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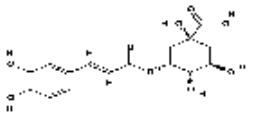

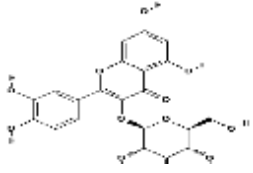

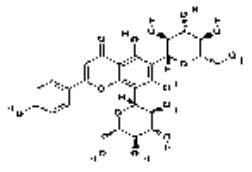

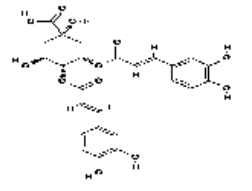

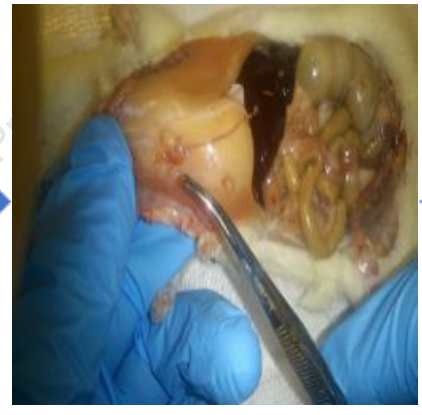

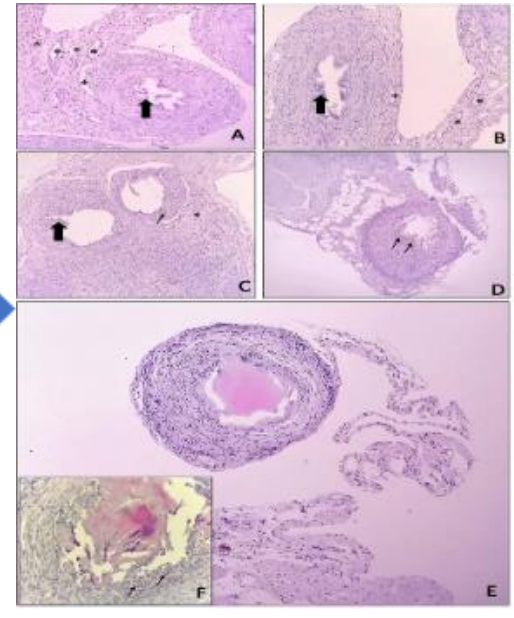

Phytochemical profiling and ameliorative effects of *Achillea cretica* L. on rat model of endometriosis

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Running Title: *Achillea cretica* for endometriosis

Authors' contribution

FB contributed in conducting the study, analyzing data, and drafting manuscript. MD, CS, and SMB contributed in phytochemical analysis. MB contributed in biochemical analysis. MA and MT contributed in revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. RR contributed in the conception of the work, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work.

Abstract

Ethnopharmacological relevance: *Achillea cretica* (*AC*) is a medicinal plant emphasized for treatment of gynecological disorders and pathological symptoms similar to endometriosis in traditional Persian medicine. Since information about its chemical constituents is limited, the aim of this study is to investigate phenolic composition of *AC* extract as well as its effect on experimental model of endometriosis. **Materials and methods**: RP-HPLC-PDA-ESI-MS/MS analysis was used for the determination of polyphenolic compounds. Endometriosis was induced in rats by suturing of uterus segments to abdominal wall of same rat, after eight weeks when the model was induced, it was followed by 28 days of treatment with 100, 200, and 400 mg/kg/day of hydroethanolic extract of the plant. Blood samples and implanted tissues were collected in the final day, and area of foci, tumor necrosis factor-α, vascular endothelial growth factor, interleukin-6, and serum total thiol molecules were measured and compared with positive group (0.2 mg/kg/day letrozole) and control group (solvent of extract: normal saline). Implanted tissue sections of the sacrificed rats were also assessed histopathologically. **Results**: Nine polyphenolic compounds were identified in *AC* extract including 7 flavonoids and 2 phenolic acids. Plant extract decreased area of foci and cytokine levels in serum and local tissue. Histopathological assessments confirmed the effectiveness of treatments by decreasing the thickness of epithelial layer and increasing the infiltration of leukocytes into this layer. Doses of 100 mg/kg and 400 mg/kg of extract showed better effects in comparison with the dose of 200 mg/kg in reduction of cytokine levels and size of implanted tissue. Extract and letrozole did not demonstrate significant effect on thiol level. **Conclusion**: *AC* aerial extract may be a favorable medicine for management of endometriosis by modulating inflammatory cytokines; however, further studies are needed for more conclusive and reliable decision about its efficacy and safety.

Keywords: endometriosis; *Achillea cretica*; chemical composition; LC/MS; inflammatory cytokine; histopathology; oxidative stress

List of Abbreviations: *AC*: *Achillea cretica*, ESI: Electrospray ionization, GAE: Gallic acid Equivalents, H&E: hematoxylin and eosin, IL: interleukin, HPLC: High Performance Liquid Chromatography, LC/MS: Liquid Chromatography/Mass Spectrophotometry, LCQ: Liquid Chromatography Quadrupole, PBS: Phosphate-buffered saline, PDA: photodiode array detector,

QE: quercetin equivalents, SD: standard deviation, TNF-α: tumor necrosis factor-α, VEGF: vascular endothelial growth factor

Introduction

Achillea cretica L. (syn: *Achillea santolina* Lindl.) is one of the 130 flowering and perennial species in the genus *Achillea* from the family Asteraceae (Compositae). This plant is distributed in Europe and Mediterranean countries such as Iraq, Jordan, Turkey, Pakistan, Egypt. It also grows in most parts of Iran. It`s perennials reach heights of 40 to 60 centimeters. The leaves are linear, pinnatifid and petiolate. This plant produces umbels of white many-stellate flowers from April to May (Mohammadhosseini et al. 2017; Saeidnia et al. 2011).

