

## EXPLORING OF THE AMELIORATIVE EFFECTS OF ETHANOLIC EXTRACT OF THE WHEATGRASS (*TRITICUM AESTIVUM* L.) ON SURGICALLY INDUCED ENDOMETRIOSIS RAT MODEL

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### Abstract

The purpose of this study was to investigate the healing effects of an ethanolic wheatgrass (WG) extract obtained from young wheat leaves on a surgically induced endometriosis rat model. 30 female *Wistar albino* rats with endometriosis were randomly organized into five groups (N = 6): Control, Letrozole a reference drug for endometriosis (0.2 mg/kg/day), WG-25 (25 mg/kg/day WG extract), WG-100 (100 mg/kg/day WG extract) and WG-400 (400 mg/kg/day WG extract). WG-100 extract and Letrozole caused a significantly decrease in adhesion score compared to the control. Additionally, treatment with WG extracts resulted in a significant reduction of endometrial cyst volumes compared to the control group. IL-6 cytokine levels in peritoneal fluid of WG-100 and Letrozole groups were importantly lower than the control. Matrix metalloproteinase 9, VEGF and kinase insert domain receptor genes were expressed in endometrial cysts. According to LC-MS/MS analysis, aconitic acid was the major phenolic acid (54.004 mg analyte/g extract) in the WG extract. Additionally, quinic acid (3.687 mg analyte/g extract) and protocatechuic acid (0.014 mg analyte/g extract) were determined in the WG extract. Moreover, the WG extract had vitamin E (5.49 ± 0.08 mg/g extract) and vitamin C (7.42 ± 0.13 mg/g extract). As conclusion, WG extract obtained from young leaves of wheat has ameliorative effects on surgically induced endometriosis due to its rich phytochemical content.

### Rezumat

Scopul acestui studiu a fost de a investiga efectele unui extract etanolic de grâu verde (WG) obținut din frunze tinere de grâu, pe un model de șobolan cu endometrioza indusă chirurgical. 30 de șobolani Albino Wistar de sex feminin cu endometrioza au fost împărțiți aleatoriu în cinci grupuri (N = 6): Martor, Letrozol, un medicament de referință pentru endometrioza (0,2 mg/kg/zi), WG-25 (25 mg/kg/zi), WG-100 (100 mg/kg/zi) și WG-400 (400 mg/kg/zi). Extractul WG-100 și letrozolul au determinat o scădere semnificativă a scorului de aderență în comparație cu grupul control. În plus, tratamentul cu extracte WG a condus la o reducere semnificativă a volumelor chisturilor endometriale în comparație cu grupul control. Nivelurile de citokine IL-6 în lichidul peritoneal din grupurile WG-100 și Letrozol au fost semnificativ mai mici decât controlul. Metalopeptidaza matriceală 9, VEGF și genele receptorului domeniului de inserție al kinazei au fost exprimate în chisturile endometriale. Conform analizei LC-MS/MS, acidul aconitic a fost acidul fenolic preponderent (54,004 mg analit/g extract) din extractul WG. În plus, acidul chinic (3,687 mg analit/g extract) și acidul protocatecuic (0,014 mg analit/g extract) au fost determinați în extractul WG. Mai mult, acesta conține vitamina E (5,49 ± 0,08 mg/g extract) și vitamina C (7,42 ± 0,13 mg/g extract). În concluzie, extractul WG obținut din frunze tinere de grâu verde are efecte amelioratoare asupra endometriozei induse chirurgical, datorită conținutului său bogat fitochimic.

