Saudi Pharmaceutical Journal 27 (2019) 889–899

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Promising activity of *Anthemis austriaca* Jacq. on the endometriosis rat model and isolation of its active constituents

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ARTICLE INFO

Article history:
Received 11 February 2019
Accepted 8 June 2019
Available online 10 June 2019

Keywords:
Anthemis austriaca
Asteraceae
Endometriosis
Flavonoids
Sterols
Rat

ABSTRACT

Anthemis austriaca Jacq. flowers are traditionally used to alleviate abdominal pain, hemorrhoids, ovary diseases and pneumonia. This study aimed to investigate the effects of *A. austriaca* flowers, which are frequently used in gynecological disorders, on the rat endometriosis model. The rat endometriosis model was used to evaluate the potential activity of the plant in endometriosis. The dried plant material was extracted with *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH), successively. The obtained extracts from *A. austriaca* flowers were applied to the rats. The adhesion scores, endometrial foci areas, and cytokine levels of the peritoneal fluids were measured on surgical induction of endometriosis in rats. The adhesion scores, endometriotic volume, and cytokine levels of the peritoneal fluids were reduced in the EtOAc, MeOH, and buserelin acetate-treated (reference) groups. The MeOH extract reduced the adhesion scores and endometrial foci areas from 3.1 to 1.1 ($p < 0.01$) and from 86.4 to 40.5 ($p < 0.01$), respectively and also the MeOH extract reduced tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), and interleukin (IL)-6 levels of the peritoneal fluids from 13.7 to 3.8 ($p < 0.01$), from 28.4 to 16.3 ($p < 0.05$) and from 50.2 to 24.3 ($p < 0.01$), respectively. Therefore, isolation studies were conducted on the EtOAc and MeOH extracts. After the MeOH extract was fractionated using RP-18 column, the obtained subfractions were evaluated again on the endometriosis rat model. Subfractions A and C of the MeOH extract displayed statistically significant activity on the endometriosis rat model. Phytochemical investigation resulted in the isolation of 4-β-D-glucopyranosyloxy-6-methyl-2*H*-pyran-2-one **(1)** from Fr. A and quercetin **(2)**, apigenin-7-*O*-(3″-*O*-acetyl)-β-D-glucopyranoside **(3)**, apigenin-7-*O*-(6″-*O*-acetyl)-β-D-glucopyranoside **(4)**, apigenin-7-*O*-β-D-glucopyranoside **(5)**, quercetin-7-*O*-β-D-glucopyranoside **(6)** from Fr. C. Moreover, β-sitosterol-3-*O*-β-D-glucopyranoside **(7)** was isolated from the EtOAc extract. As a conclusion, the MeOH extract obtained from *A. austriaca* flowers contributed to the regression of endometriosis. In addition, flavonoids and sterols of the plant were detected as the possible compounds responsible for the activity.

1. Introduction

Anthemis L. (Asteraceae) genus is represented by 52 species in the Flora of Turkey and 29 of them are endemic to Turkey (Grierson and Yavin, 1975; Güner, 2000). Flowers of this genus are used as antiseptic, anti-inflammatory, antibacterial, antispasmodic, and sedative worldwide (Vaverkova et al., 2001). In traditional folk medicine, extracts, tinctures, and salves prepared from this genus are widely used to alleviate pain and irritation, prevent ulcers, and in therapies for skin injuries, management of cystitis, and dental afflictions (Mann and Staba, 1986).

A. austriaca is indigenous to Austria, and distributed widely from Europe to Turkey. This plant is called “papatya”,

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2019.06.002
1319-0164/

"akbabatça" or "akbaşotu" in Turkey, and the flowers of this plant have been used traditionally for abdominal pain, hemorrhoids, and pneumonia (Fujita et al., 1995; Honda et al., 1996; Simsek et al., 2004). The flowers of *A. austriaca* has also been used as an infusion in folk medicine against cough and ovary diseases (Kaval et al., 2014; Tetik et al., 2013; Uysal et al., 2010). *Anthemis* species have been reported to possess anti-inflammatory, antimicrobial, antioxidant, antiviral, cytotoxic, hepatoprotective, hypoglycemic, and hypotensive activities (Al-Snafi, 2016; Baltaci et al., 2011; Barbour et al., 2004; Shahat et al., 2014). A number of compounds including xerantholide, taraxasterol acetate, taraxasterol, β-sitosterol, 1α-hydroxyxerantholide, 6α-hydroxyxerantholide, 10α-hydroxy-11β,13-dihydroxerantholide, 8α-hydroxyxeranthemolide, xeranthemolide, methyl 8α-isobutyryloxy-3-oxo-4,11(13)-guaiadien-12-oate, methyl 8α-(2-methylbutanoyloxy)-3-oxo-4,11(13)-guaiadien-12-oate, and methyl 3,8-dioxo-4,11(13)-guaiadien-12-oate have been isolated from the aerial parts of *A. austriaca* (Staneva et al., 2004).

Endometriosis is defined as the existence of endometrial glands and stroma outside of the uterine cavity. It is a common benign gynecologic disorder, affecting approximately 5% of the general population and 30% or more of infertile women. The accumulation of immune cells within the peritoneal cavity and endometriotic lesions has been recognized in women with endometriosis (Halme et al., 1987; Klein et al., 1994). In patients with endometriosis, uterine cells migrate or develop abnormally in ectopic sites such as ovaries, bladder, cervix, rectum, neck, upper arm, and axillary area. Endometriosis affects females during their reproductive years (Oyelowo, 2007). Dysmenorrhea, nonmenstrual pelvic pain, and infertility are some of the symptoms of endometriosis (Redwine, 2006). Combined oral contraceptives, oral or parenteral progestogens, synthetic steroid derivatives, gonadotropin-releasing hormone analogues are used for treating endometriosis (Dmowski, 2003; Holt et al., 2013). However, those type medicines have severe side effects. For instance, steroids have androgenic side effects and also GnRH agonists cause signs and symptoms of hypoestrogenism; hot flashes, vaginal dryness, decreased libido, and decreased bone density (Holt et al., 2013). Because of their serious side effects, those type medicines have led to researchers to the discovery of new drugs with a low side effect profile that can be used for treating endometriosis.

Therefore, the present study aimed to investigate the effects of the extracts and fractions obtained from *A. austriaca* flowers on the adhesion scores, endometrial implant volumes, and the cytokine levels of the peritoneal fluids and also determine the compound/s responsible for the activity (Fig. 1). In the light of these informations, we expected to regress the adhesion scores, endometrial implant volumes, and the cytokine levels with the treatments of the extracts and fractions. Additionally, we aimed to exhibit which types of compounds responsible for the activity through bioassay-guided fractionation.

2. Materials and methods

2.1. Plant material

The flowers of *A. austriaca* were collected from Ankara-Şerefli koçhisar roadside, opposite the Salt Lake in June 2015, and were identified by Dr. Ufuk Özbek from the Department of Botany, Faculty of Art and Science, Gazi University, Ankara, Turkey. A voucher specimen (GUEF3418) was deposited in the Herbarium of the Faculty of Pharmacy, Gazi University, Ankara, Turkey.

2.2. Preparation of the plant extracts

Shade-dried and powdered flowers of *A. austriaca* (400 g) were successively extracted using 2000 mL of *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH) at room temperature for 48 h. The extracts were filtered and evaporated to dryness under reduced pressure at 40 °C. The yields of *n*-hexane, EtOAc, and MeOH extracts were 7.6, 12.5, and 19.6%, respectively.