Various phytochemical constituents have been identified in *AC*, the major ones are essential oils and polyphenolic compounds. The main polyphenolic compounds are santoflavone, artemetin, α-santonin, β- sitosterol, lupeol and leukodin (Al-Snafi, 2013). The major components of it`s essential oil are 1, 8-cineole, fragranol, fragranyl acetate and terpinen-4-ol, caryophyllene oxide, cis-nerolidol, camphor, and linalool (El-Shazly et al. 2004; Ghani et al. 2008). Current studies have demonstrated antioxidant and free radical scavenging activity (Al-Snafi, 2016; Ardestani and Yazdanparast, 2007), antipyretic, and analgesic effects of *AC* (Zaringhalam et al. 2010).

Achillea species have been vastly used in traditional medicine of different regions (Mohammadhosseini et al. 2017). The most commonly used *Achillea* species in Iran are *A. cretica* and *A. millefolium* which are used traditionally for the treatment of urogenital and respiratory disorders and have wound healing properties (Aghili, 2008). In Europe, *A. millefolium* was used to treat various complaints including gastrointestinal, urinary, respiratory, and dermatological disorders (Lin et al., 2002). In Russian Pharmacopeia, *A. millefolium* has been recorded as hemostatic and anti-inflammatory agent (Shikov et al., 2014). In Turkey, *A. aleppica* and *A. bibersteinii* have been used as diuretic and wound healing agent and useful for treatment of gastrointestinal disorders including abdominal pains and hemorrhoids (Sezik et al., 2001). *A. wilhelmsii* has been used traditionally for treatment of gastrointestinal and pulmonary complaints in Italy and Turkey (Ma□ei et al., 1989, Cakilcioglu et al., 2011). *A. atrata* has been claimed to be effective in respiratory tract disorders in Serbia (Ristic et al., 2004). Moreover, various species of *Achillea* has been known as powerful anti-inflammatory and analgesic remedies and are used as emmenagogue, regulator of menstrual cycle, and menopausal

symptoms in folk and traditional medicine of different countries (Mohammadhosseini et al. 2017). In traditional Persian medicine, it has been claimed to have anti-inflammatory and emmenagogue properties and used for regulation of menstrual cycle and treatment of gynecological disorders and pathological symptoms similar to endometriosis (Aghili, 2008). In Unani medicine of India, *A. millefolium* is used due to its anti-inflammatory, emmenagogue, antipyretic, diuretic, and analgesic properties (Sayed and Bano, 2018). *Achillea* spp. are also well-known medicinal plants in traditional Chinese medicine due to anti-allergic, antibacterial, and anti-inflammatory properties (Yang et al., 2004).

Endometriosis, with a prevalence of 10% among women of reproductive age (Eskenazi and Warner, 1997), is an estrogen-dependent, inflammatory gynecological disorder (Zhao et al. 2015) recognized with the presence of endometrial tissue outside the uterus (Bérubé et al. 1998; Do Amaral et al. 2009), causing pelvic pain and infertility (Demirel et al. 2014), dysmenorrhea and cyclic urinary or bowel complaints (Zhao et al. 2015; Radhika et al. 2016). Conventional treatments for endometriosis include pharmacological interventions and surgery but these treatments are associated with different side effects and are not completely effective (Zhao et al. 2015). Therefore, introducing and developing new pharmacological interventions for management of this disease is necessary and medicinal plants are a precious source for this purpose (Mobli et al. 2015). In previous studies, various herbal medicines like *Uncaria tomentosa* (Nogueira Neto et al. 2011a), *Coccinia cordifolia* (Jha et al. 2010), *Viburnum opulus* (Saltan et al. 2016), *Vitex negundo* (Amuthan et al. 2015), *Copaifera langsdorffii* (Nogueira Neto et al. 2011b), *Salvia miltiorrhiza* (Zhou et al. 2012), *Alchemilla mollis* and *Alchemilla persica* (Küpeli Akkol et al. 2015), *Euterpe oleracea* (Machado et al. 2016), *A. biebersteinii* (Demirel et al. 2014) demonstrated promising effects on reducing inflammatory cytokines and lesions area in experimental rat model.

The aim of this study was to identify polyphenolic compounds of *AC* by LC/MS analysis and evaluate its effect on biochemical and histopathological features of experimental endometriosis.

Materials and methods

Plant material and extraction

The plant was purchased from a known market and was authenticated by botanist as *Achillea cretica* L. (voucher number: PMP-346). Powdered aerial parts (100 g) were consecutively extracted with 500 ml ethanol (70% v/v) at room temperature for 48 h and repeated three times. The extract was concentrated by rotary evaporator at 50°C temperature (Heidolph Rotary Evaporator, Germany).

Total Phenolic and total Flavonoid assay

The total phenolic content was calculated by the Folin-Ciocalteu method and was expressed as mg of Gallic acid Equivalents (GAE) (Singleton et al. 1999). Total flavonoid content was determined by the aluminium chloride colorimetric test and was expressed as mg of quercetin equivalents (QE) (Zhishen et al. 1999).

LC/MS analysis of AC extract

LC-Pak™Millex device (Millipore Corporation, Billerica, MA, USA) was used for the preparation of HPLC-grade water. LC/MS grade methanol and formic acid were purchased from Sigma Aldrich, Milano, Italy. The extract was solubilized in water/ methanol 50:50 v/v acidified with 0.1% formic acid to prepare the concentration of 1 mg/ml of the extract. Before of chromatographic analyses, the samples were filtrated with 0,45 and 0,22 µm RC-membrane (Minisart RC 4). All chromatographic analysis was carried out using a Thermo Finnigan Surveyor Plus HPLC apparatus consisting of a quaternary pump, a Surveyor UV–Vis photodiode array detector (PDA), and LCQ Advantage max ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), coupled through an ESI source.

The extract compounds were separated with a Kinetex® XB RP18 100 A column (150 × 4.6 mm; 5 um) equipped with analogous guard column, from Phenomenex, California, USA. The samples were analyzed under a gradient elution of 0.1% formic acid in water as phase "A" and methanol as phase "B". The elution was performed using the following condition: 0-84 min: 10%-70% phase B, 84-89 min: 70%-80 % B, 89-94 min: 80%-100% B, 94-99 min: 100 % B, 99-104 min:100 %-10 % B, 104-110 min: 10% B. The column and sample tray temperature were set at 25 and 4 °C, respectively. The flow rate was maintained at 0,3 mL/min and the sample injection volume was 5µL. Chromatograms were detected at λ 254, 280, 330 nm and all peaks were spectrophotometrically analyzed at 200–800 nm.