**Keywords:** aconitic acid, endometriosis, vitamin C, vitamin E, wheatgrass

### Introduction

Endometriosis is characterized by the presence of endometrial tissue outside of the uterus. One of the ten women at the reproductive stage was affected by endometriosis [1, 2]. Pelvic pain, dysmenorrhea, dyspareunia, irregular bleeding, and subfertility are the most common symptoms of endometriosis [3]. Metastatic tissue behaviour, angiogenesis and reduced apoptosis occur during endometriosis as well as cancer [4]. Endometriosis has become a major social and

economic problem because of its complications. Gonadotropin-releasing hormone (GnRH) agonists, oral contraceptive pills and aromatase inhibitors are used in the treatment of endometriosis [5]. Drugs used to treat endometriosis have severe side effects such as osteoporosis, hot flashes, fatigue and weight gain [5, 6]. Thus, scientists focus on alternative approaches to endometriosis treatment and the improvement of the daily comfort of women with endometriosis. Women pay attention to phytochemically rich medicinal plants, especially during the menstrual

cycle and menopause, to increase their daily comfort. Additionally, plant crude extracts and isolated bioactive compounds have ameliorative effects on endometriosis [7, 8].

Sprouts of bread wheat (*Triticum aestivum* L.) also known as wheatgrass (WG) are obtained from germinating *T. aestivum* caryopses. WG is consumed by people as tablets, capsules and freshly prepared wheatgrass juice. WG possesses anti-ulcer, anti-cancer, antioxidant and anti-arthritis activities, and it can also be used against thalassemia [9-13]. Consumption of WG juice is advised as a dietary supplement to improve endometriosis treatment [14]. Wheat sprouts have also been shown to have anti-endometrial activity [15]. WG is a rich source of vitamins (especially vitamin C and vitamin E),  $\beta$ -carotene, some amino acids and minerals. Additionally, it contains apigenin, quercetin, luteolin, abscisic acid, vanillic acid and ferulic acid [16]. Apigenin, luteolin and quercetin present in WG are phytochemicals used for the treatment of endometriosis [8, 17].

This study aimed to investigate the healing effects of the ethanolic WG extract on a surgically induced endometriosis rat model. Additionally, we aimed to determine the phenolic acid quantities of the WG extract by LC-MS/MS and to measure its vitamin C and vitamin E contents.

## Materials and Methods

### *Growth of wheat seedlings*

Caryopses of the Tosunbey bread wheat cultivar were used to grow seedlings. The caryopses (Serial number: 6546920, lot number: TR.06.20.1001.0329) were obtained from the Turkish Seed Association. Surface sterilized caryopses were sown in plastic trays with bi-layered tissue paper soaked in Hoagland's medium [18]. Plants were incubated in a controlled growth room for 14 days at a light/dark (16/8) photoperiod and  $24 \pm 1^\circ\text{C}$ . Seedlings were separated from their roots and used to make an ethanolic extract.

### *The preparation of the extract*

The young leaves of *T. aestivum* were cut into small pieces and extracted with 80 percent ethanol. Ethanol was removed from the solution by using a rotavapor (Rotavapor R-300, BUCHI) at  $40^\circ\text{C}$  under reduced pressure. The residue was frozen at  $-22^\circ\text{C}$  and the aqueous part was evaporated by the lyophilization process using a freeze dryer (Alpha 1-2 LD, Christ). Finally, the lyophilized ethanolic WG extract was obtained. The yield was calculated as 3.62% for ethanolic WG extract.

### *Animals*

Thirty female rats were supplied by Van Yüzüncü Yıl University Experimental Animal Research Unit. *Wistar albino* rats (150 - 200 g weight and 2 - 3 months old) were used in the present study. All rats were housed in  $25 \pm 2^\circ\text{C}$ , 12/12 hours light/dark

cycle. Rats were fed *ad libitum* with standard pellet food and tap water. This study was conducted according to ARRIVE guidelines and the protocol was approved by the Van Yuzuncu Yil University Animal Researches Local Ethic Committee.

### *Experimental design*

The endometriosis rat model was induced by surgery according to the auto-transplantation method with some modifications [19]. This model is composed of two stages, the first was the induction and confirmation of the endometriosis model stage (28 days) and the second was the treatment stage (28 days). The experiment duration was a total of 56 days.

