3-1-2020

The regression of endometriosis with glycosylated flavonoids isolated from Melilotus officinalis (L.) Pall. in an endometriosis rat model

Mert Ilhan
Yüzüncü Yil Üniversitesi

Zulfiqar Ali
University of Mississippi School of Pharmacy

Ikhlas A. Khan
University of Mississippi School of Pharmacy

Hakkı Taştan
Gazi Üniversitesi

Esra Küpeli Akkol
Gazi Üniversitesi

Recommended Citation

Ilhan, M., Ali, Z., Khan, I. A., Taştan, H., & Küpeli Akkol, E. (2020). The regression of endometriosis with glycosylated flavonoids isolated from Melilotus officinalis (L.) Pall. in an endometriosis rat model. Taiwanese Journal of Obstetrics and Gynecology, 59(2), 211–219. https://doi.org/10.1016/j.tjog.2020.01.008

Taiwanese Journal of Obstetrics & Gynecology 59 (2020) 211–219

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

The regression of endometriosis with glycosylated flavonoids isolated from *Melilotus officinalis* (L.) Pall. in an endometriosis rat model

Mert Ilhan [a, b, c], Zulfiqar Ali [c], Ikhlas A. Khan [c], Hakkı Taştan [d], Esra Küpeli Akkol [b, *]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Van Yüzüncü Yıl University, Tuşba, 65080, Van, Turkey
[b] Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Etiler, 06330, Ankara, Turkey
[c] National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS, 38677, USA
[d] Department of Biology, Faculty of Science, Gazi University, Etiler, 06330, Ankara, Turkey

ARTICLE INFO

Article history:
Accepted 21 October 2019

Keywords:
Endometriosis
Glycosylated flavonoids
Fabaceae
Melilotus officinalis
Rat

ABSTRACT

Objective: *Melilotus officinalis* (L.) Pall. is commonly used for treating bronchitis, painful menstruation, hemorrhoids, kidney stones, ulcers of the eyes, earache, and hardening and swelling of uterus. The European Medicines Agency reported the use of *M. officinalis* orally against stomach ache, gastric ulcer, and disorders of the liver and uterus in folk medicine. The present study aimed to appraise the activity of *M.* (L.) Pall. aerial parts in endometriosis rat model.

Materials and methods: The endometriosis rat model was used to evaluate the potential activity of *M. officinalis* aerial parts based on its folkloric usage. The aerial parts of *M. officinalis* were extracted with *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH), respectively. The adhesion scores, endometrial foci areas, and cytokine levels were measured in all treated groups. After the biological activity studies, phytochemical studies were performed on the active extract and the fractions obtained from the active extract.

Results: The MeOH extract significantly decreased the endometrial foci areas and cytokine levels in rats with endometriosis. Fractionation was performed on the MeOH extract to achieve bioactive molecules. Following the fractionation, the fractions obtained from the MeOH extract were tested. Fraction C showed the highest activity in the rat endometriosis model. Phytochemical investigation of the active fraction (Fraction C) resulted in isolation and elucidation of some quercetin and kaempferol glucoside derivatives.

Conclusion: Fraction C obtained from the MeOH extract of *M. officinalis* showed the highest activity, yielding four glycosylated flavonoids.

Introduction

Melilotus officinalis (L.) Pall. (Fabaceae) is native in Europe and Asia and known as “yellow melilot, yellow sweet clover, and medicinal sweet clover” [1,2]. *M. officinalis* is commonly used for treating hemorrhoids, bronchitis, kidney stones, painful menstruation, earache, ulcers of the eyes, and hardening and swelling of uterus [3,4]. The European Medicines Agency reported the traditional use of *M. officinalis* orally against stomach ache, gastric ulcer, and complaints of liver and uterus [5].

Biological activity studies demonstrated the antioxidant, anti-inflammatory, and antiproliferative effects of *M. officinalis* [6,7]. Some studies showed that this plant prevented skin aging, promoted tissue regeneration, and reduced fat deposition [8]. Previous studies about phytochemical profile of *M. officinalis* reported that *M. officinalis* contains kaempferol, quercetin, and coumarin derivatives [3–5,9].

Endometriosis is defined as the condition in which a tissue resembling the uterine mucous membrane, or endometrium, if found outside of the uterus. It can develop in the uterine ligaments, ovaries, pelvic peritoneum, rectovaginal septum, covering the sigmoid colon, uterus, rectum, tubes or bladder, umbilicus, laparotomy and episiotomy scars, tubal stumps, hernial sacs, appendix, cervix, vagina, vulva or lymph glands [10]. The incidence of endometriosis has increased in the last few decades. This increase is most probably

* Corresponding author. Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Etiler, 06330, Ankara, Turkey. Fax: +90 312 2235018.
E-mail address: esrak@gazi.edu.tr (E. Küpeli Akkol).

https://doi.org/10.1016/j.tjog.2020.01.008

due to the growing use of laparoscopy in gynecologic practice, making it easier to diagnose endometriosis. Endometriosis is detected in 5%–15% of laparotomies and laparoscopies performed on 30% of women with chronic pelvic pain and in up to 40% of infertile women [11].

The present study describes the effects of extracts/fractions obtained from the aerial parts of *M. officinalis* in a surgically-induced endometriosis rat model by evaluating adhesion scores of endometriotic implants, areas of endometriotic foci, and cytokine levels of the peritoneal fluids of rats as well as the identification of compounds in biologically active fractions.

Materials and methods

Plant material

M. officinalis aerial parts were collected from Kızılcahamam, Ankara in July 2013 and identified by Prof. Dr. Hayri Duman (Gazi University, Department of Biology, Faculty of Science and Art, Ankara). A voucher specimen has been kept in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey (GUEF3420).

Preparation of the plant extracts

The aerial parts of *M. officinalis* (400 g) were dried in the shade and extracted successively with *n*-hexane, ethyl acetate (EtOAc) and methanol (MeOH) for 48 h at room temperature (10 × 6L). Solvents were removed under reduced pressure at 40 °C to obtain the extracts. The yields of the *n*-hexane, ethyl acetate, and methanol extracts were 5, 11, 18%, respectively.

Animals

Non-pregnant six-week-old female Wistar Albino rats (weighing 200–250 g) were purchased from Kobay Experimental Animals Laboratory, Ankara, Turkey. The rats were housed according to the Guide for the Care and Use of Laboratory Animals. The experimental procedure was approved by the Kobay Experimental Animals Ethics Committee (Protocol number: 233). The rats were kept in polysulfone cages at appropriate temperature and humidity. The environment where the rats were housed was under light-controlled (12-h light/12-h dark) conditions at Kobay Animals Breeding and Experimental Research Center. All animals received water and chow *ad libitum* during the experimental period. The animals were quarantined for at least 2 weeks and the estrous cycle was controlled with daily assessment of vaginal cytology, and rats showing regular estrous cycles were used in this model.