Using Xcalibur software, HPLC-ESI-MS/MS analyzes were performed in negative ionization modes. The ion trap was operated in data-dependent, zoom scan, full scan (m/z 100−1000), and MSn mode.

Caffeine and gallic acid, both with the concentration of 10 μg/mL were individually dissolved in 0.1% formic acid in water/ methanol (50:50 v/v) to optimize ESI source parameters with flow injection analysis at a flow rate of 25 μL/min into the MS device. The best conditions were obtained with capillary temperature 220 °C, sheath gas 60, auxiliary gas 25 and 20, capillary voltage -26.13 V and 35 V, spray voltage 4.5 and 5 kV.

Animals

Fifty-six Wistar rats (weighing 200-250 g) were maintained in polysulfone cages under a 12h light–12h dark cycle and environmentally controlled conditions of 22±2°C and the experiment was approved by the Ethics Committee of the TUMS with code number IR.TUMS.VCR.REC.1396.2221. Among 56 rats, 20 (ten of each sex) were considered for the toxicity study and 36 female rats were kept for experimental model of endometriosis.

Acute toxicity

Acute toxicity of *A. cretica* was assessed prior to the pharmacological evaluations. For this purpose, total of 20 Wistar rats (10 of each sex) were weighted and were divided into two groups of ten (each containing five male and five female animals). The animals were administered with a single oral dose of 5,000 mg/kg of the dried extract or distilled water and then, were closely monitored for the first 24 h for any sign of toxicity, continued by a daily evaluation for a two-week period. At the end of the study, the rats were sacrificed and animal body weight, as well as the relative weight of organs including heart, lungs, liver, spleen, and kidneys were compared with the control group (Bahramsoltani et al, 2019).

Induction of endometriosis

Among 36 female rats, 6 were randomly selected as the sham group. The other 30 rats were used to establish the model of endometriosis. Induction of endometriosis was performed by surgical method described by Vernon and Wilson (1985). For each rat in the sham group, a suture without any tissue transplantation was conducted. Sham group was used to assess aseptic condition

during the experiment. Eight weeks after the surgery, a second operation was performed to check whether the disease is successfully established.

Interventions

Four days after the second surgery, thirty rats were randomly divided into five groups containing six rats in each group. The interventions include normal saline (control group) and three different concentrations of the extract (100, 200 and 400 mg/kg) and letrozole 0.2 mg/kg (positive control) and sham group were applied once a day by gavage throughout four weeks. All rats were sacrificed at the end of the procedure. Endometriotic foci area was measured by coulis instrument and compared with each other.

Histopathological investigation

The animals were euthanized 28-day post treatment and the implanted tissue were fixed in the 10% formalin for 48 h and then, processed and embedded in paraffin and the sections were stained with hematoxylin and eosin. The histological slides were evaluated using light microscopy. Any changes that confirm endometriosis were assessed in different samples. Moreover, the beneficial effects of different treatment groups on remission of endometriosis were reported. The persistence of endometrial epithelial layer in peritoneal implants was evaluated semiquantitatively based on a previous study (Keenan et al. 1999).

Determination of TNF-α, VEGF, IL-6 levels

At the end of treatment period, implanted tissue and serum samples were collected to determine tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and interleukin-6 (IL-6) levels in rats. PBS solution was added to tissue samples and homogenized with homogenizer (hielscher ultrasound technology, Germany) and supernatants used for further assessments. TNF-α, IL-6 and VEGF in the serum and implanted tissue were measured by applying enzyme linked immunosorbent assay kits [TNF-α (Diaclone F-25020 Besancon cedex-France), IL-6 and VEGF (ZellBio, Germany)] according to the manufacturer's instructions.

Determination of local antioxidant activity

Total thiol concentration in implanted tissue was calculated by the methods originally defined by Ellman (Ellman GL, 1959) and modified by Hu (HU ML, 1994). The result was expressed in mmol/L (Costa et al. 2006).

Statistical analysis

ANOVA and Tukey's post-hoc test in SPSS 15 software (SPSS Inc., Chicago, IL) were used to analyze data. Values were expressed as the mean ± standard deviation (SD) and for all comparisons, $P<0.05$ was considered as statistically significant difference.

Results

Chemical characterization of AC extract

Total phenolic and flavonoid contents of each gram of extract were equivalent to 65±0.37 mg of gallic acid and 103.78±4.68 mg of quercetin, respectively.

In addition, *AC* extract was submitted to HPLC-ESI-MS/MS analysis (Figure 1). Nine compounds were identified in the extract (Table 1) including seven flavonoids (quercetin, four apigenin derivatives, naringenin and isorhamnetin derivative), and two phenolic acids (two hydroxycinnamic acid derivatives) (Table 1).

Peak 1 with a molecular ion at *m/z* 327, was associated as 5-O-caffeoylquinic acid for the presence of a marker fragment at *m/z* 191. Peak 2 and 3 presented parent ion at *m/z* 563 were assigned as apigenin-C-pentoside-C-glucoside and apigenin-C-glucoside-C-pentoside. These two apigenins produced a fragmentation pattern with *m/z* at 383 and 353 which correspond to the apigenin aglycone linked with residue of sugar [M-H-113]-(apigenin) and [M-H-83]-(apigenin), respectively. The complete loss of glucosyl and pentosyl did not form relevant fragments to identification of compounds. Peak 4 with molecular ion at *m/z* 579, correspond to kaempferol 3-O-pentosyl hexoside, that form commonly fragments at *m/z* 429 and 447. Peak 5 was proposed to be vincentin-2 with parent ion *m/z* 593 and characteristic fragment ions at *m/z* 575, 505, 473, 383. The MS spectra of the peak 6 presented a molecular ion at 431 *m/z* could be assigned as 7-O-β-glycosyl apigenin. The MS2 showed aglycone fragment at *m/z* 269 [M-H – 162]- (apigenin), after the loss of one glycosyl unit. Peak 7 was attributed to quercetin 3-O-glucopyranoside. It has parent ion at *m/z* 463 and quercetin aglycone fragment ion at *m/z* 301. Peak 8 was identified to as

4,5-O- dicaffeoylquinic acid on basis to MS2 spectra and fragmentation reported by Clifford et al. 2005 and Carbonara et al. 2012

Peak 9 with parent ion at *m/z* 447 releasing ion fragments at *m/z* 315 [M-H]- 162 and *m/z* 299 [Aglycone – 15] at was detected as 3-O- β-glucosyl isorhamnetin.