*Endometriosis model induction and confirmation:* at this stage, a surgically induced endometriosis model was created under aseptic conditions. The rats in the pre-oestrous phase were anesthetized with ketamine HCl and xylazine HCl. The abdomens of the rats were shaved, and the same region was disinfected with an iodine solution. A three cm incision was created on the abdomen (the first operation). The right endometrium was removed and separated from the myometrium. A 15 mm piece was trimmed, and this cleaned part was opened longitudinally. The endometrium part was ligated to the periton, and the incision was sutured. Rats were fed *ad libitum* with standard pellet food and tap water for 28 days without any treatment. At the end of the 28 days, rats were labelled, and induction of the endometriosis model was confirmed by the second operation. Induction of endometrial cysts was achieved on the periton of all rats.

*Treatment stage:* Rats were treated with WG extract and letrozole, an aromatase inhibitor drug, in this stage. Carboxymethyl cellulose 0.5% (CMC) was used as a vehicle for WG extract and letrozole. Rats were fed *ad libitum* with standard pellet food and tap water during this stage (28 days). Thirty female rats with endometrial cysts were randomly organized into five groups (N = 6).

Group I (Control): Rats with endometrial cysts were treated with 0.5% CMC for 2 mL/day *via gavage* for 28 days after the second operation.

Group II (Letrozole): Rats with endometrial cysts were treated with letrozole, a reference drug, 0.2 mg/kg/day [20] *via gavage* for 28 days after the second operation.

Group III (WG-25): Rats with endometrial cysts were treated with ethanolic WG extract 25 mg/kg/day *via gavage* for 28 days after the second operation.

Group IV (WG-100): Rats with endometrial cysts were treated with ethanolic WG extract 100 mg/kg/day *via gavage* for 28 days after the second operation.

Group V (WG-400): Rats with endometrial cysts were treated with ethanolic WG extract 400 mg/kg/day *via gavage* for 28 days after the second operation.

At the end of the treatment stage, rats were sacrificed by cardiac puncture under anesthetized with ketamine

HCl and xylazine HCl. Tissues were removed and stored in an ultra-freezer.

#### *Observation of adhesion scores and measurement of endometriotic cyst volume*

Intra-abdominal adhesion scores both before and after treatment were evaluated according to Blauer's scoring system (No adhesion: 0; Thin adhesions: 1; Thick adhesion in one area: 2; Spread thick adhesion: 3; Adhesion including internal organs: 4) [21]. Length, height and width of endometrial cysts were measured to calculate the endometriotic cyst volume before (CVB) and after (CVA) treatment using the equation:

$$(\pi/6 \times \text{length} \times \text{height} \times \text{width}) [22].$$

The endometriotic cyst volume change (%) was calculated according to the formula:

$$(CVA - CVB)/CVB \times 100.$$

#### *Determination of cytokine levels*

Interleukin-6 (IL-6) levels, vascular endothelial growth factor (VEGF) and tumour necrosis factor-alpha (TNF- $\alpha$ ) levels were measured in peritoneal fluid collected before and after treatment. Elabscience (USA) provided commercially available ELISA kits to measure IL-6 (E-EL-R0015), VEGF (E-EL-R2603) and TNF- $\alpha$  (E-EL-R2856). Cytokine levels were measured according to kit procedures.

#### *RNA isolation and semi-quantitative gene expression*

All equipment was treated with diethylpyrocarbonate to prevent RNase activity. RNA was isolated from

endometrial cysts (0.1 g) using a Gene Jet RNA Purification kit (K0731, Thermo Scientific). Isolated RNAs were quantified and run on agarose gel electrophoresis. RNAs were treated with DNase I (EN0521, Thermo Scientific) to avoid DNA contamination before the construction of the cDNA library. cDNA libraries were constructed according to the protocol of the High-Capacity cDNA Reverse Transcription Kit purchased from Applied Biosystems™ (Catalog number: 4368814). cDNA synthesis was confirmed by PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene-specific primers (Table I). Semi-quantitative gene expression in endometrial cysts was performed using gene-specific primers for MMP-9, VEGF and Flk-1 genes. AMPIGENE® qPCR Green Mix (ENZ-NUC104-0200) was supplied by Enzo Life Sciences (Switzerland) for the PCR reaction mixture. An equal amount of cDNA was added to the PCR reaction. The PCR reaction conditions were optimized as initial denaturation at 95°C for 2 min, denaturation at 95°C for 5 sec, annealing and extension at 50°C for VEGF, 54°C for Flk-1 and 60°C for MMP-9 for 2 min for 40 cycles. PCR products (10 $\mu$ L) were run in an agarose gel electrophoresis system and monitored using a UV transilluminator bioimaging system. Bands were evaluated by using the ImageJ application, and the GAPDH gene was used as a housekeeping gene (Table I).