Induction of endometriosis

The endometriosis rat model was performed according to the Vernon and Wilson method with some modifications [12]. All the rats were anesthetized with intramuscular administration of the combination of 1 mL ketamine (50 mg/mL) and 1 mL xylazine (20 mg/mL). After anesthesia, the rats were placed in supine position and routine antisepsis was conducted for disinfection. A 3-cm incision was made using a scalpel. The subcutaneous and muscle layers were separated, and the abdominal cavity was opened. The right uterine horn was removed, and a 1.5-cm piece of the tissue was trimmed using microscissors. The trimmed segment was put in 0.9% sodium chloride (normal saline) and longitudinally opened. The removed endometrial tissue fragment was sutured into the abdominal wall of the same rat using USP 4/0 polyglactin suture (Lactasorb PGLA®, Orhan Boz, Turkey). The muscle layers of the abdomen were closed using USP 3/0 polyglactin suture (Lactasorb PGLA®, Orhan Boz, Turkey). Another experiment was performed under anesthesia 28 days after the first operation (Fig. 1).

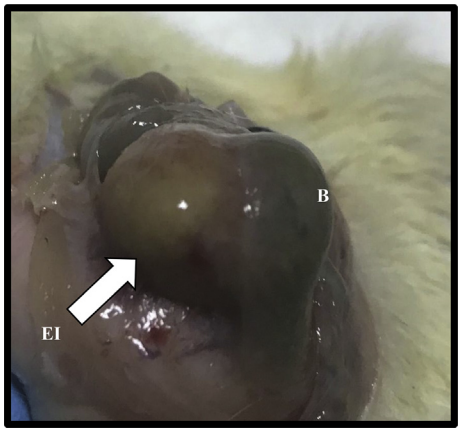

Fig. 1. Endometriotic implant view with adhesion. EI: Endometrial implant; B: Bowel.

The areas of endometrial foci were calculated by measuring their length, width, and height using a micrometer. The equation $\pi/6 \times \text{length} \times \text{width} \times \text{height}$ was used for calculating endometriotic volumes [13–15]. Furthermore, intra-abdominal adhesions were scored according to the Blauer's scoring system 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 [16]. After the all experiments, the abdomen was closed.

The administration of the extracts and fractions

Thirty rats were divided into five groups, with six rats in each group to evaluate the activity of extracts whereas thirty-six rats were randomly divided into six groups to evaluate the activity of fractions. After the second experiment, 0.5% carboxymethylcellulose (CMC) (control group), extracts of different polarity and fractions were administered orally once a day for 4 weeks. The reference group received buserelin acetate (20 mg/rat, subcutaneously) once per week. The extracts and fractions were applied to the rats at the dose of 100 mg/kg.

After giving test materials to the rats, all rats were sacrificed. The areas of endometriotic foci, intra-abdominal adhesions, and cytokine levels of the peritoneal fluids were again calculated and compared with those of the control group.

Techniques for histopathological investigation

Firstly, all endometrium tissues from the normal and 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 normal and pathological endometrium tissues were taken using Nikon Eclipse Ci with both polarizing attachment and Digital Image analysis system, which were then examined under a light microscope.

Measuring of cytokine levels

The 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) levels were quantitatively evaluated using the commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's protocol. After the scarification of animals, the peritoneal fluid was again collected, and the aforementioned process was performed. The pre- and post-treatment results were assessed.

Statistical analysis

The results were expressed as mean ± standard error of the mean (S.E.M.). The ANOVA-Dunnett's test was performed to determine the significant differences among groups using GraphPad Prism 6.0. *p* values less than 0.05 were considered significant.

Isolation procedure for the MeOH extract

The MeOH extract of the plant was subjected to silica gel column to obtain 41 fractions. The fractions were pooled to get four subfractions (A-D) after thin-layer chromatography analysis using $CHCl_3$:MeOH (9:1), $CHCl_3$:MeOH:H_2O (8:2:0.25), and EtOAc:$CHCl_3$:MeOH:H_2O (6:4:4:1) as the mobile phases. The active Fractions B and C were subjected to column chromatography over Sephadex LH-20 and RP-18 silica to obtain pure compounds. The structural elucidation of the compounds was achieved by their nuclear magnetic resonance and mass data analyses (Fig. 2).

Results

Biological activity results

The present study investigated the effects of extracts obtained from the aerial parts of *M. officinalis* in a surgically-induced endometriosis rat model by evaluating adhesion scores of endometriotic

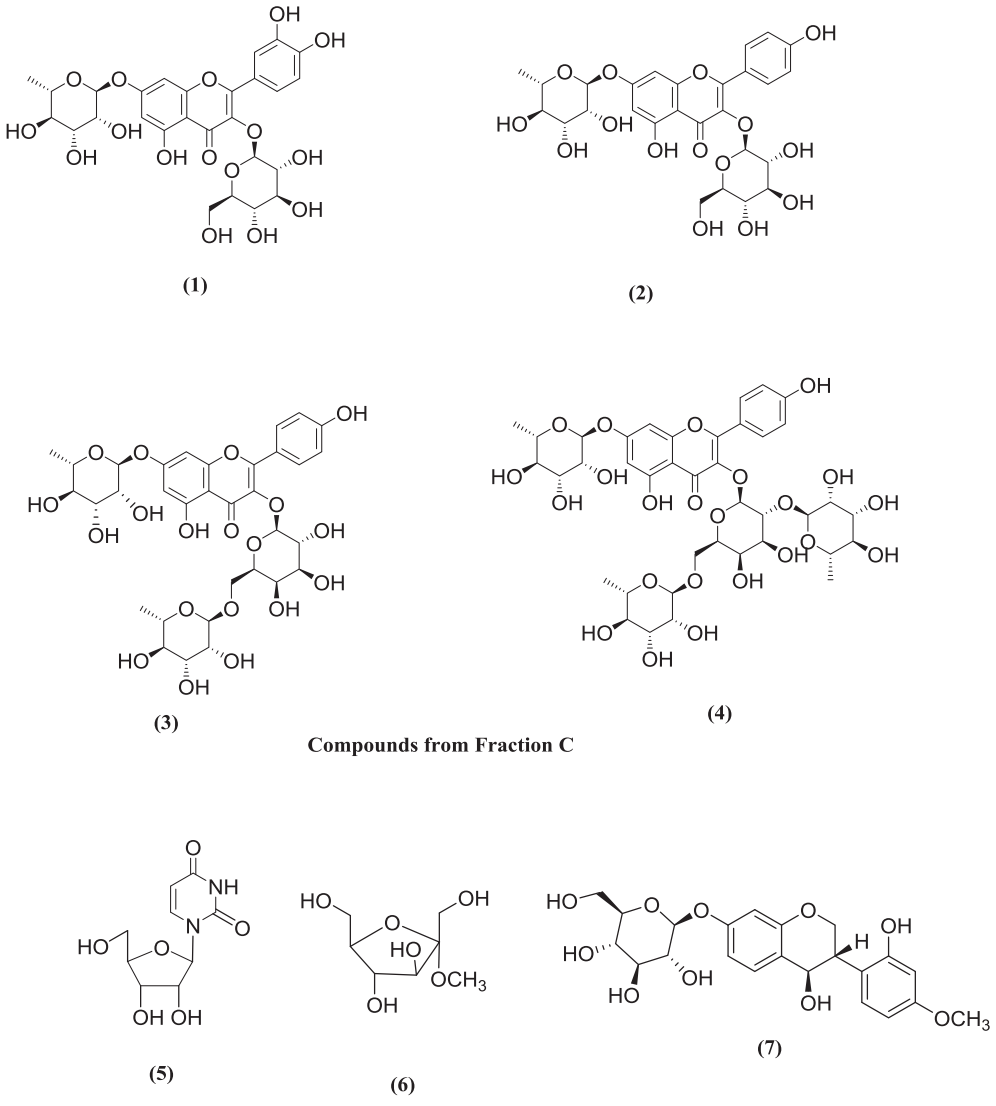

Fig. 2. Isolated compounds from active fractions of the MeOH extract of *M. officinalis*.

