

Original Research Article

Metformin and quercetin ameliorate proliferation and migration of cultured endometrial stromal cells: Potential therapeutic options for endometriosis

Eman Ibrahim Anwar^{1,2*}, Passant Mohamed Mohie², Suzan Elsharkawy³, Yasmine Amr Issa⁴, Lobna MMA Abd El Mottelib⁵, Rashed W Alweshah⁶, Ezzeldin Ahmed Hussein⁷, Samar R Saleh^{8,9}, Eman Helmy Thabet^{10,11}

¹Sulaiman AlRajhi University, College of Medicine, Department of Basic Sciences, AlBukayriyah, Qassim, Saudi Arabia, ²Department of Clinical Pharmacology, Faculty of Medicine, Alexandria University, Fahmi Abdelmeguid Street, Mowassah Campus, Alexandria 21561, ³Obstetrics & Gynecology Department, Faculty of Medicine, Alexandria University, Suez Canal Rd, Al Azarita WA Ash Shatebi, Bab Sharqi, Alexandria 5424012, ⁴Medical Biochemistry, College of Medicine, Arab Academy of Science and Technology, Al Alamein City, El Alamein, Marsa Matrouh Governorate 5060305, ⁵Department of Human Anatomy and Embryology, Faculty of Medicine, Alexandria University, Dr. Fahmi Abdelmeguid St., Mowassah campus, Alexandria 21561, ⁶Faculty of Medicine, Alexandria University, Champollion Street, Al Mesallah Sharq, Al Attarin, Alexandria Governorate 5372066, Egypt, ⁷Division of Gastroenterology, department of Medicine, Duke university school of Medicine, Durham, NC 27710, USA, ⁸Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria 21511, ⁹Bioscreening and Preclinical Trial Lab, Biochemistry Department, Faculty of Science, Alexandria University, Alexandria, ¹⁰Department of Medical Physiology, Faculty of Medicine, Alexandria University, Dr. Fahmi Abdelmeguid St., Mowassah Campus, Alexandria 21561, ¹¹Center of Excellence for Research in Regenerative Medicine and Applications (CERRMA), Faculty of Medicine, Alexandria University, Alexandria 21561, Egypt

*For correspondence: **Email:** e.ahmed@sr.edu.sa; EMAN.MOHAMED@alexmed.edu.eg

Sent for review: 30 August 2025

Revised accepted: 19 September 2025

Abstract

Purpose: Endometriosis is a medical condition characterized by an endometrial-like epithelium and stroma outside the endometrium. It is generally managed with surgical intervention and hormonal therapies. This study sought to compare the relative potency of metformin and quercetin to cisplatin and their effects on proliferative and migratory characteristics of in vitro-cultured endometrial stromal cells derived from tissue samples of patients with endometriosis.

Methods: Isolated endometrial mesenchymal stromal cells (EndMSCs) were obtained from forty patients with endometriosis at Shatby-Alexandria University Hospital, and expanded in vitro. The cells were validated using the cluster of differentiation marker (CD10) and estrogen receptor as positive markers and desmin as a negative marker. The MTT assay was conducted to assess the cytotoxicity of the three-drug formulations at different molar concentrations on EndMSCs, and their IC_{50} was determined. The following concentrations are used for Cisplatin (1 - 25 μ M), Quercetin (1 -160 μ M, and Metformin (1 - 40 mM). The markers of oxidative stress and Ki-67 expression were measured in the EndMSCs' culture media, as well as the cell migratory potential. Extracellular signal-regulated kinase (ERK) and Phospho-ERK, a central dysregulated pathway in endometriosis, were assessed in EndMSCs using Western blot assay.

Results: The calculated IC_{50} of cisplatin, quercetin, and metformin were $12.57 \pm 1.635 \mu$ M, $261.2 \pm 7.850 \mu$ M, and $92.01 \times 10^3 \pm 21.1 \mu$ M, respectively. Both metformin and quercetin-treated EndMSCs exhibited significantly reduced Ki-67 expression ($p < 0.05$ and $p < 0.01$), less cell migration profiles ($p < 0.0001$), decreased expression of pERK1/2 ($p < 0.0001$ and $p < 0.001$), with lower cell cytotoxicity (IC_{50}) ($p < 0.0001$) and oxidative stress ($p < 0.0001$) compared to cisplatin. To the best of our knowledge, no studies have compared the in vitro effects of metformin, quercetin, and cisplatin on the proliferation and migration of EndMSCs.