**Table I**

Primer sequences for semi-quantitative gene expression assay [23]

Primer Id	Sequence	Base length
VEGF-RT-F	5'ACCATGAACTTTCTGCTC3'	18
VEGF-RT-R	5'GGACGGCTTGAAGATATA3'	18
Flk-1_RT_F	5'GCACTGAATTATGGGAGA3'	18
Flk-1_RT_R	5'ATGTGATTTTCTTCTTGATG3'	20
MMP-9_RT_F	5'GTTTCTGCCCCAGTGAGAATCTC3'	23
MMP-9_RT_R	5'TGCTGGATGTCTTTTATGTGCG3'	21
GAPDH_RT_F	5'CACCACCATGGAGAAGGC3'	18
GAPDH_RT_R	5'CCATCCACAGTCTTCTGA3'	18

#### *Phytochemical analysis of the WG extract*

Phenolic bioactive compounds in WG extract were screened and quantitatively measured by LC-MS/MS (liquid chromatography-mass spectrometry) validated for 56 compounds [24]. Additionally, the vitamin C and vitamin E content of the WG extract were determined according to procedures of commercially available kits. Vitamin C and vitamin E content were determined according to the protocol of commercially available kits (E-BC-K034 for vitamin C and E-BC-K033 for vitamin E).

#### *Statistical analysis*

GraphPad Prism 8 was used for statistical analyses. Data were presented as mean  $\pm$  standard error of the mean (SEM) or standard deviation (SD). Kolmogorov-Smirnov or Shapiro-Wilk tests were carried out to

control whether the distribution is normal or not. Kruskal-Wallis test was performed for the evaluation of the adhesion scores. For the other parameters, one-way ANOVA-Dunnett's post hoc test was performed. All groups were compared to the control group.  $p < 0.05$  was considered statistically significant.

## **Results and Discussion**

Ectopic endometrial tissue that has been placed outside of the uterus and exhibits pain sensations and infertility is known as endometriosis, a chronic inflammatory disease [25]. Proliferation, apoptosis, inflammation and angiogenesis are key biological processes that are crucial for the development and survival of endometriotic lesions at ectopic locations [26]. Endometriosis is an important worldwide health

concern because of its prevalence in women of reproductive age (10%) [27]. The use of medicinal plants and phytochemicals in natural alternative therapies may present new possibilities for the treatment of endometriosis [17]. Numerous natural products with various medicinal effects have demonstrated a reduction in the size of endometriotic lesions, a reduction in pelvic adhesions, and an improvement in pelvic discomfort and ovarian function [28]. Medicinal plants and their phytochemicals are increasingly being used to treat endometriosis due to their anti-angiogenic, anti-oxidative, sedative and pain-alleviating characteristics [29]. Young leaves of wheat have antioxidant, anti-inflammatory, immunomodulatory, anticarcinogenic, diuretic, anti-aging and antibacterial effects because of their bioactive compounds [30].

Adhesion scoring is widely used for the evaluation of endometriotic cysts [31]. Adhesion scores were evaluated before and after treatment. According to the adhesion scores, there were no significant differences between groups before treatment. Adhesion scores were between  $3.125 \pm 0.398$  (Control) and  $3.417 \pm 0.193$  (WG-100) (Table II). Letrozole and WG caused a decrease in adhesion scores after treatment. The lowest adhesion score was in the Letrozole group ( $1.417 \pm 0.452$ ) and statistically significant ( $p < 0.01$ ) compared to the control group ( $3.667 \pm 0.224$ ). While the adhesion score of WG-100 treatment ( $2.000 \pm 0.302$ ) was statistically important ( $p < 0.05$ ) according to the control, WG-25 ( $2.750 \pm 0.509$ ) and WG-400 ( $2.833 \pm 0.474$ ) adhesion scores after treatment were not significant compared to the control group (Table II). Methanolic fraction of the *Urtica dioica* L. extract and its fraction C caused a decrease in adhesion scores of endometrial cysts according to the control group [31]. Treatment of the ethyl acetate fraction of the *Achillea biebersteinii* Afan. extract in rats with endometriosis resulted in the decrease in adhesion score compared to the control [32]. WG ethanolic extract (100 mg/kg) had a similar effect, according to adhesion score results.