Acute toxicity study

No death occurred during the acute toxicity study. No sign of toxicity was detected during the follow-up period. Also, there were no significant differences in the body weight of the animals, as well as the relative weight of internal organs between extract-treated animals in comparison to the control groups (data not shown).

Size of implanted tissue

The mean area foci of the test materials showed reduction of size of implanted tissue after 4 weeks of treatment and these foci were significantly decreased in the letrozole group and test groups compared with control group. No significant difference was seen between test groups and letrozole group in the foci area (Figure 2).

Histopathological study

Table 2 shows the mean score of the histopathological evaluation of the implants at the end of the treatment. The score was markedly lower in the letrozole and *AC* 400 treated animals compared to the control ($p<0.01$). There was no statistically significant difference between the *AC* 400 and letrozole groups. Although the histopathologic scores were lower in *AC* 200 in comparison with control group, the difference was not significant. The micrographs of the histopathological scores of endometriotic implants are shown in Figure 3. The epithelial layer was fully preserved in control; however, thickness of this layer was significantly diminished in letrozole and *AC* 400 treated animals. On the other hand, the infiltration of leukocytes into epithelial layer was considerably increased in letrozole and *AC* 400 treated animals.

Biochemical investigations

Figures 4, 5 and 6 demonstrate the levels of TNF-α, VEGF and IL-6 in the serum and implanted tissues.

As demonstrated in Figure 4, the levels of TNF-α in serum of test groups and letrozole group were markedly lower than those of the control group ($p<0.005$). The levels of TNF-α in the serum of all groups were higher than those of sham group. Differences between sham and *AC* 200 ($p=0.0005$) and also sham and *AC* 400 ($p=0.002$) were significant, but differences between sham and letrozole, and sham and *AC* 100 were not significant.

The levels of TNF-α in the implanted tissues of all groups were lower than those of control group. Differences between control group and letrozole group ($p=0.004$) and also control and *AC* 100 ($p=0.0001$) were significant; however, differences between control and *AC* 200 and also *AC* 400 were not significant.

As shown in Figure 5, the levels of VEGF in serum of test groups and letrozole group were significantly lower than those of the control group ($p<0.05$). The levels of VEGF in the serum of all groups were higher than those of sham group. Differences between sham group and *AC* 200 ($p=0.003$) and also sham and *AC* 400 ($p=0.001$) were significant, but differences between sham and letrozole and sham and *AC* 100 were not significant.

There was a significant difference between control group and letrozole group ($p=0.04$) and also control and *AC* 100 ($p=0.001$) in the level of VEGF; however, differences between control and *AC* 200 and also control and *AC* 400 were not significant.

As shown in Figure 6, the levels of IL-6 in the serum of test groups and letrozole group were significantly lower than those of the control group ($p<0.01$). Differences between sham group and *AC* 200 ($p=0.005$) and also sham and *AC* 400 ($p=0.03$) were significant, but differences between sham and letrozole and sham and *AC* 100 were not significant.

The levels of IL-6 in the implanted tissues of all groups except *AC* 200 were significantly lower than those of control group ($p<0.005$). The level of IL-6 in the implanted tissues of *AC* 100 was lower than letrozole group; however, the difference was not significant ($p=0.83$). About *AC* 200 and *AC* 400, both showed significantly higher level of IL-6 compared to letrozole (Figure 6). As shown in Figure 7, the levels of thiol in all groups were higher than control group, but the difference was not statistically significant in any of the groups compared to control. Moreover, there was no statistical difference between any of the *AC* groups and letrozole.

Discussion

Phytochemical investigation of *AC* revealed nine phytochemicals including 7 flavonoids and 2 phenolic acids. This is the first study reporting these phenolic compounds from *AC*. Ahmad et al. (1995) isolated two methoxylated flavones from *AC* including 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone and 7-hydroxy-3,6,3′,4-tetramethoxyflavone. Santoflavone and artemetin are other phenolic compounds reported from this species (Al-Snafi, 2013). However, the phenolic compounds identified in this study have been previously reported in some other species of the genus *Achillea*. Dall'Acqua et al. detected quercetin-3-O-glucoside, apigenin and caffeoyl quinic acid derivatives in *A. millefolium* (Dall'Acqua et al., 2011). LC/MS analysis of three *Achillea* species identified apigenin, naringenin, quercetin and quinic acid in all three species (Agar et al., 2015). Dicaffeoyl quinic acid and quercetin were among polyphenolic compounds detected in *A. multifida* (Taşkın, 2016).

The available treatments for endometriosis are based on drugs, surgery or a combination of both. Because of unfortunate results from conventional endometriosis treatments and recurrence of disease after surgery, a growing interest in complementary and alternative medicines is observed for the management of endometriosis (Bina et al. 2019). The inflammatory process of endometriosis is mainly associated with elevated levels of inflammatory cytokines including IL-1, IL-6, IL-8, IL-18 and TNF-α (Demirel et al. 2014; Dziunycz et al. 2009). Angiogenesis, the growth of blood vessels from the existing vasculature, plays a crucial role in ectopic implantation of endometrial tissue and in the development of endometric lesions. Angiogenesis implies interactions of several molecules, one of the most important of them is VEGF (Baranov et al., 2015). There are conflicting results about the role of VEGF in endometriosis. Acimovic et al. (2016) demonstrated a significantly higher value of mRNA expression for VEGF in patients with endometriosis in comparison with those without endometriosis; however, Pellicier et al. (1998) reported no significant difference in VEGF levels in the serum of affected patients compared to the control group. In the present study, hydroalcoholic extract of *AC* could reduce implanted tissue size and IL-6, TNF-α, and VEGF levels after treatment period. Different concentrations of extract reduced the size of implanted tissue similar to letrozole. Dose of 100 mg/kg and then, 400 mg/kg of extract showed better effect in reduction of cytokine levels in comparison with the dose 200 mg/kg. It should be considered that the animal model is not necesserily matched with human and offer some drawbacks like difference in biokinetics parameters or extrapolation of results to human; though, it is more reliable than *in vitro* evaluations (Saeidnia et al. 2015). On the other