Conclusion: The findings of this study indicate that both drugs are promising agents for treating endometriosis. However, quercetin demonstrated higher potency in anti-proliferative and antioxidant effects, while metformin showed an obvious modulation of pERK1/2 to ERK ratio.

Keywords: Endometriosis, Metformin, Quercetin, Endometrial stromal cells, MAPK, ERK, Ki-67, MTT

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

Tropical Journal of Pharmaceutical Research is indexed by Scopus, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Endometriosis is a chronic inflammatory gynaecological disorder characterized by the ectopic implantation of endometrial stromal and epithelial cells beyond the uterine cavity [1,2]. The "uterine origin" and "extra-uterine origin" hypotheses suggest that the aetiology of endometriosis may be due to the metaplasia of endometrial (epithelial) cells into mesenchymal cells, or epithelial-mesenchymal transition (EMT). The molecular basis behind the emergence and advancement of endometriotic lesions has been reviewed in terms of genetic, endocrine, environmental, and immunological variables [3].

Endometriosis is typically treated with surgery and hormonal therapy [4]. The efficacy of conservative treatment with preservation of ovarian function is poor, with a high recurrence rate [5]. Current pharmaceutical therapies suppress the development and activity of endometrial implants by altering the hormonal profile, which is primarily driven by estrogen. Their categories encompass combination oral contraceptives, gonadotropin-releasing hormone analogues, progestins, aromatase inhibitors, selective progesterone receptor modulators, and selective estrogen receptor modulators [6]. The side effects of chronic hormonal therapy and resistance to treatment call for non-hormonal agents targeting the endometrial cells' proliferation and migration [2].

Cisplatin, a drug frequently used for cancer treatment as a platinum-based alkylating agent, destroys rapidly dividing cells by altering the structure of DNA [7]. Cisplatin has been widely studied *in vitro* and *in vivo* on endometrial cell proliferation, migration, and autophagy and shown to reduce the amount of endometrial tissue implanted outside the uterus, which is the hallmark of endometriosis, as well as the levels of proteins associated with the condition [7].

The current study considered it a standard drug or a positive control with well-studied anti-mitogenic signalling targets on endometrial cell lines. It attempts to evaluate its anti-proliferative effect on endometrial cells against other safer drugs that share common cell signalling targets.

Metformin, an insulin sensitizer derived from *Galega officinalis*, is used to treat type 2 diabetes by activating adenosine-monophosphate-activated protein kinase (AMPK), a key regulator of cellular energy balance, and by inhibiting mammalian target of rapamycin (mTOR) signalling, which suppresses autophagy. In endometriosis, metformin may promote regression of endometriotic implants by increasing superoxide dismutase activity [8].

Among other promising non-hormonal agents is quercetin, which has an emerging role in endometriosis-related molecular targets [9]. Quercetin is a flavonoid pigment present in many plants and foods such as honey, nigella sativa seeds, onions, green tea, apples, and berries. Studies conducted both *in vitro* and *in vivo* have shown that quercetin has anti-inflammatory, anti-proliferative, and anti-migratory effects, primarily through the MAPK, PI3K, and WNT signalling pathways [10,11].

Previous research showed that quercetin and metformin have beneficial effects on experimental endometriosis. In the ectopic endometrial tissues, the application of quercetin or metformin markedly raised the mRNA level of autophagy markers and decreased the mTOR mRNA level. In addition, they do not adversely impact the production of estrogen and progesterone, in contrast to traditional hormonal medications [9].

Extracellular signal-regulated kinase (ERK) is a MAPK kinase that controls critical cellular processes necessary for endometriotic lesions to survive, including cell migration, differentiation, proliferation, and apoptosis. Aberrations in MAPK signaling have been implicated in the

pathophysiology of endometriosis, and reducing its activity could represent a potential therapeutic target [12].

Given the above, this study aimed to assess how metformin and quercetin compare to cisplatin in influencing the biomechanical and proliferative properties of *in vitro* cultured endometrial cells derived from tissue samples of endometriosis patients [1].

