as programmed cell death. In this regard, research is being done to determine which signaling pathways regulate apoptosis mechanisms and how they function [31]. Throughout an organism's life, certain tissues and cells continuously produce apoptotic cells. In order to maintain tissue homeostasis in these tissues, death (apoptosis) and regeneration (mitosis) continue in a dynamic balance [32, 33]. Two important factors in the creation of this equilibrium are the Bcl-2 gene and the Caspase-3 protein. The Caspase-3 protein promotes apoptosis, whereas the Bcl-2 gene inhibits cell death. Caspases are proteins that work well in the vertebrate apoptotic pathway. The cytosolic ICAD (inhibitor of caspase-activated deoxyribonuclease) is rendered inactive by activated caspase-3. The caspase-activated deoxyribonuclease (CAD) that inactivated ICAD typically binds to and separates. The cell undergoes apoptosis as a result of chromatin condensation and DNA breaks brought on by the released CAD entering the nucleus [34, 35]. The Bcl-2 gene of the ovarian tissue was found to be highest in the control group and significantly lower in the buckwheat groups, as shown in Fig. 6. Conversely, caspase-3 protein was found to be significantly higher in Group 2 and Group 3, despite being similar in the control and Group 1. This could be because the buckwheat used in the experiment contained bioactive compounds, particularly in its shell.

Protein kinase B, or AKT1, is a serine/threonine protein kinase that controls a variety of functions, including growth, angiogenesis, cell survival, metabolism, and proliferation. Additionally, it is essential for metabolic processes like protein synthesis and the metabolism of insulin, fats, carbohydrates, and glucose [36]. Phosphatidylinositol 3-kinase, or PI3K, affects cellular motility, embryonic development, cell communication and differentiation, cell fate-determining processes, and cell survival. By modifying cellular metabolism, including glucose metabolism, lipid metabolism, nucleotide metabolism, and amino acid metabolism, PI3K signaling encourages the growth and survival of tumor cells [37, 38]. A key regulator of cellular metabolism, growth, and

survival in response to hormones, growth factors, nutrients, energy, and stress signals is mTOR (mammalian target of rapamycin) [39, 40]. Cell proliferation, survival, motility, and metabolism are all regulated by the PI3K/AKT/mTOR pathway, a crucial signaling pathway. A crucial kinase in the PI3K/AKT signaling pathway's downstream activation is mTOR. By regulating metabolic enzymes or transcription factors, it also affects cell metabolism and nutrient transport. According to Martinez Calejman et al. [39] and Wang et al. [38], these signaling pathways assist cells in responding to their surroundings, regulating vital cellular functions, and preserving homeostasis.

In this study, the control group and Group 1 exhibited significantly higher AKT ratios compared with Groups 2 and 3. In the buckwheat-fed groups, PI3K and mTOR ratios were significantly reduced. Although the experimental diets were comparable in terms of protein, energy, vitamins, and minerals, these findings suggest that differences in the bioactive components of buckwheat may be associated with modulation of PI3K/AKT/mTOR pathway-related protein expression, rather than indicating a direct inhibitory effect. The PI3K/AKT/mTOR signaling pathway is widely studied due to its regulatory role in cellular processes related to gene expression. In this context, our results indicate that dietary factors such as buckwheat consumption may influence components of the PI3K/AKT/mTOR signaling pathway, highlighting the importance of further mechanistic and longitudinal studies to better understand the relationship between diet and signaling pathways involved in gene regulation. These findings underscore the potential relevance of dietary composition in the development of nutrition-based strategies.

Numerous genes involved in cell proliferation, survival, development, and aging are regulated by the nuclear factor kappa B (NF- κ B) family of transcription factors. Additionally, it is crucial for the control of both innate and adaptive immune responses as well as inflammation [40–42]. Through post-translational changes like phosphorylation and acetylation, metabolic enzymes and metabolites frequently control NF- κ B activity. The molecular mechanisms underlying NF- κ B function are better understood as a result of these findings. Additionally, the identification of NF- κ B signaling as a possible therapeutic target for inflammatory/immune chronic diseases depends on this new information. Cancer cells have more NF- κ B pathway regulators than normal cells. Many tumor-regulating pathways, such as Ras, EGF receptor, mTOR, Akt, STAT3, and p53, regulate NF- κ B activity in cancer cells [43, 44]. In order to guarantee a suitable cellular response, various signaling pathways converge on NF- κ B and strictly control signal transduction through a balance between activators and repressors. Numerous illnesses, such as cancer and inflammatory diseases, can arise and worsen due to any dysregulation in the NF- κ B pathway [44, 45]. Determining how nutrition affects NF- κ B

signaling pathways is crucial for illustrating how nutrition affects health. In fact, buckwheat-fed groups were found to significantly increase NF- κ B signaling in this study. Even though the study groups' nutrient ratios (crude protein, energy, vitamins, and minerals) were comparable, we can conclude that distinct outcomes were attained because of the various protein structures and bioactive components, which we refer to as bioactive substances.

5 Conclusion

Bioactive compounds were found to be abundant in the whole buckwheat used in the experiment. The corpus luteum was found to be significantly smaller than in the wheat group, and the number of cystic follicles in the ovarian tissue increased significantly as the amount of buckwheat in the rats' diets increased. Serum FSH and LH levels were found to be impacted by buckwheat. Additionally, buckwheat has distinct effects on apoptosis pathways, as evidenced by low levels of PI3K, AKT, mTOR, and Bcl-2 and high expression of NF- κ B and Caspase-3. This study shows how important dietary nutrients and bioactive compounds are in choosing healthy eating practices.

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Data Availability Data available on request from the authors.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical Approval The study was approved by the ethics committee under “decision number 35 of the Atatürk University Animal Experiments Local Ethics Committee, dated 26.02.2024 and numbered 2024/02.” The Atatürk University Scientific Research Coordination Office provided support for the study under the code “TCD-2024-13973”. The authors used ChatGPT 3.5 only to check grammar and spelling during writing.

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