hand, oxidative stress with initiating several mechanisms such as release of many inflammatory cytokines promot the disease (Saeidnia and Abdollahi, 2013). Moreover, reactive oxygen species seems to play a crucial role in the pathogenesis of endometriosis (Jackson et al. 2005; Van Langendonckt et al. 2002). In the present study, antioxidant effect of extract in animal model was assessed with total thiol method as a non-enzymatic marker of oxidation, showing similar effect to that of control and letrozole groups. So, the extract could not affect the antioxidant level at investigated doses and duration. A higher dose of the extract and longer period of treatment time (more than 4 weeks) may result is a significant improvement of antioxidant activity which should be considered for future studies. In previous studies, *Triticum aestivum* juice (Yi et al. 2011) and polysaccharide from the rhizoma of *Dioscorea opposite* (Ju et al. 2014) exhibited antioxidant activity and were effective in women with endometriosis. Antioxidant activities of hydroalcoholic extract of *AC* were explored through *in vitro* models (Ardestani and Yazdanparast, 2007). Previous studies have revealed pharmacological activities of some phytochemicals of *Achillea* species that are beneficial in endometriosis. For example, lupeol (Sánchez-Burgos et al. 2015; Geetha and Varalakshmi, 2001; Santiago and R Mayor, 2014) and β-sitosterol (Valerio and Awad, 2011) have demonstrated anti-inflammatory and antioxidant activities; apigenin has been described to have antiproliferative, anti-inflammatory, and anti-angiogenic activities (Park et al. 2017); kaempferol, naringenin and apigenin have been recognized as botanical progestins (phytoprogestins) which are capable of activating progesterone receptor signaling (Toh et al. 2012); so beneficial effects of *AC* on endometriosis may be attributed to the above-mentioned chemical constituents.

As this is a preliminary study on the effects of *Achillea cretica* in the animal model of endometriosis, the popular markers assessed in the diseases including VEGF, IL-6, and TNF-α (Ilhan et al., 2019) were considered to evaluate the effectiveness of the plant. There are several other biomarkers, histopathological techniques, and hormonal evaluations, as well as gene and protein expression assessments which can be used in *in vitro* or *in vivo* models of endometriosis in order to identify the mechanism of this plant in the treatment of endometriosis. Also, bioassay-guided fractionation of the extract can further clarify the main pharmacologically active molecule(s) of the extract which can lead to preparation of enriched herbal supplements with a higher concentration of the active ingredients.

Conclusion

The extract from aerial part of *AC* may be a favorable medicine for the management of endometriosis by modulating inflammatory cytokines and oxidative stress; however, *in vitro* and preclinical mechanistic studies, as well as clinical trials are needed to confirm the safety and efficacy of this plant as an alternative or adjuvant therapy in patients with endometriosis.

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Conflicts of interest

There are no conflicts of interest.

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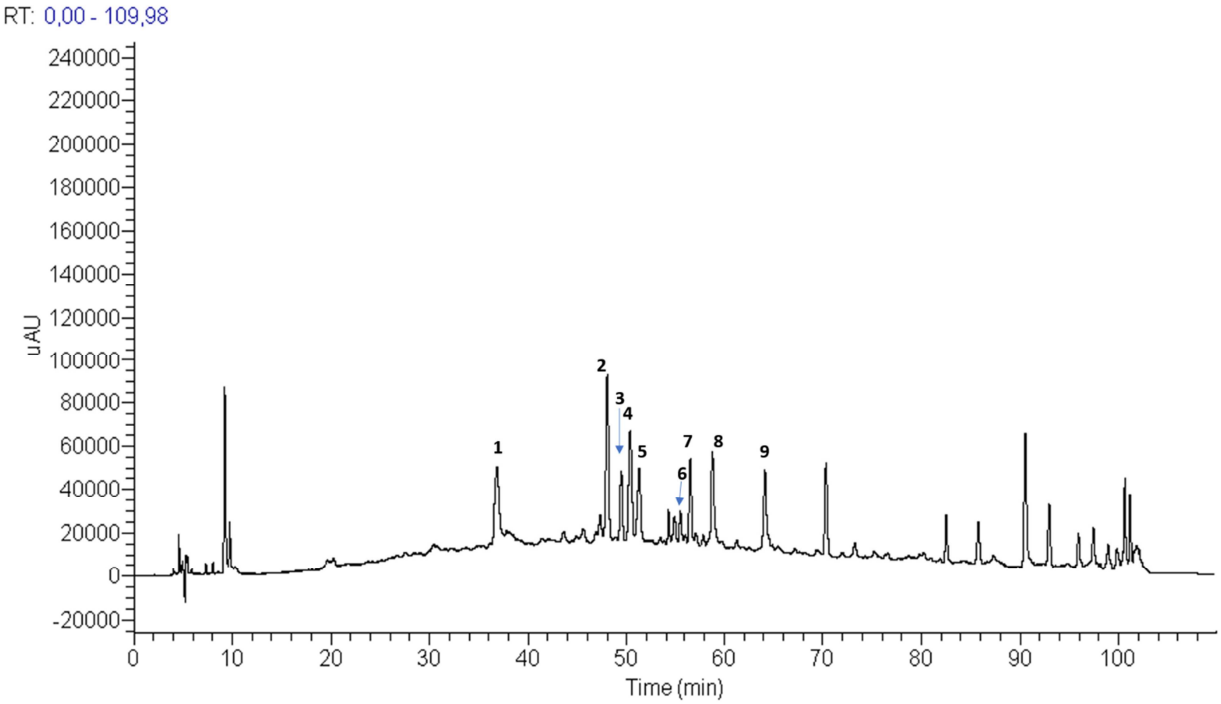