METHODS

The procedures adhered to the national ethical standards and international guidelines of the Declaration of Helsinki and were approved by the Alexandria University ethical authorities on 15 November 2018 (protocol no. 0304146). The subjects provided written informed consent.

Sample collection

The sample size was calculated using Power Analysis and Sample Size Software (PASS 2020) by NCSS, LLC, Kaysville, Utah, USA, available at ncss.com/software/pass. The estimated minimum sample size is 40. Human endometrium tissues were obtained from the Obstetrics and Gynaecology Department at El Shatby University Hospital in Alexandria, Egypt, using a Z-test with a 5 % significance level, 20 % effect size, and 5 % precision.

In the operating theatre, samples were taken from endometriosis patients, and then immediately transported in Dubelco's Minimum Essential Medium (DMEM) high glucose, 5 % fetal bovine serum (FBS), 1 % Penicillin-Streptomycin-Amphotericin solution (PSA) on ice to the Centre of Excellence for Research in Regenerative Medicine and Applications (CERRMA), Faculty of Medicine, Alexandria University, located in Alexandria, Egypt. The isolation of endometrial stroma mesenchymal stem cells (EndMSCs) and culture were performed in a clean lab using sterile equipment and reagents. All tissue or cell manipulation was conducted within a sterile laminar flow hood.

Inclusion criteria

1. Age in the range of 20 to 40 years
2. Patients indicated for operative laparoscopy to excise endometriotic chocolate cysts due to infertility or period pains.

Exclusion criteria

1. History of recent hormonal treatment (e.g., moderate-dose hormonal pills, estrogen antagonists, testosterone derivatives, GnRH agonist (GnRHa), aromatase inhibitors, dienogest, or other progestins).
2. Suspected malignancy cases

Endometrial stromal cell isolation (EndMSCs) and culture

EndMSCs isolation was performed using a previously established protocol [13]. The sample of the endometriotic lesion was transferred in sterile Hank's balanced salt solution (HBSS). The sample was rinsed with HBSS to remove any blood or debris, centrifuged for five minutes at 600 g, and the supernatant was discarded. The pelleted sample was minced into 1 mm² fragments on a 100 mm plastic tissue culture dish. The minced tissue was then moved to a 50 mL Falcon tube and digested using collagenase type II 250U/mL (Roche, cat#11088866001) in basal media for two hrs on a shaking rotor at 37°C. The digested sample was pipetted vigorously to disaggregate any residual tissue fragments and then sequentially filtered through 100 µm and 40 µm strainers. The flow through containing the EndMSCs was washed and cultured in DMEM, high glucose (Biowest, supplemented with 5 % FBS (#SH30070-03, Thermo Fisher Scientific, Waltham, MA, USA), 100 IU ml⁻¹ penicillin, and 100mg ml⁻¹ streptomycin (#15140, Thermo Fisher Scientific) in a 37 °C humidified incubator with 5% CO₂. Media was changed every two days upon confluency, and cells were passaged using 0.25% (w/v) trypsin/ethylene diamine tetraacetic acid (EDTA) (#2500056, Thermo Fisher Scientific), and expanded in T75 flasks. Experiments were conducted on passages 3 – 5 (Figure 1).

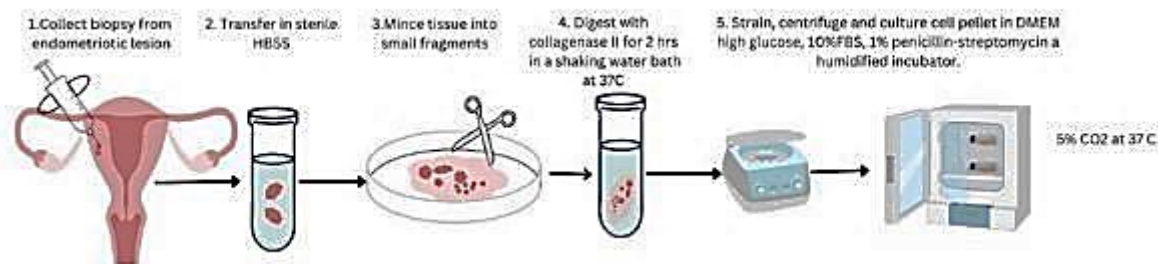


Figure 1: Schematic diagram of the steps of isolation and culture of endometrial stromal cells (EnDMSc) from endometriotic lesions

Characterization studies

Phase contrast microscopy

The growth and morphology of the isolated EndMSCs were monitored, and images were captured using an inverted Phase-contrast microscope (Olympus, Japan).