2.3. Animals

Six-week-old female, non-pregnant, Wistar albino rats (weighing 200–250 g) were obtained from Laboratory Experimental Animals, Kobay, Turkey. All animals were hospitalized in accordance with the Guide for the Care and Use of Laboratory Animals, and the experiment was approved by the Experimental Animal Ethics Committee of Kobay (Protocol number: 233). The animals were housed in polysulfone cages at 21–24 °C, 40–45% humidity, and light-controlled (12-h light/12-h dark) conditions at Laboratory Animals Breeding and Experimental Research Center, Kobay (Ankara, Turkey). The animals were maintained on a standard pellet diet and water *ad libitum* during the experimental period. The rats were quarantined for at least 2 weeks. The estrous cycle was followed by a daily assessment of vaginal cytology, and rats exhibiting regular 4- to 5-day estrous cycles were used in this study.

2.4. Preparation of surgically induced rat endometriosis by uterine autotransplantation

2.4.1. First operation

The endometriosis rat model was conducted according to the Vernon and Wilson method (Vernon and Wilson, 1985). All the rats

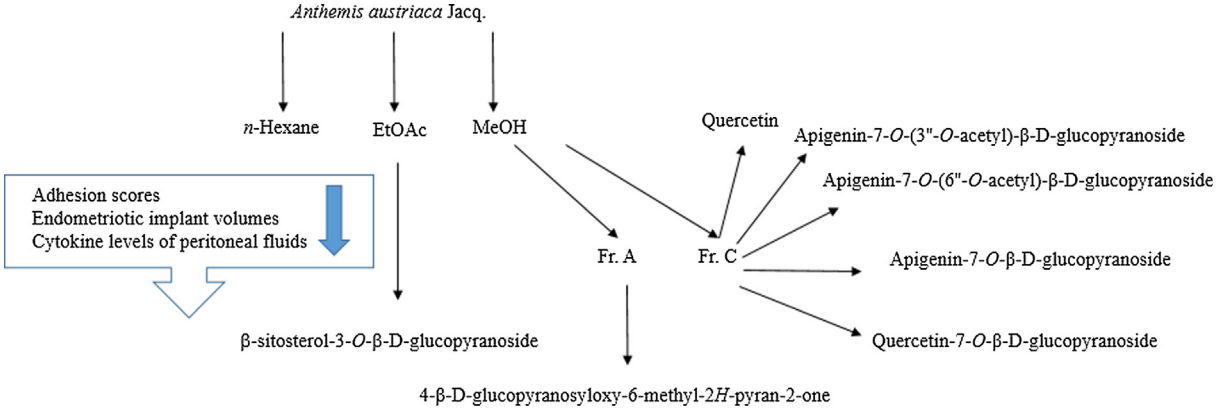

Fig. 1. Flowchart of the present study.

were anesthetized with intramuscular administration of the combination of 1 mL ketamine (50 mg/mL) and 1 mL xylazine (20 mg/mL). For the experimental procedure, the rats were placed in a supine position, and iodine was applied to their abdomens for disinfection. An incision of 3 cm was made using a scalpel blade. The subcutaneous and muscle layers were separated, and the abdominal cavity was opened. The right uterine horn was ligated and resected. A 15-mm piece of the tissue was trimmed using microscissors. This section was placed in saline, longitudinally opened and the endometrium layer was separated from the myometrium. The trimmed piece of the endometrium was transplanted onto the inner surface of the abdominal wall with the serosal surface apposed and secured, just next to a vessel using USP 4/0 polyglactin sutures (Lactasorb PGLA®, Orhan Boz, Turkey). The muscle layers of the abdomen were closed using USP 3/0 polyglactin sutures (Lactasorb PGLA®, Orhan Boz, Turkey).

2.4.2. Second operation

Twenty-eight days after the first operation, another experiment was performed under anesthesia. The endometriotic foci areas and adhesions were evaluated in the intra-abdominal area. The areas of the endometrial foci were calculated by measuring their length, width, and height using a micrometer. For the calculation of endometriotic volume, the equation $\pi/6 \times$ length $\times$ width $\times$ height was used (Altintas et al., 2008). Intra-abdominal adhesions were evaluated according to the Blauer's scoring system (Blauer and Collins, 1988) as follows: 0, no adhesion; 1, thin adhesions; 2, thick adhesions in one area; 3, widespread thick adhesions; and 4, adhesions of the internal organs to the abdominal wall. The abdomen was closed using the same procedure. In order to minimize adhesion formation, 2 mL of saline were administered into the abdominal cavity before the closing abdominal wall (Uzunlar et al., 2014).