Figure 1. LC/MS chromatogram of *Achillea cretica* extract

Table 1. LC/MS fragmentations of *Achillea cretica* extract

Peak number	RT	UV	[M-H]-	[M-H]- FRAM	Compounds	References
1	36.84	327	353	191 (100); 179 (4) 135 (2)	5-O-Caffeoylquinic acid (Neochlorogenic acid)	Vitalini et al., 2016
2	48.03	271; 334	563	473 (40); 443 (30); 383 (20); 353 (10)	Apigenin-C-pentoside-C-glucoside	Younesi et al., 2016
3	49.56	271; 338	563	473 (20); 443 (30); 383 (2); 353 (>2)	Apigenin--C-glucoside-C-pentoside	Younesi et al., 2016
4	50.37	270; 348	579	447 (100); 429 (2);	Kaempferol-3-O-hexosyl pentoside	Ibrahim et al., 2015
5	51.37	271; 350	593	575 (10) ;503 (30); 473 (40); 383 (20);	Apigenin-6,8-di-C-glycoside (vicenin-2)	Ibrahim et al., 2015
6	55.56	271; 289 338	431	311 (100)	7- O-beta Glucosylapigenin	Vitalini et al., 2016
7	57.89	270; 349	463	301 (20); 179 (20);	Quercetin-3-O-glucoside (Isoquercitrin)	Ibrahim et al., 2015
8	58.8	220; 328	515	353 (100); 191 (100) ;172 (80)	4,5-Di-O-caffeoylquinic acid (Isochlorogenic acid C)	Vitalini et al., 2016
9	64.14	254; 302; 351	477	315 (20); 299 (<5)	3-O-beta-Glucosyl-isorhamnetin	Vitalini et al., 2016

Table 2. Histopathological score in groups after treatment

Groups	**Score**
Negative control	3
Positive control	0.4±0.54
Achillea cretica 100	2.6±0.44
Achillea cretica 200	2.1±0.32
Achillea cretica 400	0.6±0.51

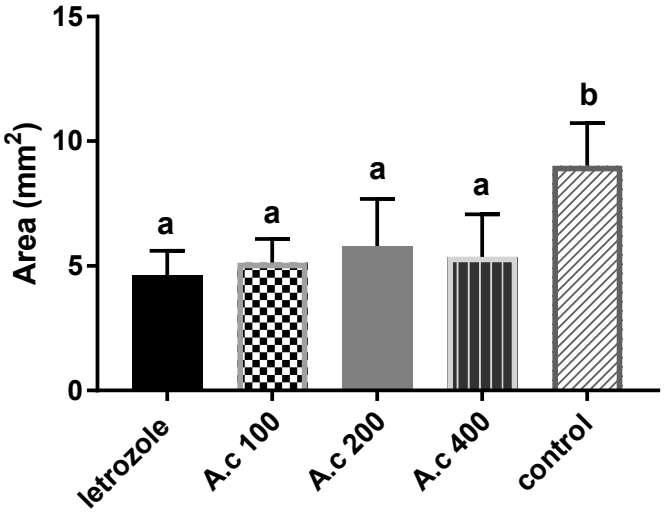

Figure 2. Area of implanted tissue after treatment with *Achillea cretica* extract. A.c 100, *Achillea cretica* at dose of 100 mg/kg/day; A.c 200, *Achillea cretica* at dose of 200 mg/kg/day; A.c 400, *Achillea cretica* at dose of 400 mg/kg/day. [a] Significantly different from the control group. [b] Significantly different from the letrozole group