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

Groups	Adhesion scores	
	Pre-treatment	Post-treatment
Control	3.1 ± 1.1	3.7 ± 1.0
n-Hexane extract	3.3 ± 0.9	2.6 ± 0.8
EtOAc extract	3.2 ± 0.7	2.2 ± 0.7
MeOH extract	2.9 ± 1.1	1.8 ± 1.1
Reference	3.6 ± 0.5	**0.0 ± 0.0*****
Control	3.4 ± 0.8	3.8 ± 0.5
Fr. A	3.6 ± 0.3	2.4 ± 1.3
Fr. B	3.1 ± 0.5	**1.9 ± 0.9***
Fr. C	3.1 ± 0.6	**0.9 ± 0.1****
Fr. D	2.7 ± 1.0	2.6 ± 0.7
Reference	3.5 ± 0.7	**0.0 ± 0.0*****

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; S.E.M.: Standard Error of Mean.

Table 2
Comparison of the pre-treatment and the post-treatment endometriotic implant volumes.

Material	Volume of Endometrioma (mm^3) ± S.E.M.	
	Pre-treatment	Post-treatment
Control	98.4 ± 10.7	112.3 ± 14.2
n-Hexane extract	94.5 ± 9.6	86.9 ± 8.2
EtOAc extract	86.1 ± 12.3	71.8 ± 9.9
MeOH extract	81.9 ± 9.9	**54.4 ± 7.2****
Reference	96.2 ± 8.7	**23.5 ± 3.8*****
Control	99.1 ± 10.3	105.3 ± 9.4
Fr. A	98.3 ± 9.9	85.1 ± 8.4
Fr. B	92.6 ± 12.4	**64.9 ± 9.1***
Fr. C	96.2 ± 11.9	**48.3 ± 6.1*****
Fr. D	86.3 ± 8.5	85.7 ± 9.1
Reference	96.8 ± 9.6	**25.3 ± 4.2*****

**: $p < 0.01$; ***: $p < 0.001$; S.E.M.: Standard Error of Mean.

implants, areas of endometriotic foci, and cytokine levels of the peritoneal fluids of rats. Statistically significant difference was not found in adhesion scores among the extract-treated groups compared with the control group (Table 1).

Taking into consideration endometriotic implant volumes, the application of the MeOH extract significantly decreased endometriotic implant volumes from 81.9 to 54.4 mm^3. Nevertheless, no statistically significant difference was observed between groups treated with other extracts and the control group (Table 2).

In addition to adhesion scores and endometriotic implant volumes, the cytokine levels of the peritoneal fluids were examined. The application of MeOH extract significantly decreased TNF-α, VEGF, and IL-6 levels to 5.2, 17.4, and 42.5 pg/mL, respectively. Although the application of *n*-hexane and EtOAc extract decreased the cytokine levels, no statistical difference was noted compared with the control group (Table 3).

In addition to the MeOH extract, the reference group (buserelin acetate) also significantly reduced the adhesion scores, endometriotic implant volumes, and cytokine levels of the peritoneal fluids.

After the MeOH extract was found to display the remarkable activity in the rat endometriosis model, it was fractionated using a silica column to obtain four main fractions (Frs. A-D). The activities of the four main fractions were also tested in the rat endometriosis model. Among the fractions obtained from the MeOH extract, Fractions B and C significantly decreased adhesion scores to 1.9 and 0.9, respectively (Table 1). Furthermore, the endometriotic implant volumes decreased to 64.9 and 48.3 mm^3 in groups treated with Fractions B and C, respectively (Table 2). Fraction C also significantly decreased TNF-α, VEGF, and IL-6 levels of the peritoneal fluids, whereas Fraction B significantly decreased the only IL-6 level of the peritoneal fluids (Table 3).

Quercetin-3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**1**), kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**2**), kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside (**3**), and kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside (**4**) were isolated and identified from Fraction C, which showed higher activity compared to those of other three fractions. In addition to Fraction C, Fraction B also significantly decreased adhesion scores and endometriotic implant volumes. Uridine (**5**), methyl-α-D-fructofuranoside (**6**), and melilofficinaside (**7**) were isolated and identified from Fraction B.

According to histopathological analyses, whereas a number of endometrial glands (G) and mononuclear cell infiltration (MCI) were observed in the control groups, the decrease in G and MCI was detected in the reference, MeOH extract, Frs. B and C treated groups. The severity of the lesions was reduced in the *n*-hexane, EtOAc, MeOH, and reference groups, respectively (Fig. 3). Histopathological findings showed that the severity of the lesions was reduced in the Fr D, Fr. A, Fr. B, Fr. C, and reference groups, respectively (Fig. 4).

NMR data of isolated compounds

Quercetin-3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**1**) — ^{1}H-NMR (400 MHz, DMSO): δ 7.71 (1H, dd, $J = 8.4, 2.1$ Hz, H-6′), 7.59 (1H, d, $J = 2.1$ Hz, H-2′), 6.84 (1H, d, $J = 8.4$ Hz, H-5′), 6.79

Table 3
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	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Control	12.6 ± 4.5	9.3 ± 3.8	24.6 ± 5.9	26.2 ± 6.3	52.2 ± 9.3	54.7 ± 12.1
n-Hexane extract	9.3 ± 2.7	8.5 ± 2.4	22.5 ± 7.1	19.2 ± 7.2	51.4 ± 11.4	46.2 ± 11.4
EtOAc extract	11.2 ± 3.8	8.4 ± 2.5	26.7 ± 9.3	19.7 ± 11.0	56.3 ± 10.9	47.8 ± 9.3
MeOH extract	10.1 ± 2.5	**5.2 ± 0.9***	28.1 ± 8.2	**17.4 ± 4.3***	53.5 ± 15.8	**42.5 ± 8.1***
Reference	11.3 ± 3.4	**1.9 ± 0.4*****	21.5 ± 8.4	**9.6 ± 2.3*****	51.2 ± 10.8	**24.3 ± 7.4*****
Control	16.1 ± 4.7	13.4 ± 6.6	31.4 ± 9.4	28.7 ± 8.3	56.4 ± 10.4	59.1 ± 11.8
Fr. A	17.8 ± 9.5	16.3 ± 8.1	24.7 ± 5.9	24.5 ± 9.1	59.3 ± 10.1	55.1 ± 13.6
Fr. B	14.3 ± 8.9	13.6 ± 6.9	22.5 ± 10.5	19.8 ± 8.6	54.1 ± 7.3	**45.6 ± 8.3***
Fr. C	15.3 ± 8.2	**3.5 ± 0.9****	26.4 ± 7.5	**12.3 ± 4.6****	56.6 ± 11.4	**33.9 ± 6.3****
Fr. D	18.3 ± 7.6	19.6 ± 8.8	29.4 ± 9.6	22.5 ± 6.2	52.5 ± 13.7	49.6 ± 11.1
Reference	17.2 ± 6.1	**1.2 ± 0.8*****	27.1 ± 7.0	**8.8 ± 1.1*****	55.3 ± 9.0	**22.5 ± 6.4*****

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; S.E.M.: Standard Error of Mean.