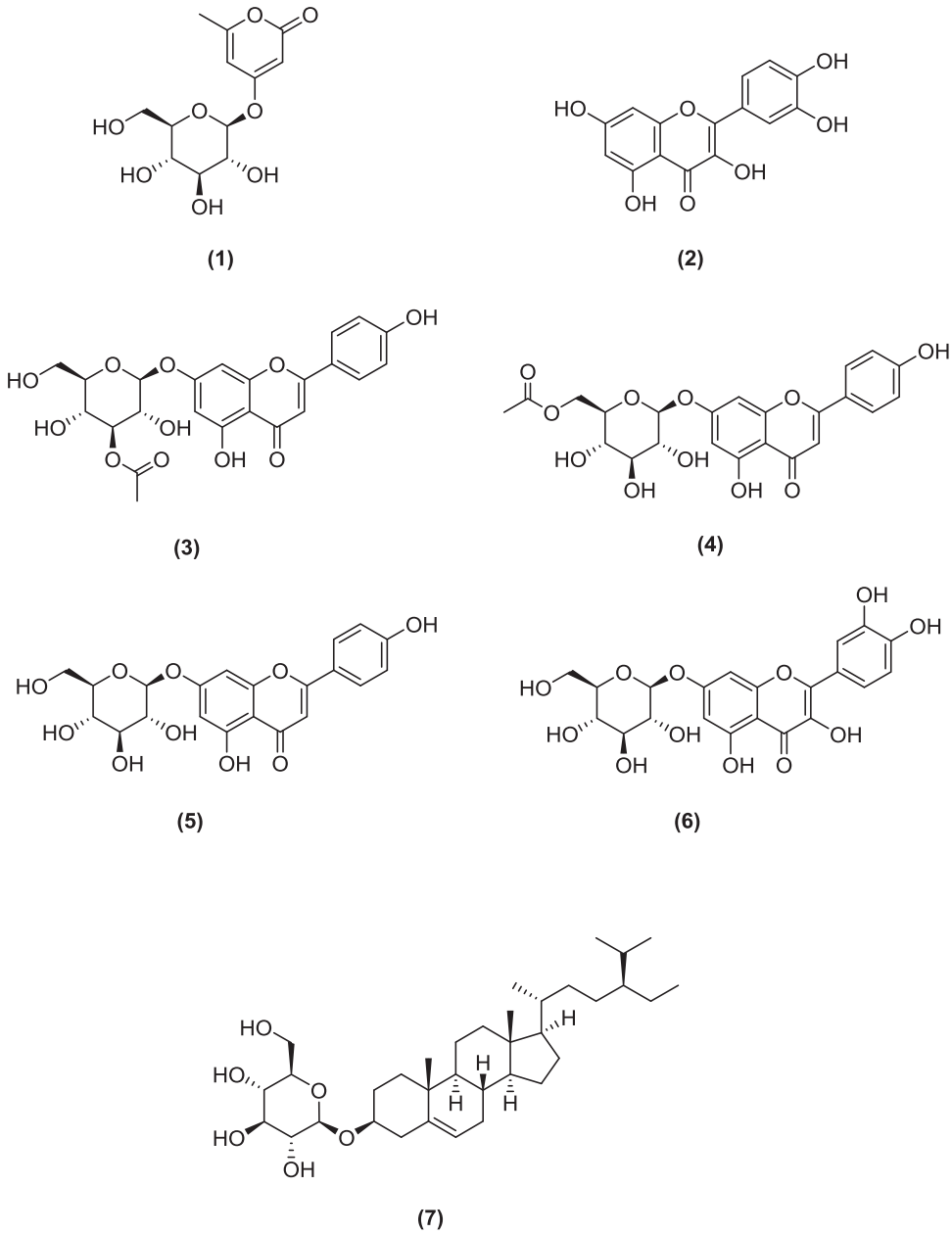

Fig. 2. Isolated compounds from the active extracts.

2.4.3. Treatment procedure

Thirty rats were divided randomly into five groups to evaluate the activities of the extracts, including six rats in each group. Thirty-six rats were randomly divided into six groups to evaluate the activities of the fractions. Three days after the second experiment, 0.5% carboxymethylcellulose (control group) and different polarity extracts (test materials) and the fractions obtained from MeOH extract were administered orally once a day for 4 weeks. The estrous cycle was evaluated by daily vaginal cytology during the experiment. Extracts and fractions were administered to the rats at the dose of 100 mg/kg. Buserelin acetate (20 mg/rat) was subcutaneously injected to the rats once a week (Demirel et al., 2014; Küpeli Akkol et al., 2015). All rats were sacrificed at the end of the management process. The intra-abdominal adhesions, endometriotic foci areas and cytokine levels of the peritoneal fluids were again evaluated and compared using previous findings.

2.5. Techniques for histopathological investigation

Firstly, all endometriotic tissues from the experimental groups were fixed with 10% formaldehyde. All tissues were detected using the Thermo Scientific Excelsior (ES) machine. The tissues were embedded in paraffin wax and blocks were prepared using the HistoCentre 2 machine. Subsequently, sections of 3.5 μm thickness were made from paraffin-embedded blocks using a Leica RM2255 microtome. The sections were stained with hematoxylin–eosin (HE) using the Shandon Varistan machine. Photographs of pathological endometriotic tissues were taken using Nikon Eclipse Ci with both polarizing attachment and Digital Image analysis system, which were then examined under a light microscope using. In the histopathological analysis, the severity of lesions in the implants were determined according to the presence of endometrial glands (Elgamal et al., 2016).

2.6. Detection of cytokine levels

In the second exploratory laparotomy, peritoneal fluid was collected to detect the tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), and interleukin (IL)-6 levels in rats. TNF-α, VEGF (Cusabio® USA; catalog numbers CSB-E11987r and CSB-E04757r), and IL-6 (Bio Source® International, Nivelles, Belgium; catalog number MBS701221) in the peritoneal fluid were quantitatively assessed using the commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. After the animals were sacrificed, peritoneal fluid was again collected and the aforementioned process was performed. The pre- and post-treatment results were compared and statistically evaluated.

2.7. Statistical analysis

The results were given as mean ± standard error of the mean (S.E.M.). The analysis of variance test was used to determine significant differences among groups. Statistical analysis was performed using the GraphPad Prism 6.0. Lilliefors adjusted Kolmogorov-Smirnov test was used to test whether the variables used in the study were normally distributed. Statistical differences between the treated and the control groups were evaluated by ANOVA-Dunnett's test and p values less than 0.05 were considered significant.

2.8. Isolation studies

2.8.1. Isolation procedure for MeOH extract

The MeOH extract of the plant (20 g) was subjected to vacuum liquid chromatography using RP-18 silica with elution of H_2O: MeOH (100:0, 2 L), (90:10, 1 L), (80:20, 1 L), (70:30, 2 L), (60:40, 2 L), (50:50, 2 L), (40:60, 2 L), (30:70, 2 L), (20:80, 2 L), (10:90, 1 L), (0:100, 1 L) and then acetone (1 L) to obtain 29 fractions. The resulting fractions were pooled to four fractions (A–D) on the basis of thin layer chromatography (TLC) profile, developed by using EtOAc:$CHCl_3$:MeOH:H_2O (15:8:4:1) solvent mixture. Compound **1** was precipitated from Fr. A which is one of the active fractions. Fr. C, which is the other active fraction, was subjected to silica column to obtain other pure compounds. Structure elucidation of isolated compounds was achieved by their NMR and mass data analyses (Fig. 2).

2.8.2. Isolation procedure for EtOAc extract

EtOAc extract of the plant (4 g) was subjected to silica gel column chromatography using hexanes:$CHCl_3$ (1:1) and (0:1) and $CHCl_3$:MeOH (19:1) solvent systems to isolate β-sitosterol-3-*O*-β-D-glucopyranoside.

Table 1
Intraabdominal adhesion scores of the endometriotic implants of extracts and fractions from MeOH extract prepared from *Anthemis austriaca*.

Groups	Adhesion scores		
	Pre-treatment	Post-treatment	FCV
Control	3.2 ± 0.7	3.5 ± 0.6	1.09
***n*-Hexane extract**	2.8 ± 0.7	2.0 ± 0.4	0.71
EtOAc extract	2.9 ± 0.8	**1.4 ± 0.5***	0.48
MeOH extract	3.1 ± 0.7	**1.1 ± 0.3****	0.35
Reference	3.0 ± 0.8	**0.0 ± 0.0*****	0.00
Control	3.5 ± 1.3	3.8 ± 0.8	1.09
Fr. A	2.9 ± 0.7	**1.9 ± 0.7***	0.66
Fr. B	3.5 ± 0.8	3.3 ± 1.0	0.94
Fr. C	3.1 ± 0.9	**1.0 ± 0.2****	0.32
Fr. D	3.5 ± 0.7	3.3 ± 0.5	0.94
Reference	2.6 ± 0.7	**0.0 ± 0.0*****	0.00

S.E.M.: Standard Error of Mean; FCV: Fold Change Value.
Bold and asterisk indicate statistical significance.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

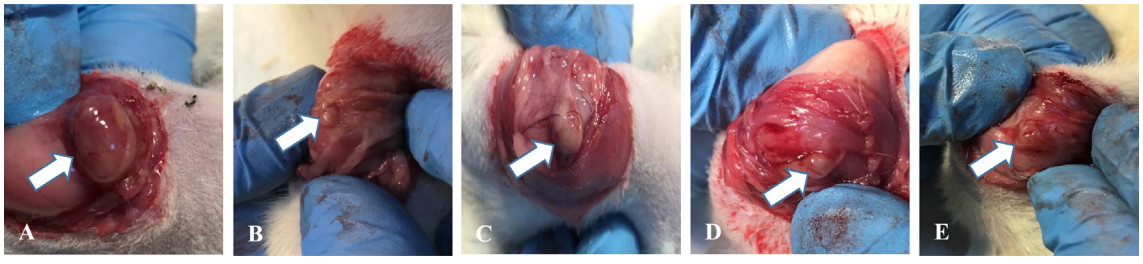

Fig. 3. Endometriotic implants after treatment procedure. (A): Control group; (B): MeOH extract treated group; (C): Fr. A treated group; (D) Fr. C treated group; (E): Reference group (Buserelin acetate).