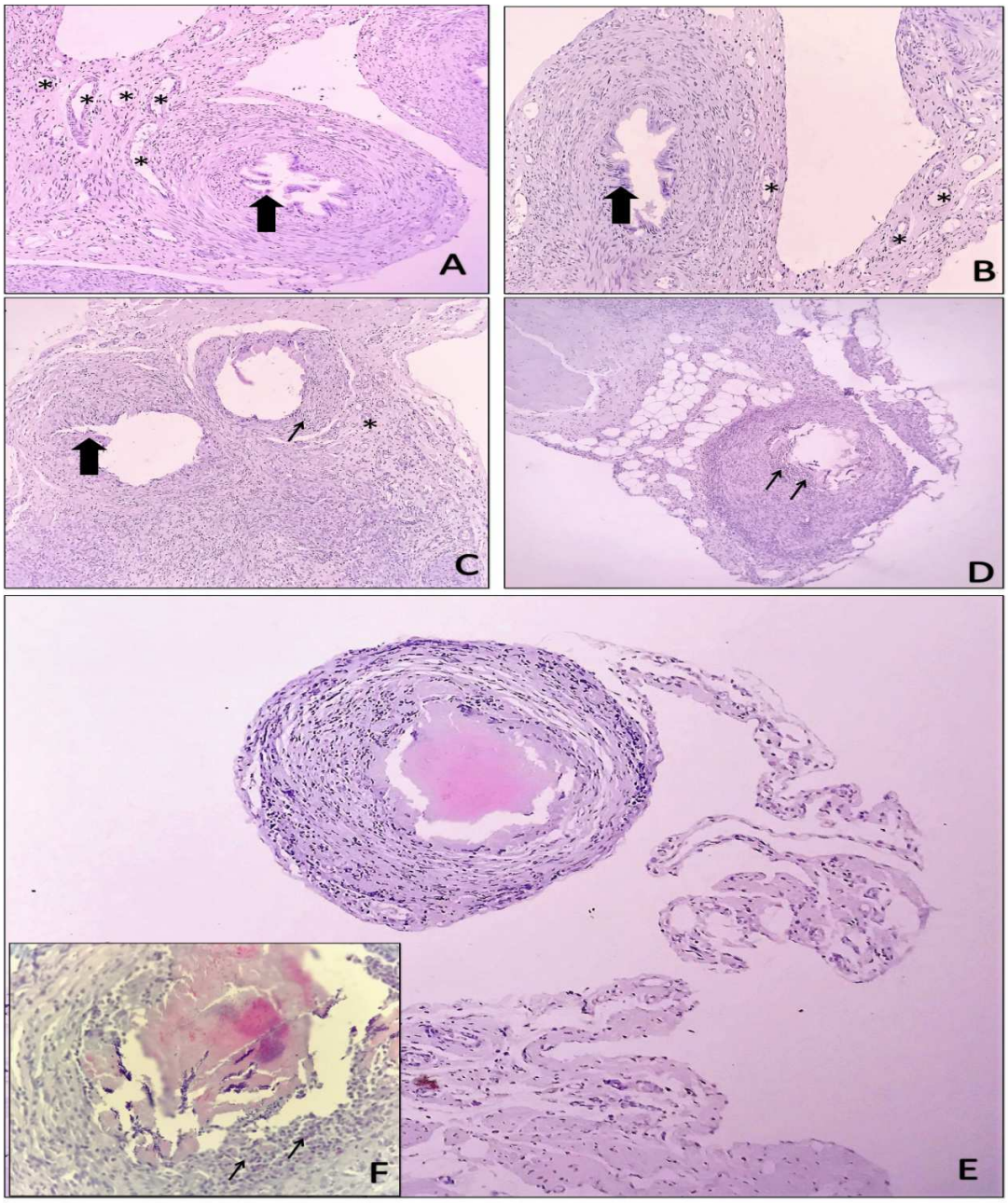

Figure 3. Histopathologic micrographs of endometrial implants, A. Negative control, B. *Achillea cretica* 100, C. *Achillea cretica* 200, D. *Achillea cretica* 400, E. Letrozole 0.2 mg/kg , F. High magnification of Letrozole group (X400). Thick arrows: epithelial layer, Thin arrows: leukocyte infiltration, asteroids: glands, H&E, X200.

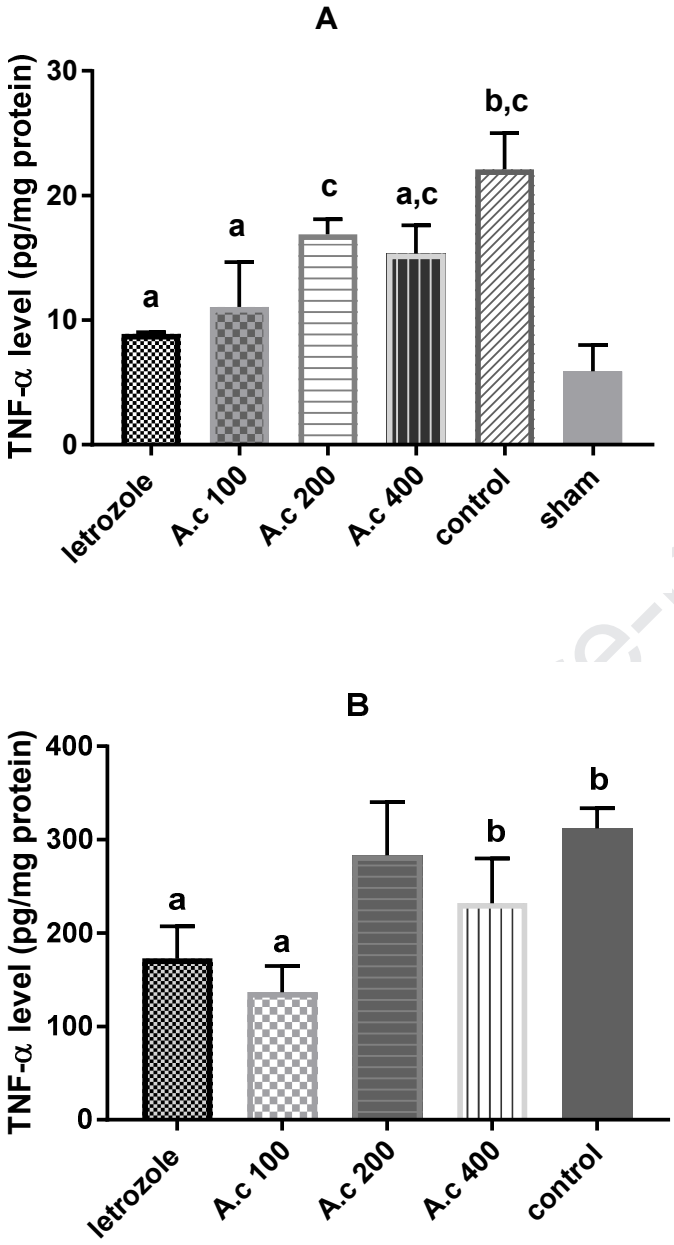

Figure 4. Tumor necrosis factor-alpha (TNF-α) levels in serum (A) and tissue (B) samples. A.c 100, *Achillea cretica* at dose of 100 mg/kg/day; A.c 200, *Achillea cretica* at dose of 200 mg/kg/day; A.c 400, *Achillea cretica* at dose of 400 mg/kg/day. [a] Significantly different from the control group. [b] Significantly different from the letrozole group. [c] Significantly different from the sham group.