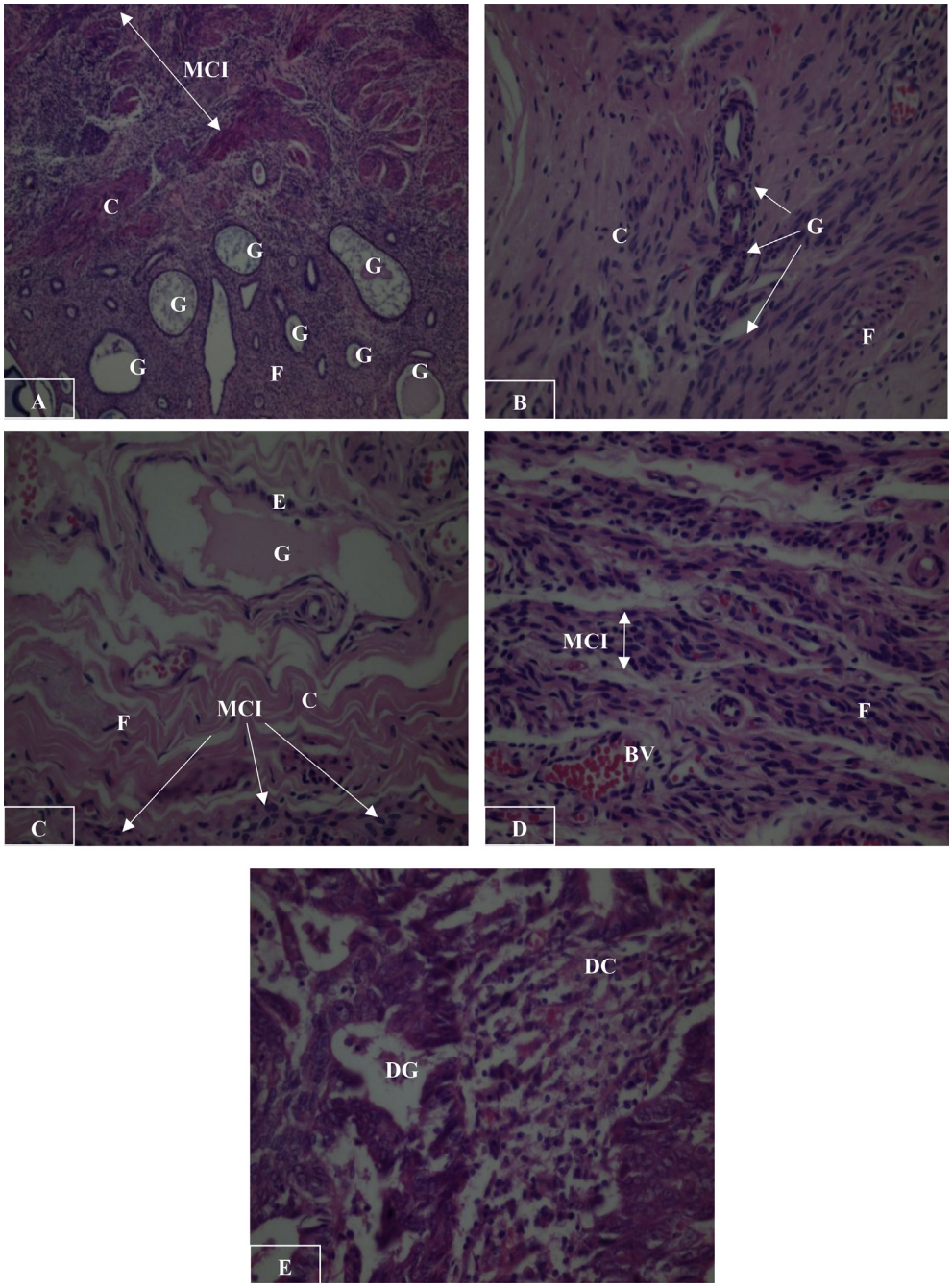

Fig. 3. Histopathological views of all extracts-treated groups. (A): Control group, Original magnification was 10×, HE (B): *n*-Hexane extract-treated group, Original magnification was 10×, HE (C): EtOAc extract-treated group, Original magnification was 40×, HE (D): MeOH extract-treated group, Original magnification was 40×, HE (E): Reference group, Original magnification was 40×, HE Arrow pointed abbreviation: G: Endometrial gland; F: Fibroblast; MCI: Mononuclear cell infiltration; C: Collagen; E: Endometrial gland epithelium; BV: Blood Vessel; DG: Degenerative endometrial glands; DC: Degenerative collagen fibers.

(1H, d, J = 2.1 Hz, H-8), 6.44 (1H, d, J = 2.1 Hz, H-6), 5.55 (1H, d, J = 2.0 Hz, H-1‴), 5.41 (1H, d, J = 7.7 Hz, H-1″), 3.85–3.02 (the protons of sugars), 1.13 (3H, d, J = 6.1 Hz, H-6‴); ^{13}C-NMR (100 MHz, DMSO) δ 177.7 (C-4), 161.6 (C-7), 160.9 (C-5), 156.8 (C-2), 155.9 (C-9), 148.7 (C-4′), 144.9 (C-3′), 133.8 (C-3), 122.1 (C-6′), 121.0 (C-1′), 116.1 (C-2′), 115.3 (C-5′), 105.6 (C-10), 101.7 (C-1″), 99.4 (C-6), 98.5 (C-1‴), 94.4 (C-8), 75.9 (C-5″), 73.2 (C-3″), 71.7 (C-2″), 71.2 (C-4‴), 70.3 (C-5‴), 70.1 (C-3‴), 69.9 (C-4″), 68.0 (C-2‴), 60.2 (C-6″), 18.0 (C-6‴). HRESIMS: m/z = 611.1606 $[M+H]^+$ (calcd. 611.1612 for $C_{27}H_{31}O_{16}$).

Kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (2) — ^{1}H-NMR (500 MHz, CD_3OD): δ 8.11 (2H, d, J = 8.7 Hz, H-2′, 6′), 6.89 (2H, d, J = 8.7 Hz, H-3′, 5′), 6.75 (1H, d, J = 2.1 Hz, H-8), 6.46 (1H, d, J = 2.1 Hz, H-6), 5.57 (1H, d, J = 1.8 Hz, H-1‴), 5.20 (1H, d, J = 7.7 Hz, H-1″), 4.02–3.45 (the protons of sugars), 1.25 (3H, d, J = 6.2 Hz, H-6‴); ^{13}C-NMR (125 MHz, CD_3OD) δ 179.8 (C-4), 163.6 (C-7), 162.8 (C-5), 161.8 (C-4′), 159.6 (C-2), 158.0 (C-9), 135.7 (C-3), 132.5 (C-2′, 6′), 122.5 (C-1′), 116.2 (C-3′, 5′), 107.3 (C-10), 104.6 (C-1″), 100.6 (C-6), 99.8 (C-1‴), 95.6 (C-8), 77.2 (C-5″), 74.9 (C-3″), 73.6 (C-2″), 73.0 (C-4‴), 72.0 (C-4″), 71.7 (C-3‴), 71.3 (C-2‴), 70.0 (C-5‴),

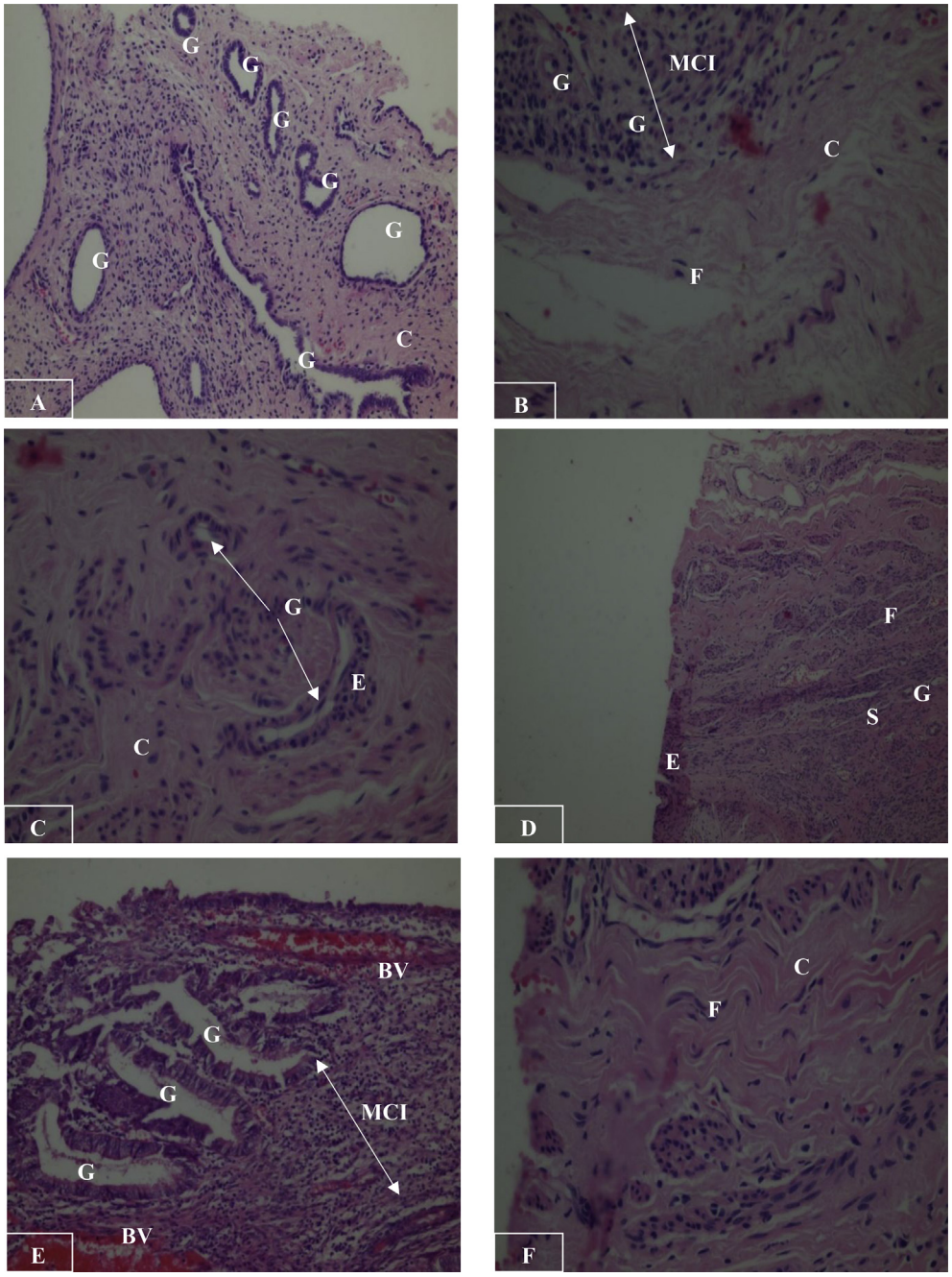

Fig. 4. Histopathological views of all fractions-treated groups. (A): Control group, Original magnification was 10×, HE (B): Fraction A-treated group, Original magnification was 40×, HE (C): Fraction B-treated group, Original magnification was 40×, HE (D): Fraction C-treated group, Original magnification was 4×, HE (E): Fraction D-treated group, Original magnification was 10×, HE (F): Reference group, Original magnification was 40×, HE Arrow pointed abbreviation: G: Endometrial gland; F: Fibroblast; MCI: Mononuclear cell infiltration; C: Collagen; E: Endometrial gland epithelium; BV: Blood Vessel; DG: Degenerative endometrial glands; DC: Degenerative collagen fibers; S: Stroma.

62.0 (C-6″), 18.1 (C-6‴). HRESIMS: m/z = 595.1657 $[M+H]^+$ (calcd. 595.1663 for $C_{27}H_{31}O_{15}$).

Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside (**3**) — ^{1}H-NMR (400 MHz, DMSO): δ 8.10 (2H, d, J = 8.8 Hz, H-2′, 6′), 6.88 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.81 (1H, d, J = 2.2 Hz, H-8), 6.45 (1H, d, J = 2.2 Hz, H-6), 5.56 (1H, d, J = 1.8 Hz, H-1‴′), 5.36 (1H, d, J = 7.7 Hz, H-1″), 4.41 (1H, d, J = 1.6 Hz, H-1‴), 3.86–3.09 (the protons of sugars), 1.13 (3H, d, J = 6.1 Hz, H-6‴′), 1.07 (3H, d, J = 6.1 Hz, H-6‴); ^{13}C-NMR (100 MHz, DMSO) δ 177.7 (C-4), 161.7 (C-7), 160.8 (C-5), 160.3 (C-4′), 157.1 (C-2), 156.1 (C-9), 133.6 (C-3), 131.1 (C-2′, 6′), 120.7 (C-1′), 115.2 (C-3′, 5′), 105.6 (C-10), 99.4 (C-6), 94.7 (C-8), 7-rha: 98.5 (C-1), 71.7 (C-4), 70.3 (C-3), 70.1 (C-2), 69.9 (C-5), 17.8 (C-6), 3-gal: 101.9 (C-1), 73.7 (C-5), 73.0 (C-3), 71.1 (C-2), 68.3 (C-4), 65.3 (C-6), Rha (C-6 of gal): 100.1 (C-1), 72.0 (C-4), 70.6 (C-3), 70.4 (C-2), 68.0 (C-5), 17.9 (C-6). HRESIMS: m/z = 741.2229 $[M+H]^+$ (calcd. 741.2242 for $C_{33}H_{41}O_{19}$).

Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside (4) — ^{1}H-NMR (500 MHz, CD_3OD): δ 8.09 (2H, d, J = 8.9 Hz, H-2′, 6′), 6.87 (2H, d, J = 8.9 Hz, H-3′, 5′), 6.73 (1H, d, J = 2.2 Hz, H-8), 6.46 (1H, d, J = 2.2 Hz, H-6), 5.60 (1H, d, J = 7.7 Hz, H-1″), 5.56 (1H,

d, J = 1.7 Hz, H-1‴″), 5.23 (1H, d, J = 1.6 Hz, H-1‴′), 4.52 (1H, d, J = 1.6 Hz, H-1‴), 4.01–3.27 (the protons of sugars), 1.26 (3H, d, J = 6.1 Hz, H-6‴′), 1.17 (3H, d, J = 6.3 Hz, H-6‴″), 0.99 (3H, d, J = 6.3 Hz, H-6‴); ^{13}C-NMR (125 MHz, CD_3OD) δ 179.5 (C-4), 163.3 (C-7), 162.9 (C-5), 162.9 (C-4′), 159.5 (C-2), 157.9 (C-9), 134.5 (C-3), 132.4 (C-2′, 6′), 121.7 (C-1′), 116.9 (C-3′, 5′), 107.4 (C-10), 100.4 (C-6), 95.6 (C-8), 7-rha: 99.9 (C-1), 73.6 (C-4), 72.1 (C-3), 71.7 (C-2), 71.2 (C-5), 18.1 (C-6), 3-gal: 100.8 (C-1), 77.5 (C-2), 75.8 (C-3), 75.4 (C-5), 70.7 (C-4), 67.1 (C-6), Rha (C-6 of gal): 101.8 (C-1), 73.9 (C-4), 72.2 (C-3), 72.1 (C-2), 69.7 (C-5), 17.9 (C-6), Rha (C-2 of gal): 102.6 (C-1), 74.0 (C-4), 72.4 (C-2), 72.3 (C-3), 69.9 (C-5), 17.6 (C-6). HRESIMS: m/z = 887.2826 $[M+H]^+$ (calcd. 887.2821 for $C_{39}H_{51}O_{23}$).

Uridine (5) — ^{1}H-NMR (400 MHz, CD_3OD): δ 8.01 (1H, d, J = 8.0 Hz, H-6), 5.90 (1H, d, J = 4.7 Hz, H-1′), 5.70 (1H, d, J = 8.0 Hz, H-5), 4.18 (1H, t, J = 4.7 Hz, H-2′), 4.15 (1H, t, J = 4.7 Hz, H-3′), 4.01 (1H, m, H-4′), 3.84 (1H, dd, J = 12.2, 2.7 Hz, H-5′b), 3.73 (1H, dd, J = 12.2, 3.2 Hz, H-5′a); ^{13}C-NMR (100 MHz, CD_3OD) δ 166.2 (C-4), 152.5 (C-2), 142.8 (C-6), 102.7 (C-5), 90.8 (C-1′), 86.4 (C-4′), 75.8 (C-2′), 71.4 (C-3′), 62.3 (C-6′). HRESIMS: m/z = 451.1613 $[M+H]^+$ (calcd. 451.1604 for $C_9H_{13}N_2O_6$).

Methyl-α-D-fructofuranoside (6) — ^{1}H-NMR (400 MHz, CD_3OD): δ 4.05 (1H, d, J = 4.1 Hz, H-3), 3.92 (1H, m, H-4), 3.87 (1H, m, H-5), 3.78 (1H, dd, J = 12.0, 1.8 Hz, H-6b), 3.74 (1H, dd, J = 12.0, 5.1 Hz, H-6a), 3.66 (1H, d, J = 11.9 Hz, H-1b), 3.65 (1H, d, J = 11.9 Hz, H-1a), 3.33 (3H, s, OCH_3); ^{13}C-NMR (100 MHz, CD_3OD) δ 109.2 (C-2), 84.6 (C-5), 82.6 (C-3), 78.9 (C-4), 62.8 (C-6), 60.5 (C-1), 48.9 (OCH_3). HRESIMS: m/z = 195.0859 $[M+H]^+$ (calcd. 195.0868 for $C_7H_{15}O_6$).

Melilofficinaside (7) — ^{1}H-NMR (500 MHz, pyridine): δ 7.55 (1H, dd, J = 8.3 Hz, 1.9 Hz, H-5), 7.21 (1H, d, J = 8.1 Hz, H-6′), 7.11 (1H, dd, J = 8.3 Hz, 2.3 Hz, H-6), 7.09 (1H, d, J = 2.3 Hz, H-8), 6.71 (1H, d, J = 2.3 Hz, H-3′), 6.59 (1H, dd, J = 8.1 Hz, 2.3 Hz, H-5′), 5.67 (1H, d, J = 7.2 Hz, H-1″), 5.58 (1H, dd, J = 7.8 Hz, 1.9 Hz, H-4), 4.28 (1H, m, H-2), 3.72 (1H, m, H-2), 3.67 (3H, s, OCH_3), 3.57 (1H, m, H-3); ^{13}C-NMR (125 MHz, pyridine) δ 162.0 (C-4′), 161.7 (C-2′), 160.2 (C-7), 157.5 (C-9), 132.9 (C-5), 125.8 (C-6′), 120.2 (C-1′), 115.1 (C-10), 111.5 (C-6), 107.0 (C-5′), 105.6 (C-8), 97.6 (C-3′), 79.2 (H-4), 67.2 (C-2), 55.8 (C-4′-OCH_3), 40.4 (C-3); sugar moiety: 102.4 (C-1″), 79.3 (C-3″), 78.8 (C-5″), 75.2 (C-2″), 71.5 (C-4″), 62.6 (C-6″). HRESIMS: m/z = 451.1613 $[M+H]^+$ (calcd. 451.1604 for $C_{22}H_{27}O_{10}$).

Discussion

M. officinalis is commonly used for the treating ulcers of the eyes, earache, bronchitis, hemorrhoids, kidney stones, and painful menstruation, swelling and hardening of uterus [3,4]. The European Medicines Agency reported the use of *M. officinalis* orally against stomach ache, gastric ulcer, and disorders of liver and uterus in folk medicine [5].

Previous studies reported that *M. officinalis* has some biological activities such as the antioxidant, antiproliferative and anti-inflammatory effects [6,7]. Pastorino et al. also showed that *M. officinalis* promoted tissue regeneration, prevented skin aging, and reduced fat deposition [8].

Endometriosis is a disease defined by the availability and growth of endometrial tissue outside the uterus, especially into the peritoneum. Some biological changes, such as local inflammatory–reparative phenomena and involvement of peripheral blood mononuclear cells, are observed in the peritoneal fluid of women with endometriosis [17]. Pizzo et al. (2002) reported that the peritoneal fluid levels of TNF-α were extremely high in early stage [18]. The other study demonstrated that the levels of cytokines including VEGF, IL-6, IL-1β and TNF-α were higher in women with endometriosis, indicating their role in the pathogenesis of endometriosis [19]. However, no significant difference was observed in the serum levels of IL-5, IL-7, and IL-12 between women with endometriosis and healthy women [20]. Koyama et al. (1993) found that the levels of IL-5 and IL-6, but not IL-1, were related to endometriosis [21]. Li et al. (2017) showed that disorders involving IL-6 and IL-6 receptors were correlated with the etiology of endometriosis. An increase in the IL-6 soluble receptor in the peritoneal fluid promoted the development of endometriosis by enhancing the bioactivity of IL-6 [22]. In the light of these informations, the cytokine levels in the peritoneal fluids were evaluated in the present study. According to the results, the cytokine levels decreased in the MeOH extract–treated and reference groups.