3. Results

In the present study, the effects of the extracts obtained from *A. austriaca* were investigated on surgically-induced endometriosis in rats by evaluating the adhesion scores, endometrial foci areas and cytokine levels of the peritoneal fluids. The endometriotic implants were observed to be in cystic and vascularized formation during the second operation (Fig. 3). The adhesion scores, mean endometriotic implant volume values, and cytokine levels of the test materials are presented in Tables 1 and 2. According to the Blauer's scoring system, the adhesion scores of the MeOH, EtOAc extract–treated and reference groups significantly decreased from 3.1, 2.9, 3.0 to 1.1 ($p < 0.01$), 1.4 ($p < 0.05$) and 0.0 ($p < 0.001$), respectively (Table 1). The endometriotic implant volumes significantly decreased in the reference, MeOH and EtOAc extract–treated groups from 95.0, 86.4, 83.9 mm^3 to 24.5 ($p < 0.001$), 40.5

Table 2
Peritoneal TNF-α, VEGF and IL-6 levels before and after treatment in all groups.

Material	Peritoneal TNF-α level (pg/ml)			Peritoneal VEGF level (pg/ml)			Peritoneal IL-6 level (pg/ml)		
	Pre-treatment	Post-treatment	FCV	Pre-treatment	Post-treatment	FCV	Pre-treatment	Post-treatment	FCV
Control	9.4 ± 3.1	8.9 ± 2.0	0.95	23.8 ± 5.4	26.1 ± 7.2	1.10	51.4 ± 10.2	50.3 ± 8.7	0.98
***n*-Hexane extract**	8.9 ± 2.3	7.6 ± 1.9	0.85	22.8 ± 7.1	20.9 ± 10.1	0.92	58.6 ± 15.2	53.1 ± 14.3	0.91
EtOAc extract	9.5 ± 1.4	**5.3 ± 1.4***	0.56	24.1 ± 8.5	**15.2 ± 4.7***	0.63	52.7 ± 9.6	**42.2 ± 9.0***	0.80
MeOH extract	13.7 ± 4.2	**3.8 ± 0.7****	0.28	28.4 ± 7.4	**16.3 ± 7.6***	0.57	54.4 ± 14.6	**30.1 ± 9.3****	0.55
Reference	9.1 ± 0.9	**2.4 ± 0.6*****	0.26	20.1 ± 6.8	**9.9 ± 1.5*****	0.49	50.2 ± 11.3	**24.3 ± 9.8*****	0.48
Control	9.9 ± 2.6	10.4 ± 3.5	1.05	26.2 ± 6.7	28.2 ± 4.6	1.08	52.7 ± 13.1	55.4 ± 11.8	1.05
Fr. A	9.4 ± 3.5	6.4 ± 2.3	0.68	28.4 ± 8.5	**16.1 ± 4.1***	0.57	57.5 ± 13.4	**44.7 ± 9.1***	0.78
Fr. B	10.1 ± 2.1	9.7 ± 2.4	0.96	26.6 ± 7.9	26.8 ± 9.3	1.01	58.4 ± 12.6	56.1 ± 11.5	0.96
Fr. C	13.7 ± 3.2	**5.7 ± 1.3***	0.42	27.9 ± 6.5	**14.8 ± 5.2***	0.53	55.9 ± 9.8	**38.0 ± 7.4****	0.68
Fr. D	12.6 ± 5.4	13.9 ± 5.2	1.10	22.3 ± 9.6	21.8 ± 8.1	0.98	50.1 ± 10.4	52.4 ± 8.5	1.05
Reference	12.9 ± 4.1	**2.0 ± 0.5*****	0.16	25.7 ± 7.8	**10.2 ± 0.7*****	0.40	53.6 ± 9.7	**20.7 ± 5.6*****	0.39

S.E.M.: Standard Error of Mean; FCV: Fold Change Value.
Bold and asterisk indicate statistical significance.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

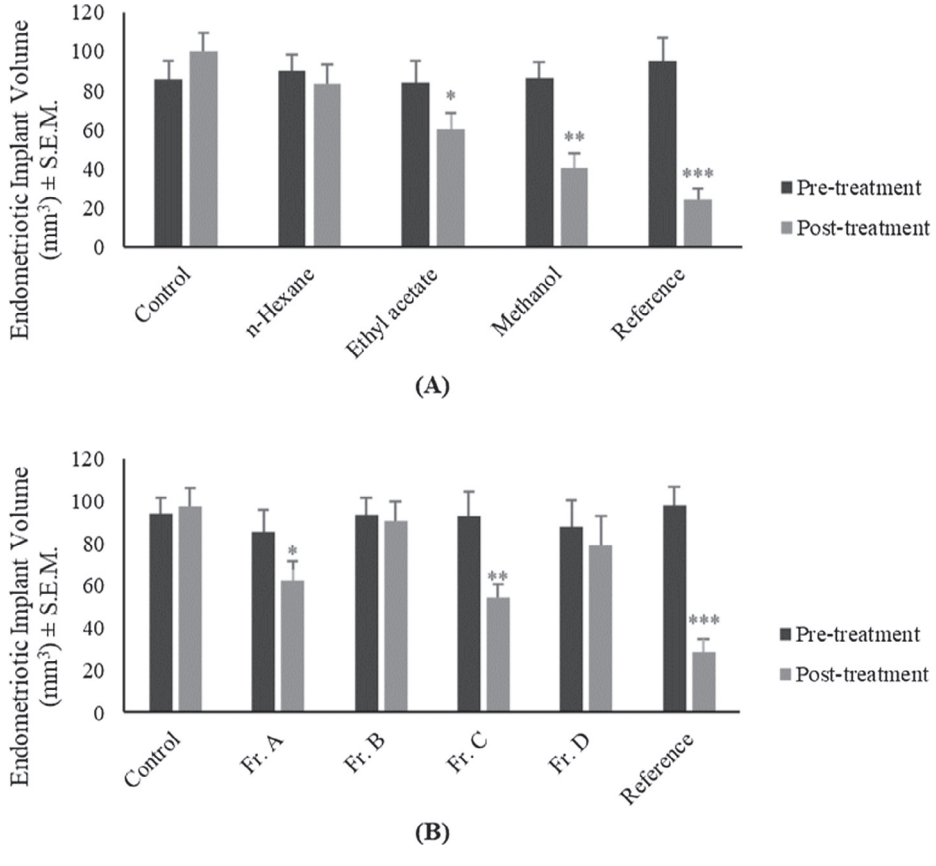

Fig. 4. Endometriotic implant volumes of all treated groups.

($p < 0.01$), and 60.3 ($p < 0.05$) mm^3, respectively (Fig. 4A). Significant differences were detected the peritoneal TNF-α, VEGF, and IL-6 levels in the MeOH extract–treated and reference groups compared to those of the control group (Table 2). After the MeOH extract treatment, the levels of TNF-α, VEGF, and IL-6 decreased from 13.7, 28.4, 54.4 pg/mL to 3.8 ($p < 0.01$), 16.3 ($p < 0.05$), 30.1 pg/mL ($p < 0.01$), respectively. After the EtOAc extract treatment, the levels of TNF-α, VEGF, and IL-6 significantly decreased from 9.5, 24.1, 52.7 pg/mL to 5.3 ($p < 0.05$), 15.2 ($p < 0.05$), 42.2 pg/mL ($p < 0.05$), respectively. Furthermore, the cytokine levels were reduced significantly in the reference group.