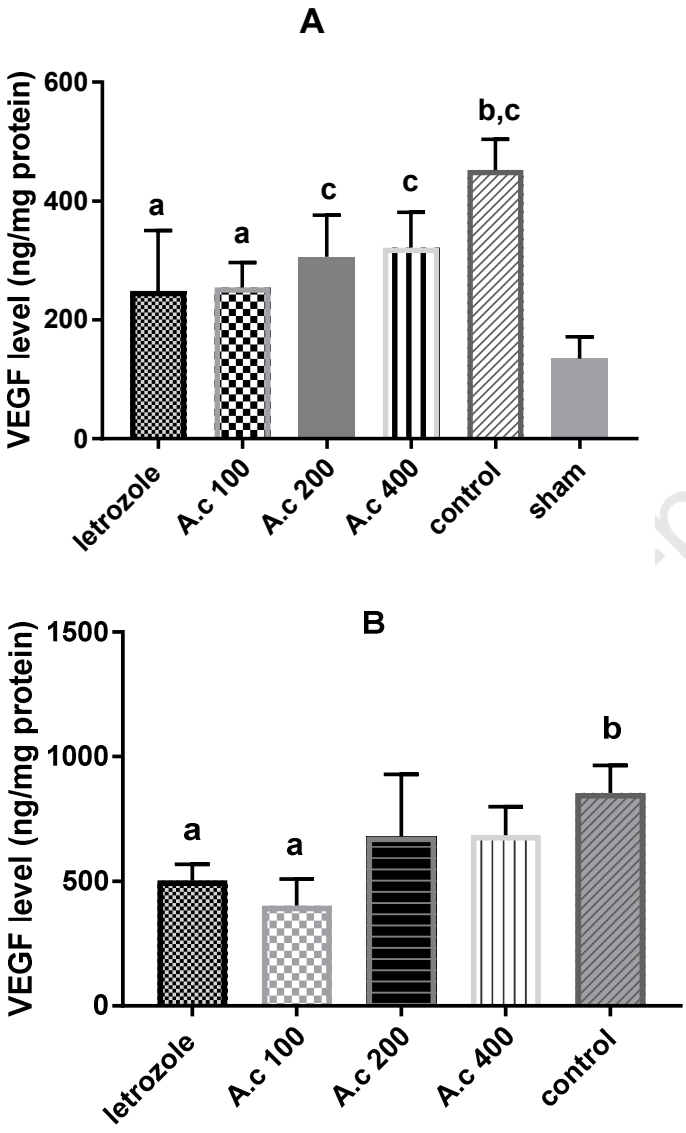

Figure 5. Vascular endothelial growth factor (VEGF) levels in serum (A) and implanted tissue (B) samples. A.c 100, *Achillea cretica* at dose of 100 mg/kg/day; A.c 200, *Achillea cretica* at dose of 200 mg/kg/day; A.c 400, *Achillea cretica* at dose of 400 mg/kg/day. [a] Significantly different from the control group. [b] Significantly different from the letrozole group. [c] Significantly different from the sham group.

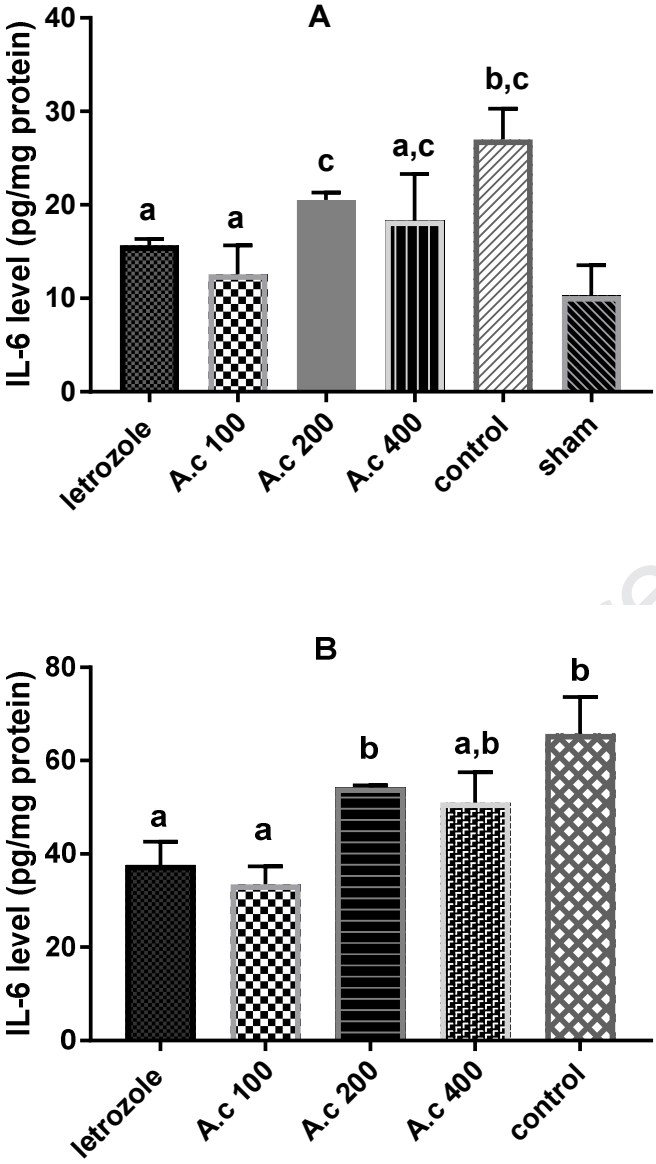

Figure 6. Interleukin-6 (IL-6) levels in serum (A) and implanted tissue (B) samples. A.c 100, *Achillea cretica* at dose of 100 mg/kg/day; A.c 200, *Achillea cretica* at dose of 200 mg/kg/day; A.c 400, *Achillea cretica* at dose of 400 mg/kg/day. [a] Significantly different from the control group. [b] Significantly different from the letrozole group. [c] Significantly different from the sham group.

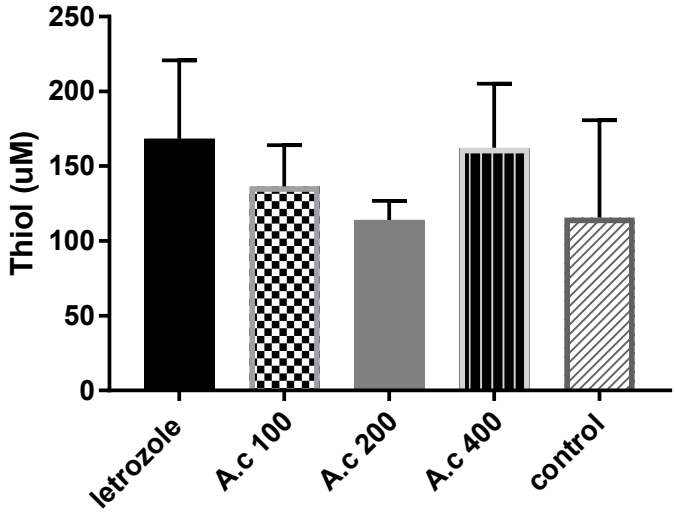

Figure 7. Antioxidant activity of letrozole, test and control group in implanted tissues. A.c 100, *Achillea cretica* at dose of 100 mg/kg/day; A.c 200, *Achillea cretica* at dose of 200 mg/kg/day; A.c 400, *Achillea cretica* at dose of 400 mg/kg/day.