Sex steroids such as progesterone and estrogen are essentially produced in the ovaries and these type hormones cause the growth of endometrial tissue, basically by stimulating and inhibiting cell proliferation. Furthermore, estrogen plays a significant role in the regulation of cyclic gonadotropin release and in folliculogenesis [23]. 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 [24]. Furthermore, flavonoids decrease the expression and secretion of cytokines [25]. Another study showed that apigenin inhibited TNF-α-induced cell proliferation and reduced the mitogenic activity and inflammatory response in endometriotic stromal cells [26]. Apigenin was similarly stated to inhibit the proliferation and tumorigenesis of human ovarian cancer A2780 cells *in vitro* and might serve as a substitute compound for treating endometrial cancer in postmenopausal women [27].

Endometriotic lesions are qualified by a deep vascularization that occurs through angiogenesis process [28–31]. VEGF which is one of the most potent angiogenic factors is postulated to be involved in the progress of the ectopic lesions in endometriosis [32,33]. Vascularization and VEGF and its receptor expression are mainly high in deeply infiltrating endometriosis. Those situations supported that the antiangiogenic therapy contribute to the regression of endometriosis. Kim (2003) searched the anti-angiogenic potential of flavonoids. Their results reported that the flavonoids displayed antiangiogenic effect preventing VEGF/basic fibroblast growth factor-induced matrix metalloproteinase (MMP)-1 and the activation of pro-MMP-2 [34]. Another study conducted by Wu et al. (2012) showed that the flavonoid extract including some quercetin and kaempferol derivatives were active in inhibiting expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule induced by TNF-α in human umbilical vein endothelial cells [35]. Previous studies reported that quercetin showed antiangiogenic effect by decreasing the phosphorylation and mRNA expression of VEGF receptor (VEGFR)-2, the expression of cyclooxygenase (COX)-2 and the secretion of MMP-2 and MMP-9 [36–40]. Kaempferol also displayed antiangiogenic effect by reducing VEGF secretion, VEGF mRNA and protein expression, MMP-2 and MMP-9 activity [41–43]. Another studies which is about the antiangiogenic effects of flavonoids exhibited that apigenin inhibited Smad2/3 and Src/FAK/AKT pathways, IL-6/STAT3 pathway, mRNA and protein expression of IL-6, IL-8 and decreased MMP-2 and -9 activities [44–46].

In the present study, Fraction C obtained from the MeOH extract showed the highest activity in the rat endometriosis model, and four glycosylated flavonoids were isolated from this fraction. Based on the findings of previous studies and the present study, it was suggested that the MeOH extract of the aerial parts of *M. officinalis* could be used to treat endometriosis due to its glycosylated flavonoids.

Conclusions

This novel study described the role of *M. officinalis* against endometriosis. The present study proved the traditional use of the aerial parts of *M. officinalis* in endometriosis. Four flavonoid glycosides (**1**–**4**) were isolated as the main components of the active fraction, which might be responsible for the activity of plant extract in the endometriosis rat model. In addition, compounds **5**–**7** were isolated from Fraction B which showed moderate activity. Therefore, the effect of the extract could be attributed to glycosylated flavonoids. Furthermore, the authors think that the action mechanism of glycosylated flavonoids is due to their antiangiogenic properties. In further studies, we are planning to conduct the dose-effect studies in the rat endometriosis model.

Funding/support statement

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

Mert Ilhan: Isolation, Biological activity studies (eczmertilhan@gmail.com)

Zulfiqar Ali: Isolation studies, Structure elucidation of isolated compounds (zulfiqar@olemiss.edu).

Ikhlas A Khan: Isolation studies, Structure elucidation of isolated compounds (khan@olemiss.edu).

Hakkı Taştan: Histopathological studies (hakkitastan@gazi.edu.tr).

Esra Küpeli Akkol: Study design, Biological activity studies (esrak@gazi.edu.tr).

Declaration of Competing Interest

The authors have no conflicts of interest relevant to this article.