**Table II**

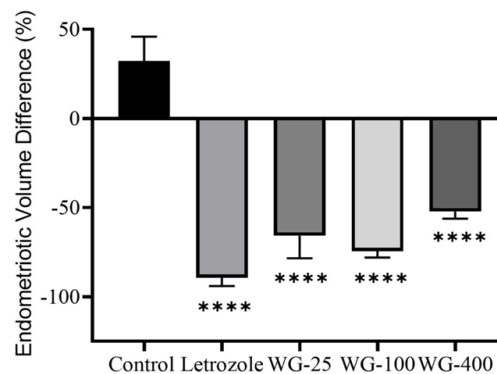
Adhesion scores of endometrial implants before and after treatment

	Before treatment	After treatment
Control	$3.125 \pm 0.398$	$3.667 \pm 0.224$
Letrozole	$3.167 \pm 0.322$	$1.417 \pm 0.452^{**}$
WG-25	$3.250 \pm 0.279$	$2.750 \pm 0.509$
WG-100	$3.417 \pm 0.193$	$2.000 \pm 0.302^*$
WG-400	$3.250 \pm 0.329$	$2.833 \pm 0.474$

Data were presented as mean  $\pm$  SEM. \* for  $p < 0.05$  and \*\* for  $p < 0.01$  compared to control in the same column

According to endometriotic cyst volume change results, Letrozole which was used as a reference drug and WG extracts caused significant decreases compared to the control group ( $p < 0.0001$ ) (Figure 1). The most

effective treatment was determined in the Letrozole group (84.4% decrease). Endometriotic cyst volume percent was increased in the control group (32.2% increase). WG-100, WG-25 and WG-400 extracts caused 74.5%, 65.8% and 52.2% decrease in endometriotic cyst volume, respectively (Figure 1).