Colony-forming unit

In passage 3, the colony-forming potential (CFU) of EndMSCs was assessed. Specifically, 100 cells were seeded onto a 60 mm culture dish and incubated for 14 days. Following incubation, the cells were washed, fixed, and stained for five minutes at room temperature with crystal violet (Sigma-Aldrich, USA) at 3% (w/v) in methanol. After staining, the dye was removed, and the cells were rinsed with distilled water.

The CFU was calculated according to the following equation (Eq. 1):

$$PE (\%) = (N.CI/NC)100 \dots\dots\dots(1)$$

Where PE is the plating efficiency, NCI. is the number of colonies, and NC is the number of cells plated.

All visible colonies were identified, and colonies with five or more cells were counted using a phase-contrast inverted microscope. A CFU exceeding 40 % was considered suitable for cells of mesenchymal stem cell type [13].

Immunocyto staining

To confirm the phenotype of the isolated cells, endometrial stromal cell markers were further analyzed via immunocytochemistry using CD10, ER, and desmin antibodies. EndMSCs were grown on a cover slip in a 6-well plate until confluent, then fixed with 4 % PFA for 10 min at 37 °C. They were permeabilized with 0.1 % Triton

and blocked with 2 % bovine serum albumin (BSA).

After blocking, the cells were incubated with primary antibodies for 3 h at room temperature, then washed three times with PBS for 5 min each. Subsequently, they were incubated with secondary antibodies for 45 min. The nuclei were stained with counterstain, and the cells were mounted for imaging.

Drug preparation and dosage

The molar concentration of the studied drugs was calculated using the GraphPad molarity calculator. The following concentrations are used for Cisplatin (1 - 25 μ M) [14], Quercetin (1 -160 μ M) [9], and Metformin (1 - 40 mM) [14]. The drugs were purchased from Sigma-Aldrich with the product 's code (CAS 15663-27-1, Q4951, M-072), respectively.

Cytotoxicity study

The 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to assess the cytotoxic effects of the drug formulations on EndMSCs using a standard protocol [10]. EndMSCs were plated in 96-well plates at a density of 7×10^3 cells per well and incubated overnight in a humidified environment with 5% CO₂ at 37 °C. Once confluence was reached, the medium was replaced with fresh medium containing progressively higher molar concentrations of cisplatin, quercetin, and metformin. Following a 48-hour incubation, MTT solution (5 mg/mL in media) was introduced to each well and incubated for 4 hours at 37 °C in the dark with 5 % CO₂. After four hours, the media containing the MTT solution was removed, and 100 μ L of DMSO was added to each well. The plates were then shaken gently on an orbital shaker (Heidolph Instruments, Schwabach, Germany) for ten minutes to dissolve the formazan crystals. The absorbance at the wavelength maximum of 570 nm (A570 nm) was

recorded using an automated microplate reader (BioTek® Instruments, VT, USA). The data were used to generate a dose-response curve, which facilitated calculations. Cell viability was expressed as a percentage using Eq 2.

$$\text{Cell viability (\%)} = (T/C)100 \dots\dots\dots(2)$$

where T is the treated and C is the untreated control, with 100% indicating untreated control cells.

All experiments were performed in triplicate. The 50 % growth inhibitory concentration (IC₅₀) was then determined by non-linear regression analysis (Figure 3), using GraphPad Prism-10 software [10].

Proliferation studies (Ki-67)

EndMSCs were fixed with 4 % PFA for 10 min at 37 °C, permeabilized using 0.1 % Triton X-100 for 15 min at room temperature, and then blocked for 1 hour with 2 % BSA. They were subsequently incubated with Alexa Fluor 488-conjugated anti-rabbit Ki67 antibody (IgG, Cell Signaling Technology, USA, cat# 11882S). Evaluation and data analysis were carried out using BD FACS Calibur flow cytometry.