After fractionation of the MeOH extract, Frs. A and C showed significant activity compared to the control group. The adhesion scores and endometriotic foci areas decreased significantly from 2.9 to 1.9 ($p < 0.05$) and from 85.4 to 62.2 mm^3 ($p < 0.05$), respectively with the treatment of Fr. A, whereas the adhesion scores and endometriotic foci areas decreased significantly from 3.1 to 1.0 ($p < 0.01$) and from 92.9 to 54.4 mm^3 ($p < 0.01$), respectively with the treatment of Fr. C (Table 1 and Fig. 4B). After the Fr. A treatment, the levels of TNF-α, VEGF, and IL-6 significantly decreased from 9.4 to 6.4 (non-significant), from 28.4 to 16.1 ($p < 0.05$) and from 57.5 to 44.7 pg/mL ($p < 0.05$), respectively. After the Fr. C treatment, the levels of TNF-α, VEGF, and IL-6 significantly decreased from 13.7 to 5.7 ($p < 0.05$), from 27.9 to 14.8 ($p < 0.05$) and from 55.9 to 38.0 pg/mL ($p < 0.01$), respectively (Table 2). In addition, there was not seen any side effect or pathological change in the animals.

The structures of isolated compounds were elucidated by their NMR and mass data. The molecular ion peak of compound **1** in positive ion mode was $m/z = 289.0909$ showing the molecular formula $C_{12}H_{17}O_8$. According to the NMR (Table 3) and mass data, compound **1** was identified as a 4-β-D-glucopyranosyloxy-6-methyl-2*H*-pyran-2-one (Gafner et al., 1998). The molecular ion peak of compound **2** in positive ion mode was $m/z = 303.0523$ showing the molecular formula $C_{15}H_{11}O_7$. According to the NMR (Table 4) and mass data, compound **2** was identified as a quercetin (Kyriakou et al., 2012). The molecular ion peak of compound **3** in positive ion mode was $m/z = 475.1245$ showing the molecular formula $C_{23}H_{23}O_{11}$. According to the NMR (Table 4) and mass data, compound **3** was identified as a apigenin-7-*O*-(3″-*O*-asetil)-β-D-glucopyranoside (Xie et al., 2014). The molecular ion peak of compound **4** in positive ion mode was $m/z = 475.1208$ showing the molecular formula $C_{23}H_{23}O_{11}$. According to the NMR (Table 4) and mass data, compound **4** was identified as a apigenin-7-*O*-(6″-*O*-asetil)-β-D-glucopyranoside (Svehlikova et al., 2004). The molecular ion peak of compound **5** in positive ion mode was $m/z = 433.1129$ showing the molecular formula $C_{21}H_{21}O_{10}$. According to the NMR (Table 5) and mass data, compound **5** was identified as a apigenin-7-*O*-β-D-glucopyranoside (Li et al., 2009a). The molecular ion peak of compound **6** in positive ion mode was $m/z = 465.1094$ showing the molecular formula $C_{21}H_{21}O_{12}$. According to the NMR (Table 5) and mass data, compound **6** was identified as a quercetin-7-*O*-β-D-glucopyranoside (Legault et al., 2011). According to the NMR data (Table 6), compound **7** was identified as a β-sitosterol-3-*O*-β-D-glucopyranoside (Rai et al., 2006).

Table 3
^1H and ^{13}C NMR data of compound **1** (CD_3OD, ^1H: 400 MHz, ^{13}C: 100 MHz).

C/H	δ_C (ppm)	δ_H (ppm)/*J*
2 (C=O)	167.5	q
3 (CH)	91.7	5.69 (1H, d, *J* = 2.1 Hz)
4 (C)	171.5	q
5 (CH)	101.5	6.13 (1H, d, *J* = 2.1 Hz)
6 (C)	164.8	q
7 (CH_3)	19.7	2.25 (3H, s)
1″ (CH)	100.8	5.04 (1H, d, *J* = 7.3 Hz)
2″ (CH)	77.6	3.49 (1H, m)
3″ (CH)	74.3	3.44 (1H, m)
4″ (CH)	70.9	3.38 (1H, m)
5″ (CH)	78.5	3.49 (1H, m)
6″$_A$ (CH_2)	62.2	3.88 (1H, dd, *J* = 12.1; 2.2 Hz)
6″$_B$ (CH_2)	62.2	3.69 (1H, dd, *J* = 12.1; 5.5 Hz)

q: quaterner.

Table 4
^1H and ^{13}C NMR data of compound **2**, **3**, and **4**.

	2 (CD_3OD, ^1H: 500 MHz, ^{13}C: 125 MHz)		**3** (CD_3OD, ^1H: 400 MHz, ^{13}C: 100 MHz)		**4** (CD_3OD, ^1H: 400 MHz, ^{13}C: 100 MHz)	
C/H	δ_C (ppm)	δ_H (ppm)/*J*	δ_C (ppm)	δ_H (ppm)/*J*	δ_C (ppm)	δ_H (ppm)/*J*
2 (C)	147.9	q	166.5	q	166.8	q
3 (CH)	137.3	q	104.1	6.65 (1H, s)	104.1	6.63 (1H, s)
4 (C=O)	177.3	q	183.6	q	184.0	q
5 (C)	162.5	q	163.4	q	162.9	q
6 (CH)	99.2	6.17 (1H, d, *J* = 2.0 Hz)	101.2	6.48 (1H, d, *J* = 2.1 Hz)	101.1	6.48 (1H, d, *J* = 2.1 Hz)
7 (C)	165.6	q	164.2	q	164.6	q
8 (CH)	94.4	6.38 (1H, d, *J* = 2.0 Hz)	96.1	6.81 (1H, d, *J* = 2.1 Hz)	96.2	6.75 (1H, d, *J* = 2.1 Hz)
9 (C)	158.2	q	158.7	q	158.8	q
10 (C)	104.5	q	107.2	q	107.1	q
1′ (C)	124.1	q	123.0	q	122.9	q
2′ (CH)	115.9	7.73 (1H, d, *J* = 2.0 Hz)	129.6	7.87 (2H, d, *J* = 7.5 Hz)	129.6	7.85 (2H, d, *J* = 8.9 Hz)
3′ (CH)	146.2	q	117.1	6.92 (2H, d, *J* = 7.5 Hz)	117.1	6.92 (2H, d, *J* = 8.9 Hz)
4′ (C)	148.8	q	162.8	q	163.1	q
5′ (CH)	116.2	6.88 (1H, d, *J* = 8.4 Hz)	117.1	6.92 (2H, d, *J* = 7.5 Hz)	117.1	6.92 (2H, d, *J* = 8.9 Hz)
6′ (CH)	121.6	7.63 (1H, dd, *J* = 8.4; 2.0 Hz)	129.6	7.87 (2H, d, *J* = 7.5 Hz)	129.6	7.85 (2H, d, *J* = 8.9 Hz)
1″ (CH)			101.3	5.18 (1H, d, *J* = 7.9 Hz)	101.5	5.04 (1H, d, *J* = 7.4 Hz)
2″ (CH)			73.0	3.63 (1H, m)	74.7	3.50 (1H, m)
3″ (CH)			78.7	5.09 (1H, m)	77.7	3.51 (1H, m)
4″ (CH)			69.4	3.59 (1H, m)	71.6	3.39 (1H, m)
5″ (CH)			78.1	3.64 (1H, m)	75.6	3.77 (1H, m)
6″$_A$ (CH_2)			62.1	3.94 (1H, dd, *J* = 12.0; 2.1 Hz)	64.8	4.46 (1H, dd, *J* = 11.9; 2.1 Hz)
6″$_B$ (CH_2)			62.1	3.75 (1H, dd, *J* = 12.0; 4.2 Hz)	64.8	3.49 (1H, dd, *J* = 11.9; 4.7 Hz)
Acetyl						
C=O			172.5	q	172.7	q
CH_3			21.1	2.15 (3H, s)	20.8	2.06 (3H, s)

q: quaterner, d: doublet, m: multiplet, s: singlet.