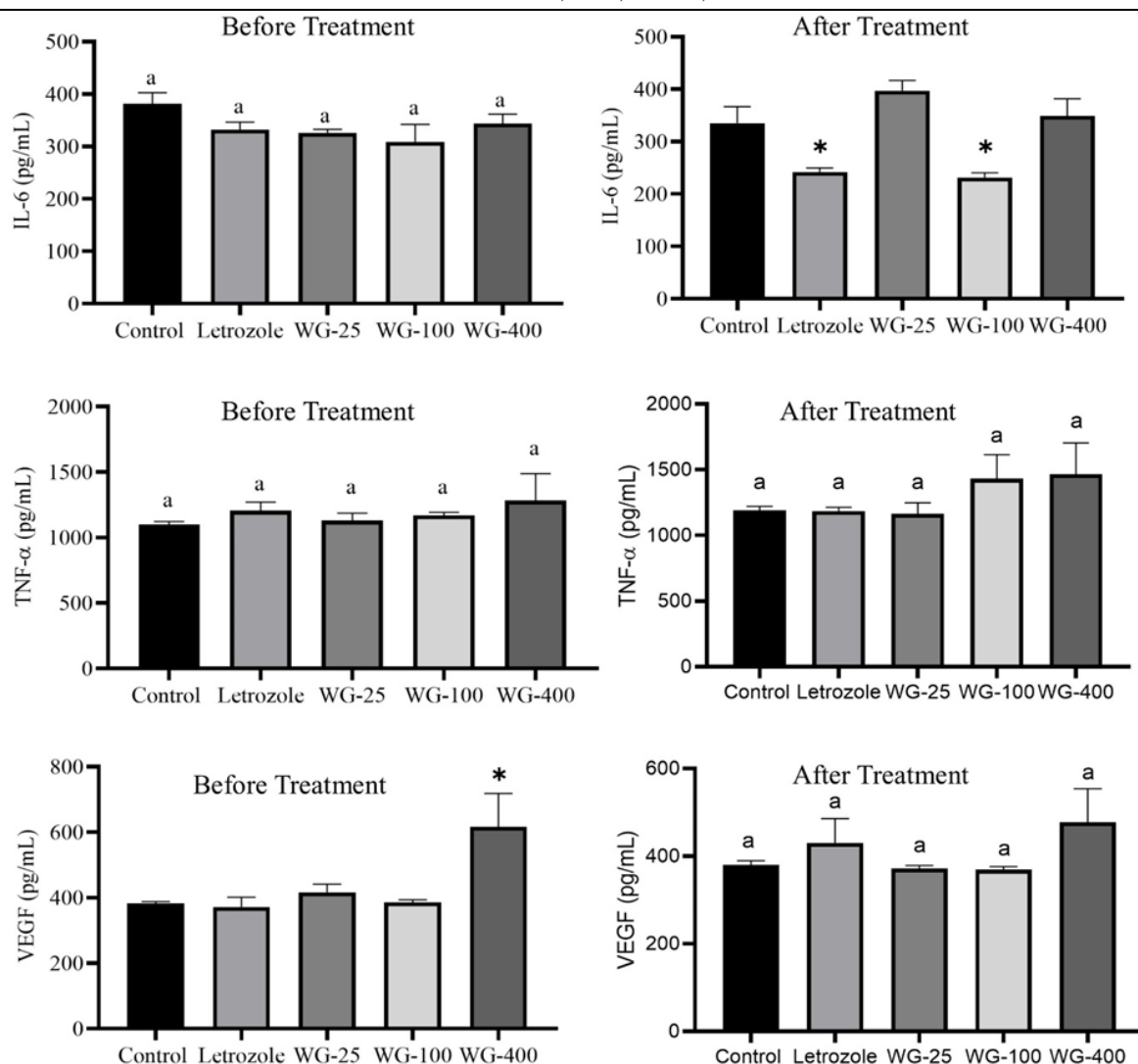
**Figure 1.**

Endometriotic volume change percent

\*\*\*\* indicates  $p < 0.0001$  according to Control

Methanolic extract of *Urtica dioica* L. and its fraction C caused a decrease in the volume of endometrial cysts [31]. Additionally, endometrial cyst volume was decreased in the reference group compared to the control in the same study. Hydro-methanolic extracts of the aerial parts of *Alchemilla mollis* (Buser) Rothm. and *Alchemilla persica* Rothm. plants caused a decrease in endometrial cyst volumes [33]. Ethyl acetate fraction of the *Achillea biebersteinii* Afan. extract-treated rats with endometriosis had less endometrial cyst volume than the control rats [32]. Similarly, ethanolic WG extract caused a decrease in endometrial cyst volume in the current study.

IL-6 levels increase peritoneal fluid in women with endometriosis [34]. VEGF is an important factor in the regulation of both normal and abnormal angiogenesis [35]. Cytokines including IL-6, TNF- $\alpha$  and VEGF were evaluated. They present valuable data to evaluate endometriosis pathogenesis [36, 37]. High levels of those cytokines were reported in the peritoneal fluid of women with endometriosis [38]. IL-6 and TNF- $\alpha$  levels in letrozole and WG extract-treated groups were not significantly different compared to the control group. Decreases in IL-6 levels in Letrozole and WG-100 groups after treatment were found statistically important compared to control ( $p < 0.05$ ) (Figure 2). While the VEGF level of WG-400 was significantly higher than the control before treatment, it was not different from the control after treatment. While hydro-methanolic extract of the aerial parts of *Alchemilla mollis* (Buser) Rothm. caused decreases in levels of IL-6, TNF- $\alpha$  and VEGF, their levels were not affected by *Alchemilla persica* Rothm. aerial extract even if it reduced endometrial cyst volume [33].