Migration studies

To evaluate the anti-migratory effects of quercetin and metformin-treated EndMSCs compared to cisplatin, EndMSCs were cultured in 12-well plates at a density of $1 \times 10^{4.5}$ cells per well to form a confluent monolayer [10]. A sterile 200-μL pipette tip was gently used to scratch the cell monolayer, forming a straight, cell-free track. The cells were washed with PBS and kept in serum-free media. The next day, the scratched cells were treated with sub-IC₅₀ doses of quercetin, metformin, and cisplatin.

Wells treated with cisplatin served as a positive control. The track closure was evaluated and recorded at 0, 24, 48, and 72 h. Wound images were taken with an inverted microscope (Olympus, Japan) at 100x magnification, capturing five images per well. The horizontal gap of the track was measured using ImageJ (version 1.53C, NIH, US) to calculate the cell migration rate and track closure percentage, based on Eq 3.

$$\text{Closure (\%)} = 100 - \{(A_t/A_0)100\} \dots\dots\dots (3)$$

Where A_t is the track area at time, t, and A₀ is its initial area.

Oxidative stress markers

Malondialdehyde (MDA) and total antioxidant capacity (TAC) are biomarkers of oxidative stress measured in cell lysate using a colorimetric assay (Biodiagnostic, Egypt: cat # MD 25 29 & cat # TA 25 13, respectively). MDA indicates lipid peroxidation. The reaction with thiobarbituric acid (TBA) produces an MDA-TBA₂ conjugate that absorbs light at 532 nm, resulting in a red-pink color. The TAC was determined by reacting antioxidants in the sample with a specific amount of added hydrogen peroxide. Samples were mixed with reagents according to the manufacturer's protocol, and the absorbance of the blanks and samples was measured immediately at 505 nm. TAC concentration was calculated in mM/L using Eq 4.

$$\text{TAC} = (\text{Abs. Bl.} - \text{Abs. S.}) \times 3.33 \dots\dots\dots (4)$$

Where TAC is total antioxidant capacity, Abs Bl is the absorbance of the blank, and Abs S is the absorbance of the sample.

Western blot assay

Cell lysates were prepared on ice using RIPA buffer containing 10 mM Tris-HCl, 1 % Triton X-100, 0.1 % SDS, 1 mM EDTA, 150 mM NaCl, 5 mM NaF, and 1 mM PMSF. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C. Fifty micrograms of protein were loaded onto 12 % SDS-PAGE gels and transferred to nitrocellulose membranes. The membrane was blocked with 5 % BSA for 1 hour at room temperature, then incubated overnight at 4 °C with primary antibodies against ERK1/2, pERK1/2, and β-actin at specified dilutions. After five TBST washes, it was incubated with secondary antibody (anti-rabbit IgG, AP-linked, 1:3000) for 2.5 h. The membrane was then developed with NBT/BCIP, and bands were quantified using ImageJ.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 10.4.2. All data are presented as mean ± SD from biological triplicates. Differences between groups were assessed with one-way ANOVA, followed by multiple Tukey tests (for three or more groups).

RESULTS

Endometrial stromal cells (EndMSCs) morphology

The primary cultures obtained from digesting the biopsies from endometriosis patients contained

mostly flat adherent cells of mixed populations. Under phase-contrast microscopy, the cultured stromal cells adhered to plastic and grew as a monolayer of tightly packed spindle cells of consistent shape and morphology (Figure 2 A). The cells were passaged (P) until P5 (Figure 2 B). EndMSCs, when seeded in low densities, were able to grow in colonies at P3 (Figure 2 A). Additionally, EndMSCs showed CFU potential. They grew in separate colonies when plated in low seeding densities (Figure 2 C). The CFU potential was calculated to be 50 %.

Immunocytochemical characteristics of EndMSCs

Immunoprofile of the cultured endometrial stromal cells, positive immunostaining for estrogen receptor (ER), CD10 (common acute

lymphoblastic leukemia antigen), and negative for the desmin muscle marker (Figure 2 D – F). These markers were maintained in culture up to passage 5.