Table 5
^{1}H and ^{13}C NMR data of compound **5** and **6.**

	5 (DMSO, ^{1}H: 400 MHz, ^{13}C: 100 MHz)		6 (CD_3OD, ^{1}H: 400 MHz, ^{13}C: 100 MHz)	
C/H	**δ_C (ppm)**	**δ_H (ppm)/*J***	**δ_C (ppm)**	**δ_H (ppm)/*J***
2 (C)	164.3	q	148.2	q
3 (CH)	103.1	6.86 (1H, s)	136.5	q
4 (C=O)	182.0	q	177.3	q
5 (C)	161.4	q	161.5	q
6 (CH)	99.5	6.44 (1H, d, *J* = 2.1 Hz)	100.2	6.44 (1H, d, *J* = 2.0 Hz)
7 (C)	163.0	q	164.4	q
8 (CH)	94.9	6.83 (1H, d, *J* = 2.1 Hz)	95.5	6.72 (1H, d, *J* = 2.0 Hz)
9 (C)	157.0	q	156.2	q
10 (C)	105.4	q	105.9	q
1′ (C)	121.1	q	124.0	q
2′ (CH)	128.6	7.95 (2H, d, *J* = 8.8 Hz)	116.2	7.75 (1H, d, *J* = 2.0 Hz)
3′ (CH)	116.0	6.94 (2H, d, *J* = 8.8 Hz)	146.2	q
4′ (C)	161.1	q	148.7	q
5′ (CH)	116.0	6.94 (2H, d, *J* = 8.8 Hz)	116.2	6.87 (1H, d, *J* = 7.8 Hz)
6′ (CH)	128.6	7.95 (2H, d, *J* = 8.8 Hz)	121.9	7.64 (1H, dd, *J* = 7.8; 2.0 Hz)
1″ (CH)	99.5	5.07 (1H, d, *J* = 7.2 Hz)	101.6	5.07 (1H, d, *J* = 7.7 Hz)
2″ (CH)	73.1	3.44–3.15	74.7	3.56–3.36
3″ (CH)	76.5		77.8	
4″ (CH)	69.6		71.3	
5″ (CH)	77.2		78.3	
$6''_A$ (CH_2)	60.6	3.72 (1H, dd, *J* = 10.9; 2.8 Hz)	62.4	3.94 (1H, dd, *J* = 11.8; 1.9 Hz)
$6''_B$ (CH_2)	60.6	3.49 (1H, dd, *J* = 10.9; 5.6 Hz)	62.4	3.73 (1H, dd, *J* = 11.8; 5.6 Hz)

q: quaterner, d: doublet.

Table 6
^{1}H and ^{13}C NMR data of compound **7** [$CDCl_3$:CD_3OD (1:1), ^{1}H: 400 MHz, ^{13}C: 100 MHz].

C/H	δ_C (ppm)	δ_H (ppm)/*J*
1 (CH_2)	37.9	–
2 (CH_2)	30.1	–
3 (CH)	79.6	–
4 (CH_2)	40.4	–
5 (C)	141.0	q
6 (CH)	122.5	5.60 (1H, bs)
7 (CH_2)	32.5	–
8 (CH)	31.6	–
9 (CH)	50.8	–
10 (C)	37.3	q
11 (CH_2)	21.6	–
12 (CH_2)	39.2	–
13 (C)	42.9	q
14 (CH)	57.4	–
15 (CH_2)	24.8	–
16 (CH_2)	28.8	–
17 (CH)	56.7	–
18 (CH_3)	12.2	0.93 (3H, s)
19 (CH_3)	19.7	1.26 (3H, s)
20 (CH)	36.7	–
21 (CH_3)	19.1	1.17 (3H, d, *J* = 6.3 Hz)
22 (CH_2)	34.5	–
23 (CH_2)	26.6	–
24 (CH)	46.5	–
25 (CH)	29.7	–
26 (CH_3)	19.3	1.05 (3H, d)
27 (CH_3)	20.1	1.07 (3H, d)
28 (CH_2)	23.6	–
29 (CH_3)	12.2	1.09 (3H, t)
1′ (CH)	101.8	4.64 (1H, d, *J* = 7.8 Hz)
2′ (CH)	74.2	3.45 (1H, t)
3′ (CH)	77.2	3.63 (1H, m)
4′ (CH)	70.9	3.62 (1H, m)
5′ (CH)	76.7	3.52 (1H, m)
$6'_A$ (CH_2)	62.3	4.10 (1H, dd, *J* = 12.0; 2.5 Hz)
$6'_B$ (CH_2)	62.3	3.96 (1H, dd, *J* = 12.0; 5.1 Hz)

q: quaterner, bs: broad singlet, d: doublet, s: singlet, t: triplet, m: multiplet.

In the histopathological analyses, whereas a number of endometrial glands and mononuclear cell infiltration (MCI) were observed in the control groups, the decrease in endometrial glands and MCI was detected in the reference, MeOH extract, Frs. A and C treated groups (Figs. 5 and 6). Endometriotic implants reveal special histological characteristics. Mononuclear cell infiltration occurrs into subjacent fibromuscular tissue along the loose connective tissue septa in endometriosis. Hyperplasia of the fibromuscular tissue around the implant is generally observed and perivascular mononuclear inflammatory cells are usually present (Cornillie et al., 1990). Histopathological findings showed that the severity of lesions for the extracts was reduced in the *n*-hexane, EtOAc, MeOH, and reference groups, respectively (Fig. 5) on the other hand the severity of the lesions for the fractions was reduced subjectively in the Fr. D, Fr. B, Fr. A, Fr. C, and reference groups, respectively (Fig. 6). The results are presented with microscopical figures.

4. Discussion

Anthemis genus has diverse biological effects including antimicrobial, antioxidant, antiviral, cytotoxic, (Barbour et al., 2004; Shahat et al., 2014) and phytochemical content including sesquiterpene lactones, enzymes, sterols, essential oils, and flavonoids (Saroglou et al., 2010; Staneva et al., 2008; Theodori et al., 2006). The flowers of *A. austriaca* has been used as an infusion in folk medicine against cough and ovary diseases (Kaval et al., 2014; Tetik et al., 2013; Uysal et al., 2010). Based on its folkloric usage, it has been aimed to determine the effects of *A. austriaca* on the treatment of endometriosis in this study.