**Figure 2.**

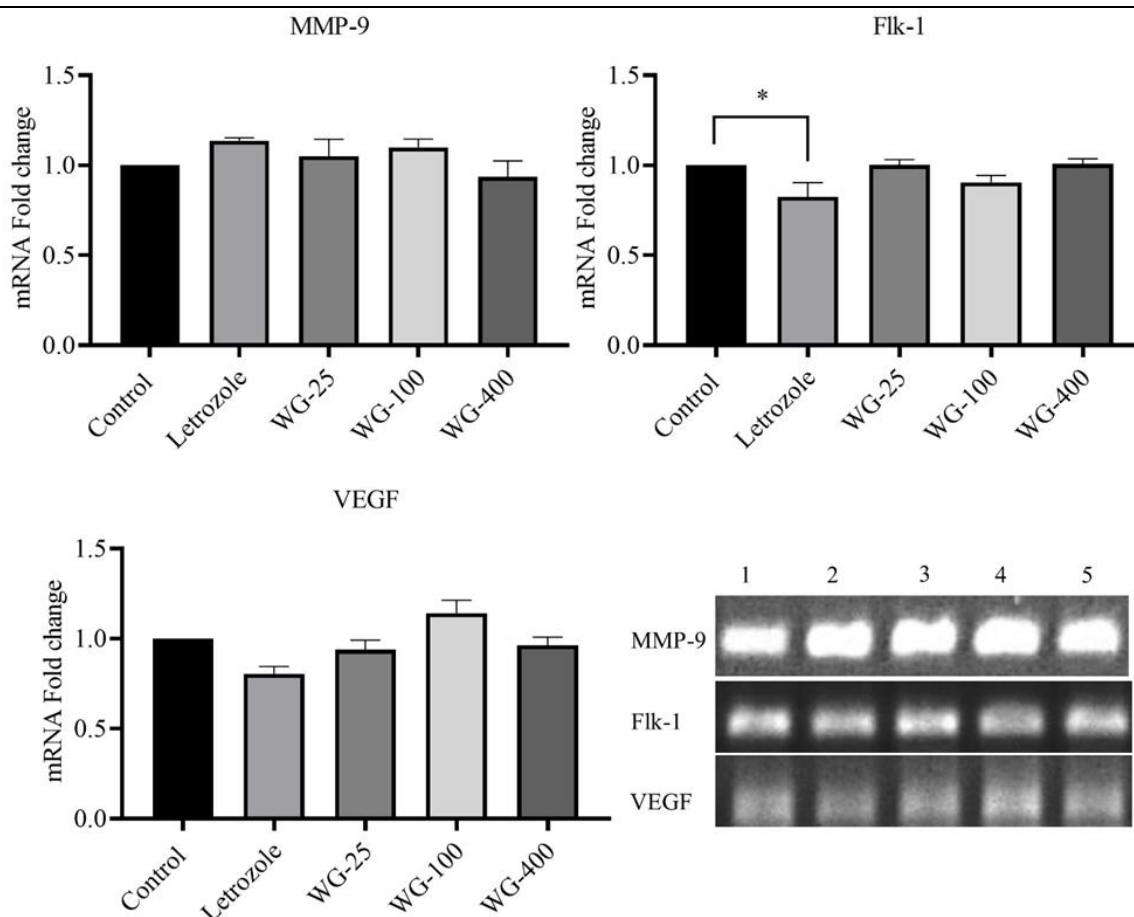
Cytokine levels before and after treatment

Asterisk (\*) on the columns indicates  $p < 0.05$  according to Control

In a gene expression profiling study, it was reported that the expression of 71 genes was upregulated and that 45 genes were downregulated in the endometriosis tissue according to normal uterine [39]. Expression of VEGF, Flk-1 and MMP-9 genes in endometriosis tissue was high and similar to cancer disease [23]. Additionally, an increase in MMP-9 gene expression was determined in women with endometriosis [40]. The expression of VEGF, MMP-9 and Flk-1 genes was determined in the endometrial cyst tissue in the current study (Figure 3). According to semi-quantitative gene expression results, expression of the Flk-1 gene in the Letrozole group was lower than the control group.