Cell cytotoxicity

The MTT assay was used to identify the IC_{50} values of cisplatin, quercetin, and metformin on each EndMSC line. As shown in Figure 3, upon exposure to the studied drugs for 48 h, the calculated IC_{50} of cisplatin was $12.57 \pm 1.635 \mu M$, quercetin was $261.2 \pm 7.850 \mu M$, and that of metformin was $92.01 \times 10^3 \pm 21.1 \mu M$. Cisplatin is considered the positive control. Both metformin and quercetin demonstrated significantly higher toxicity concentrations on EndMSCs than cisplatin ($p < 0.0001$).

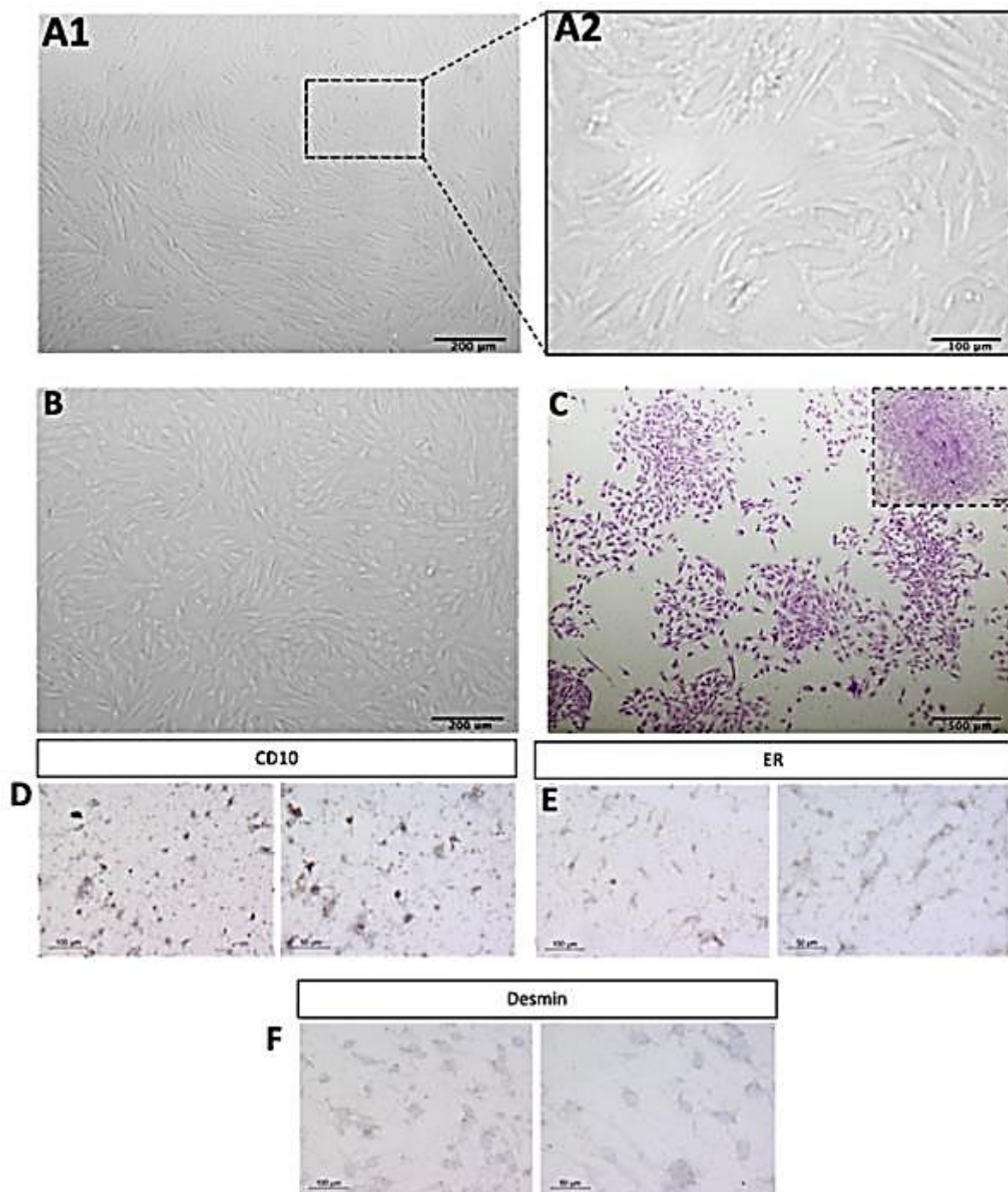