References

[1] Chamberlain DF. *Melilotus* L. In: Davis PH, editor. Flora of Turkey and the east Aegean Islands. Edinburgh: University of Edinburgh; 1970. p. 448–52.
[2] Udayama M, Kinjo J, Yoshida N, Nohara T. A new oleanene glucuronide having a branched-chain sugar from *Melilotus officinalis*. Chem Pharm Bull 1998;46(3):526–7.
[3] Hamid A, Raina AK. Ethnobotanical uses of plants in and around Kanji wildlife sanctuary, North West Himalaya. Int J Sci Res 2014;3(11):538–45.
[4] Kang SS, Lim CH, Lee SY. Soyasapogenols B and E from *Melilotus officinalis*. Arch Pharm Res (Seoul) 1987;10(1):9–13.
[5] EMEA, European Medicines Agency. Committee on herbal medicinal products (HMPC). *Melilotus officin*alis (L.) Lam. London, UK: herba; 2008.
[6] Miliauskas G, Venskutonis PR, van Beek TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem 2004;85(2):231–7.
[7] Trouillas P, Calliste CA, Allais DP, Simon A, Marfak A, Delage C, et al. Antioxidant, anti-inflammatory and antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. Food Chem 2003;80(3):399–407.
[8] Pastorino G, Marchetti C, Borghesi B, Cornara L, Ribulla S, Burlando B. Biological activities of the legume crops *Melilotus officinalis* and *Lespedeza capitata* for skin care and pharmaceutical applications. Ind Crops Prod 2017;96:158–64.
[9] Ilhan M, Ali Z, Khan IA, Kupeli Akkol E. A new isoflavane-4-ol derivative from *Melilotus officinalis* (L.) Pall. Nat Prod Res 2019;33(13):1856–61.
[10] Daniel JC, Boyce PKW. Surgical induction of endometriosis. In: Daniel JC, editor. Methods in mammalian reproduction. New York: Academic Press; 1978. p. 545–55.
[11] Bedaiwy MA, Falcone T. Endometriosis and adenomyosis. In: Sokol AI, Sokol ER, editors. General gynecology. Philadelphia, PA, USA: Mosby; 2007. p. 321–45.
[12] Vernon MW, Wilson EA. Studies on the surgical induction of endometriosis in the rat. Fertil Steril 1985;44(5):684–94.
[13] Altintas D, Kokcu A, Tosun M, Cetinkaya MB, Kandemir B. Comparison of the effects of cetrorelix, a GnRH antagonist, and leuprolide, a GnRH agonist, on experimental endometriosis. J Obstet Gynaecol Res 2008;34(6):1014–9.
[14] Demirel MA, Suntar I, Ilhan M, Keles H, Kupeli Akkol E. Experimental endometriosis remission in rats treated with *Achillea biebersteinii* Afan.: histopathological evaluation and determination of cytokine levels. Eur J Obstet Gynecol Reprod Biol 2014;175:172–7.
[15] Kupeli Akkol E, Demirel MA, Bahadir Acikara O, Suntar I, Ergene B, Ilhan M, et al. Phytochemical analyses and effects of *Alchemilla mollis* (Buser) Rothm. and *Alchemilla persica* Rothm. in rat endometriosis model. Arch Gynecol Obstet 2015;292(3):619–28.
[16] Blauer KL, Collins RL. The effect of intraperitoneal progesterone on postoperative adhesion formation in rabbits. Fertil Steril 1988;49(1):144–9.
[17] Arici A, Oral E, Attar E, Tazuke SI, Olive DL. Monocyte chemotactic protein-1 concentration in peritoneal fluid of women with endometriosis and its modulation of expression in mesothelial cells. Fertil Steril 1997;67(6):1065–72.
[18] Pizzo A, Salmeri FM, Ardita FV, Sofo V, Tripepi M, Marsico S. Behaviour of cytokine levels in serum and peritoneal fluid of women with endometriosis. Gynecol Obstet Investig 2002;54(2):82–7.
[19] Markowska J, Kowalska M, Gogacz M, Lubin J, Markowski M, Kaminska J. Cytokines and endometriosis. Clin Exp Obstet Gynecol 2004;31(4):269–70.
[20] Malutan AM, Drugan T, Costin N, Ciortea R, Bucuri C, Rada MP, et al. Pro-inflammatory cytokines for evaluation of inflammatory status in endometriosis. Cent Eur J Immunol 2015;40(1):96–102.
[21] Koyama N, Matsuura K, Okamura H. Cytokines in the peritoneal fluid of patients with endometriosis. Int J Gynaecol Obstet 1993;43(1):45–50.
[22] Li S, Fu X, Wu T, Yang L, Hu C, Wu R. Role of interleukin-6 and its receptor in endometriosis. Med Sci Monit 2017;23:3801–7.
[23] Parente Barbosa C, Bentes De Souza AM, Bianco B, Christofolini DM. The effect of hormones on endometriosis development. Minerva Ginecol 2011;63(4):375–86.
[24] Collins-Burow BM, Burow ME, Duong BN, McLachlan JA. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and-independent mechanisms. Nutr Cancer 2000;38(2):229–44.
[25] Leyva-Lopez N, Gutierrez-Grijalva EP, Ambriz-Perez DL, Heredia JB. Flavonoids as cytokine modulators: a possible therapy for inflammation-related diseases. Int J Mol Sci 2016;17(6):921.
[26] Suou K, Taniguchi F, Tagashira Y, Kiyama T, Terakawa N, Harada T. Apigenin inhibits tumor necrosis factor alpha-induced cell proliferation and prostaglandin E2 synthesis by inactivating NFkappaB in endometriotic stromal cells. Fertil Steril 2011;95(4):1518–21.
[27] Li ZD, Hu XW, Wang YT, Fang J. Apigenin inhibits proliferation of ovarian cancer A2780 cells through Id1. FEBS Lett 2009;583(12):1999–2003.
[28] Ceyhan ST, Onguru O, Baser I, Gunhan O. Expression of cyclooxygenase-2 and vascular endothelial growth factor in ovarian endometriotic cysts and their relationship with angiogenesis. Fertil Steril 2008;90(4):988–93.
[29] Clinical practice Giudice LC. Endometriosis. N Engl J Med 2010;362(25):2389–98.
[30] Laschke MW, Giebels C, Menger MD. Vasculogenesis: a new piece of the endometriosis puzzle. Hum Reprod Update 2011;17(5):628–36.
[31] McLaren J. Vascular endothelial growth factor and endometriotic angiogenesis. Hum Reprod Update 2000;6(1):45–55.
[32] Laschke MW, Elitzsch A, Vollmar B, Vajkoczy P, Menger MD. Combined inhibition of vascular endothelial growth factor (VEGF), fibroblast growth factor and platelet-derived growth factor, but not inhibition of VEGF alone, effectively suppresses angiogenesis and vessel maturation in endometriotic lesions. Hum Reprod 2005;21(1):262–8.
[33] Laschke MW, Menger MD. *In vitro* and *in vivo* approaches to study angiogenesis in the pathophysiology and therapy of endometriosis. Hum Reprod Update 2007;13(4):331–42.
[34] Kim MH. Flavonoids inhibit VEGF/bFGF-induced angiogenesis *in vitro* by inhibiting the matrix-degrading proteases. J Cell Biochem 2003;89(3):529–38.
[35] Wu WB, Hung DK, Chang FW, Ong ET, Chen BH. Anti-inflammatory and anti-angiogenic effects of flavonoids isolated from *Lycium barbarum* Linnaeus on human umbilical vein endothelial cells. Food Funct 2012;3(10):1068–81.
[36] Pratheeshkumar P, Budhraja A, Son YO, Wang X, Zhang Z, Ding S, et al. Quercetin inhibits angiogenesis mediated human prostate tumor growth by targeting VEGFR-2 regulated AKT/mTOR/P70S6K signaling pathways. PLoS One 2012;7:e47516.
[37] Maurya AK, Vinayak M. Quercetin attenuates cell survival, inflammation, and angiogenesis via modulation of AKT signaling in murine T-Cell lymphoma. Nutr Cancer 2017;69:470–80.
[38] Pilatova M, Stupakova V, Varinska L, Sarissky M, Mirossay L, Mirossay A, et al. Effect of selected flavones on cancer and endothelial cells. Gen Physiol Biophys 2010;29:134–43.
[39] Scoditti E, Calabriso N, Massaro M, Pellegrino M, Storelli C, Martines G, et al. Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: a potentially protective mechanism in atherosclerotic vascular disease and cancer. Arch Biochem Biophys 2012;527:81–9.
[40] Lai WW, Hsu SC, Chueh FS, Chen YY, Yang JS, Lin JP, et al. Quercetin inhibits migration and invasion of SAS human oral cancer cells through inhibition of

NF-κB and matrix metalloproteinase-2/-9 signaling pathways. Anticancer Res 2013;33:1941–50.
[41] Luo H, Rankin GO, Juliano N, Jiang BH, Chen YC. Kaempferol inhibits VEGF expression and *in vitro* angiogenesis through a novel ERK-NFkappaB-cMyc-p21 pathway. Food Chem 2012;130:321–8.
[42] Luo H, Rankin GO, Liu L, Daddysman MK, Jiang BH, Chen YC. Kaempferol inhibits angiogenesis and VEGF expression through both HIF dependent and independent pathways in human ovarian cancer cells. Nutr Cancer 2009;61:554–63.
[43] Li C, Zhao Y, Yang D, Yu Y, Guo H, Zhao Z, et al. Inhibitory effects of kaempferol on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-9. Biochem Cell Biol 2015;93:16–27.
[44] Lamy S, Akla N, Ouanouki A, Lord-Dufour S, Beliveau R. Diet-derived polyphenols inhibit angiogenesis by modulating the interleukin-6/STAT3 pathway. Exp Cell Res 2012;318:1586–96.
[45] Wang J, Liao Y, Fan J, Ye T, Sun X, Dong S. Apigenin inhibits the expression of IL-6, IL-8, and ICAM-1 in DEHP-stimulated human umbilical vein endothelial cells and *in vivo*. Inflammation 2012;35:1466–76.
[46] Mirossay L, Varinská L, Mojžiš J. Antiangiogenic effect of flavonoids and chalcones: an Update. Int J Mol Sci 2018;19:e27.