This study investigated the activity of *A. austriaca* flowers on the rat endometriosis model. The results displayed that the MeOH extract and Fr. C obtained from the MeOH extract remarkably decreased the adhesion scores, endometriotic implant volumes and cytokine levels. Through bioactivity guided fractionation, five flavonoids namely quercetin, apigenin-7-*O*-(3″-*O*-asetil)-β-D-glucopyranoside, apigenin-7-*O*-(6″-*O*-asetil)-β-D-glucopyranoside, apigenin-7-*O*-β-D-glucopyranoside, quercetin-7-*O*-β-D-glucopyranoside were isolated from Fr. C which was the most active fraction.

In studies investigating the connection between endometriosis and estrogen levels, it has been reported that the increase in estrogen levels leads to the growth of endometrial lesions, and the suppression of estrogen levels contribute to the healing of the

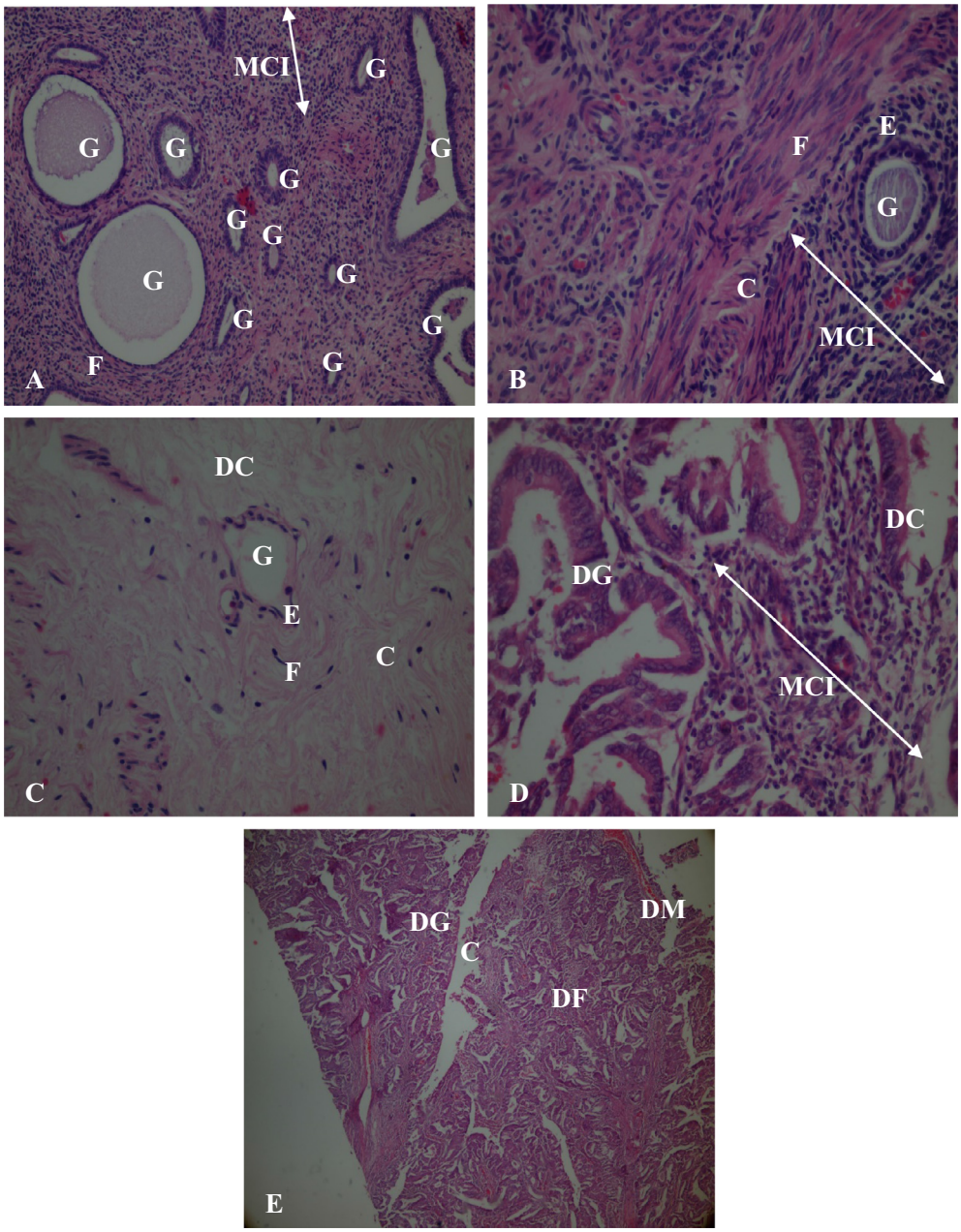

Fig. 5. Histopathological views of all extracts treated groups. (A) Control group; F: fibroblast cells, G: endometrial gland, MCI: monuclear cells infiltration, (B) *n*-Hexane extract treated group; C: collagen fibers, F: fibroblast cells, G: endometrial gland, E: endometrial gland epithelium, MCI: monuclear cells infiltration, (C) EtOAc extract treated group; G: endometrial gland, E: endometrial gland epithelium, C: collagen fibers, F: fibroblast cells, DC: degenerative collagen fibers, (D) MeOH extract treated group; DG: degenerative endometrial gland, DC: destruction connective tissue, MCI: mononuclear cells infiltration, (E) Buserelin acetate group; DF: degenerative functional layer, DG: degenerative endometrial gland, DM: degenerative myometrium, C: separation of connective tissue.

endometrial lesions (Kitawaki et al., 2002). Moreover, a similar study performed by Bedaiwy et al. found that endometriotic implants use estrogen to maintain their viability (Bedaiwy et al., 2017).

Kettel et al. (1996) reported that the increase of endometriotic implant volumes is stimulated by estrogen and progesterone. In case of their absence, the symptoms of endometriosis usually abate. It has been proven that mifepristone which is antiprogesterogenic agent appears to be effective in improving the symptoms and contributing regression of endometriosis in the absence of significant side effects in the same study (Kettel et al., 1996).

As mentioned before, there is a relation between ovarian steroids such as estrogen and progesterone and endometriosis. Based on the previous studies, we considered that the extracts and fractions obtained from *A. austriaca* contributed to the regression of endometriosis by reducing the levels of estrogen and progesterone.

Endometriosis causes inflammatory changes in the peritoneal fluid. For instance, it can give rise to the proliferation of macrophages and phagocytic dysfunction and also release proinflammatory and angiogenic factors (Taylor and Lebovic, 2014). Wu and Ho (2003) reported that in case of endometriosis, the levels of TNF-α, VEGF, and IL-6 increase whereas the levels of IL-8, IL-13, and interferon-γ do not show any change. IL-6 was reported to be crucial in the regulation of ovarian steroid creation and early implantation events. Therefore, this cytokine can be an important factor