Phenolic acids were determined and quantified by LC-MS/MS. Wheat seedlings have aconitic acid in their leaves [41]. Aconitic acid was determined to be

the major phenolic acid in WG extract (54.004 mg analyte/g extract) (Table III). Quinic acid (3.687 mg analyte/g extract) and protocatechuic acid (0.014 mg analyte/g extract) were other phenolics in the WG extract. Aconitic acid, an organic acid produced by higher plants, has two isomers in nature; cis-aconitic acid and trans-aconitic acid [42]. Cis-aconitic acid is converted to itaconate by the cis-aconitate decarboxylase enzyme one of the most highly upregulated genes during proinflammatory [43]. Antioxidant and anti-inflammatory effects of itaconate have been reported [44]. Hydro-alcoholic extract of *Echinodorus grandiflorus* leaves having trans-aconitic acid as a major compound had TNF- $\alpha$  inhibition [45]. Metabolic changes in human endometrial stromal cells were induced in oxygen deficiency conditions by reducing the isocitric acid and suppressing cis-aconitic and citric acid [46].

**Figure 3.**

Expression levels of MMP-9, Flk-1 and VEGF genes

Asterisk (\*) on the columns indicates  $p < 0.05$  according to Control. Gel figure definitions: 1: Control. 2: Letrozole. 3: WG-25. 4: WG-100. 5: WG-400

**Table III**

Phytochemicals in the WG extract

Bioactive compound	Quantity
Aconitic acid (mg analyte/g extract)	54.004
Quinic acid (mg analyte/g extract)	3.687
Protocatechuic acid (mg analyte/g extract)	0.014
Vitamin C (mg/g extract)	$7.42 \pm 0.13$
Vitamin E (mg/g extract)	$5.49 \pm 0.08$

Vitamin E and vitamin C content of the WG extract were detected at  $5.49 \pm 0.08$  mg/g and  $7.42 \pm 0.13$  mg/g, respectively (Table III). Vitamins are essential for human metabolism and disruptions in vitamin metabolism, especially those of vitamin A, vitamin C, vitamin D and vitamin E, are related to endometriosis [47]. Oxidative stress, characterized by an imbalance between oxidants and antioxidants [48], occurs during endometriosis [49]. Macromolecules that are highly important for biological activities such as lipids, proteins and nucleic acids are targets for oxidants both of reactive oxygen and nitrogen species [50]. Vitamin C and vitamin E are powerful antioxidants to prevent oxidative stress in endometriosis [47]. Women with endometriosis had a low level of vitamin E in their serum [51]. Vitamin C and E supplements effectively

minimized the severity of dysmenorrhea and improved dyspareunia and the intensity of pelvic inflammation [52]. Additionally, the consumption of nutrients with high amounts of vitamin C, vitamin E, folate and thiamine had a protective role against endometriosis [53]. Therefore, supplementation with vitamins C and E was linked to a reduction in the levels of oxidative stress indicators in endometriosis patients [54]. Intravenous vitamin C treatment prevented implant induction and endometrial cyst volumes in a surgically induced endometriosis rat model [55]. In the current study, similarly, treatments of WG extract rich in vitamin C and vitamin E caused a decrease in endometrial cysts and adhesions in rats with endometriosis.

## Conclusions

Although the WG-25 and WG-400 treatments reduced endometriotic cyst volume, they did not reduce adhesion scores. Letrozole and WG-100 therapies, on the other hand, reduced both adhesion scores and endometriotic cyst volume. Additionally, IL-6 levels were discovered to decrease in Letrozole and WG-100 treatments, according to the cytokine findings. Furthermore, molecular biology findings (decrease in Flk-1 gene expression) suggest that Letrozole is more effective than wheatgrass in healing endometriosis. Aconitic acid was the major compound in the WG extract according to LC-MS/MS analysis and WG extract was rich with vitamin C and vitamin E. Because of their high phytochemical content, young wheat leaves were found to have a healing effect on the surgically induced endometriosis rat model.

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## Conflict of interest

The authors declare no conflict of interest.

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