Original Article

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

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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.

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**Introduction**

*Melilotus officinalis* (L.) Pall. (Fabaceae) is native in Europe and Asia and known as “yellow mellilot, 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...
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 Kızılcabaham, 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 muscular layers were separated, and the abdominal cavity was opened. The right uterine horn was removed, and a 1.5-cm piece of the tissue thickness were made from 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.

**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.

**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.

**Fig. 1.** Endometriotic implant view with adhesion. EI: Endometrial implant; B: Bowel.
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 CHCl₃:MeOH (9:1), CHCl₃:MeOH:H₂O (8:2:0.25), and EtOAc:CHCl₃:MeOH:H₂O (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.
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 (Fr. 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 mm³ 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 melillocin acids (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)**, 1H-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 1

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume of Endometrioma (mm³) ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98.4 ± 10.7</td>
</tr>
<tr>
<td>n-Hexane extract</td>
<td>94.5 ± 9.6</td>
</tr>
<tr>
<td>EtOAc extract</td>
<td>86.1 ± 12.3</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>81.9 ± 9.9</td>
</tr>
<tr>
<td>Reference</td>
<td>96.2 ± 8.7</td>
</tr>
<tr>
<td>Control</td>
<td>99.1 ± 10.3</td>
</tr>
<tr>
<td>Fr. A</td>
<td>98.3 ± 9.9</td>
</tr>
<tr>
<td>Fr. B</td>
<td>92.6 ± 12.4</td>
</tr>
<tr>
<td>Fr. C</td>
<td>96.2 ± 11.9</td>
</tr>
<tr>
<td>Fr. D</td>
<td>86.3 ± 8.5</td>
</tr>
<tr>
<td>Reference</td>
<td>96.8 ± 9.6</td>
</tr>
</tbody>
</table>

*: 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.

<table>
<thead>
<tr>
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<tr>
<td>Reference</td>
<td>96.8 ± 9.6</td>
</tr>
</tbody>
</table>

**: p < 0.01; ***: p < 0.001; S.E.M.: Standard Error of Mean.
(1H, d, J = 2.1 Hz, H-8), 6.44 (1H, d, J = 2.0 Hz, H-1”), 5.55 (1H, d, J = 2.1 Hz, H-6), 5.41 (1H, d, J = 7.7 Hz, H-1”), 3.85 e 3.02 (the protons of sugars), 1.13 (3H, d, J = 6.1 Hz, H-6”); 13C-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 C27H31O16).

**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.
62.0 (C-6”), 18.1 (C-6”). HRESIMS: m/z = 595.1657 [M+H]+ (calcd. 595.1663 for C27H31O15).

Kaempferol 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyl-7-O-α-L-rhamnopyranoside (3) — 1H-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”);
13C-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 C33H41O19).

Kaempferol 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-7-O-α-L-rhamnopyranoside (4) — 1H-NMR (500 MHz, CD3OD): 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, 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”);
13C-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’),
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 growth of endometrial tissue outside the uterus, especially into the peritoneal cavity, are observed in the peritoneal cavity of women with 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. 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. Collins-Burrow 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 progression 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 antiangiogenic 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/ ATK 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.

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Declaration of Competing Interest

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

References