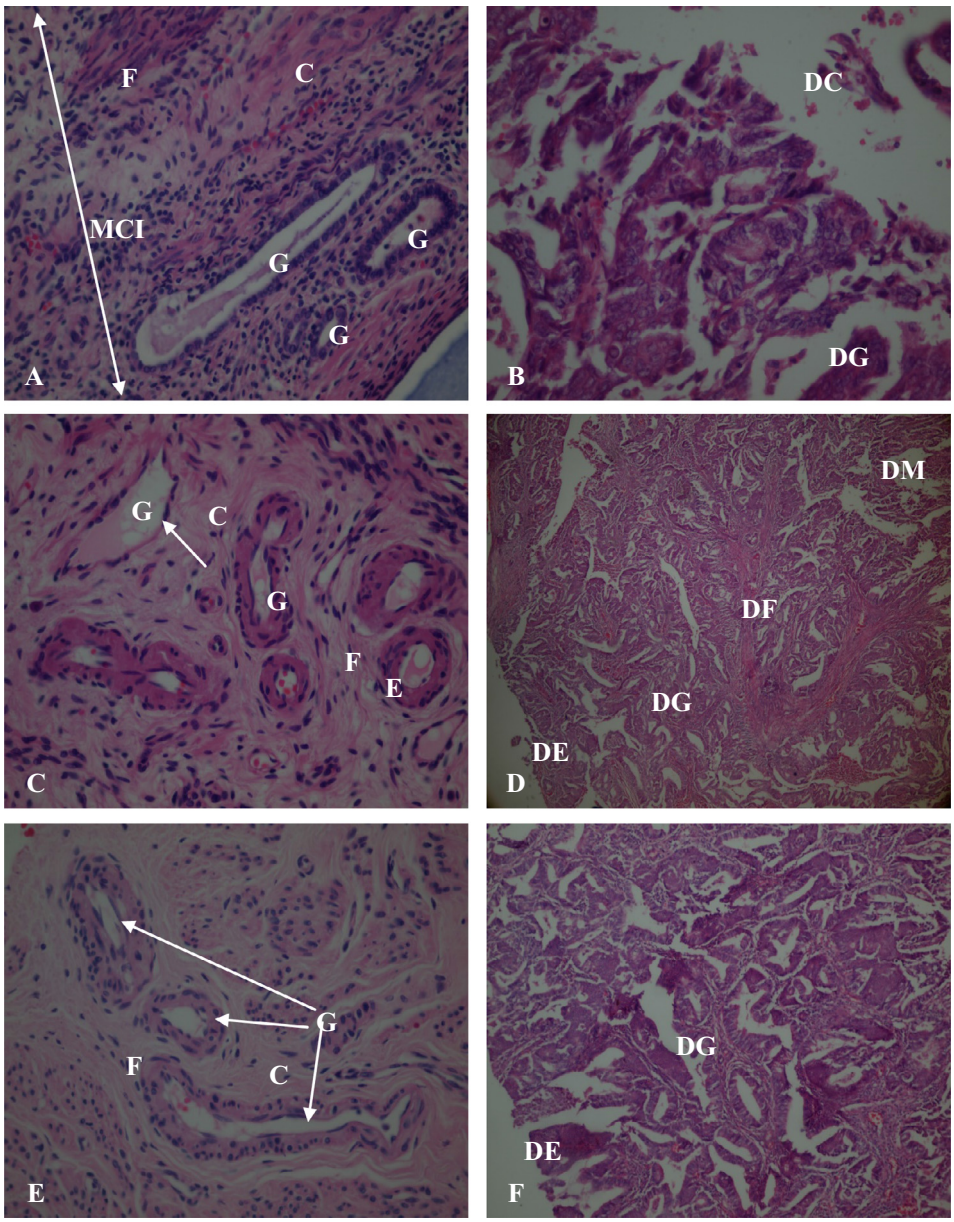

Fig. 6. Histopathological views of all fractions obtained from the MeOH extract treated groups. (A) Control group; C: regular collagen fibers, F: fibroblast cells, G: endometrial gland, MCI: monuclear cells infiltration, (B) Fr. A treated group; DC: degenerative collagen fibers, DG: degenerative endometrial gland, (C) Fr. B treated group; F: fibroblast cells, G: endometrial gland, C: collagen fibers, E: endometrial gland epithelium, (D) Fr. C treated group; DF: degenerative fuctional layer, DG: degenerative endometrial gland, DM: degenerative myometrium, DE: degenerative endometrial epithelium, (E) Fr. D treated group; F: fibroblast cells, G: endometrial gland, C: collagen fibers, (F) Buserelin acetate group; DE: degenerative endometrial epithelium, DG: degenerative endometrial gland.

for the infertility in women with endometriosis (Punnonen et al., 1996). The presence of VEGF has also been verified in human endometrium, and it may be important in both physiological and pathological angiogenesis (McLaren et al., 1996; Shifren et al., 1996). Oral contraceptives, androgenic agents, progestins, gonadotropin-releasing hormone (GnRH) analogs are used in the medical treatment of endometriosis (Waller and Shaw, 1993; Valle and Sciarra, 2003).

Collins-Burow et al. (2000) reported that some flavonoids possess antiestrogenic activity as well as estrogenic activity. The antiestrogenic activities of flavonoids such as apigenin, luteolin, kaempferide are more than their estrogenic activities (Collins-Burow et al., 2000). Furthermore, flavonoids decrease cytokine expression and secretion (Leyva-Lopez et al., 2016). Another study showed that apigenin inhibited TNF-α-induced cell proliferation and reduced the mitogenic activity and inflammatory response in endometriotic stromal cells (Suou et al., 2011). Apigenin was similarly stated to inhibit proliferation and tumorigenesis of human ovarian cancer A2780 cells *in vitro* (Li et al., 2009b) and might be a substitute compound for treating endometrial cancer and postmenopausal women (Shukla and Gupta, 2007). In the light of these informations, the flavonoids especially apigenin derivatives could be effective by regulating cytokine levels on the endometriosis.

Moreover, in this study, β-sitosterol-3-*O*-β-D-glucopyranoside was isolated from the EtOAc extract, which was the second active extract. Fraile et al. (2012) and Bouic and Lamprecht (1999) showed that plant sterols, such as β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside, have immunomodulating properties. Thus, the activity of EtOAc extract could be related to β-sitosterol-3-*O*-β-D-glucopyranoside.

At the time of clinical presentation, most women already have established endometriosis, it is hardly possible to give experimental evidence for physiological roles in the pathogenesis of this disease in humans. In addition, ethical considerations limit the performance of controlled experiments, and it is not possible to monitor the disease progression without performing repeated laparoscopies. Thus, research into the fundamental mechanisms by which menstrual endometrium adheres, invades and establishes a functional vasculature to persist in an ectopic site, as well as the development of new therapeutical approaches, is best performed in experimental animal models (Grümmer, 2006). Despite of these advantages of animal models, they have some limitations such as small sample size, the applicability to humans.

5. Conclusions

In this study, the effects of *A. austriaca* flowers were investigated in the rat endometriosis model. According to the biological activity results, the EtOAc and MeOH extracts of the plant showed statistically significant activity. However, the activity of the MeOH extract was higher than that of the EtOAc extract. Phytochemical studies led to the isolation of 5 flavonoids and 1 lactone from the MeOH extract and β-sitosterol-3-*O*-β-D-glucopyranoside from the EtOAc extract. Therefore, according to the results of the present study, flavonoids and sterols have been detected as the possible compounds responsible for the activity. Moreover, there is only one publication about the phytochemical ingredient of *A. austriaca* in the literature (Staneva et al., 2004). So, the presence of isolated compounds from *A. austriaca* in the plant has been reported first time in the present study.

Acknowledgements

This study was supported by 2214/A International Research Fellowship Program, provided by "The Scientific and Technological Research Council of Turkey (TUBITAK)" and the Scientific Research Project of Gazi University, Grant No.: 02/2017-21.

Authors' contributions

MI: Isolation studies and Biological Activity Studies.
ZA: Isolation Studies and Structure Elucidation.
IAK: Isolation Studies and Structure Elucidation.
HT: Histopathological analysis.
EKA: Biological Activity Studies

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