Figure 2: characteristics of endometrial stromal cells. Under phase contrast microscopy, passages 3 (A) and 5 (B) of EndMSC show 75 % confluency; scale bar 200 μm. The EndMSCs were adherent and exhibited spindle-shaped morphology under higher magnification, scale bar 100 μm (A2). EndMSCs demonstrated colony-forming potential when plated in low seeding densities (C). Immunohistochemical staining of the cultured EndMSCs showed positive expression for the stromal marker CD10 (D), the estrogen receptor (ER) (E), and negative expression for the muscle marker desmin (F). Scale bars 100 and 50 μm

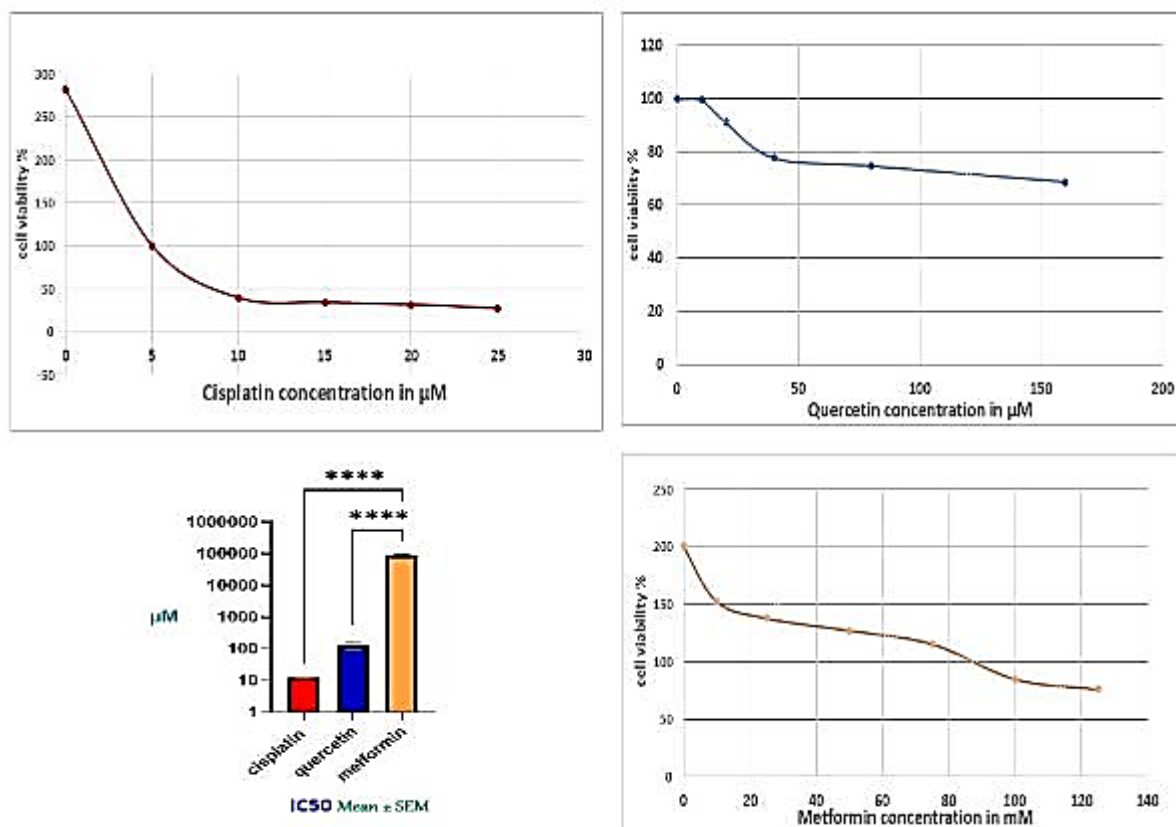


Figure 3: The EndMSCs' viability curves and IC_{50} values (mean in $\mu M \pm SEM$) for cisplatin, quercetin, and metformin based on MTT assay

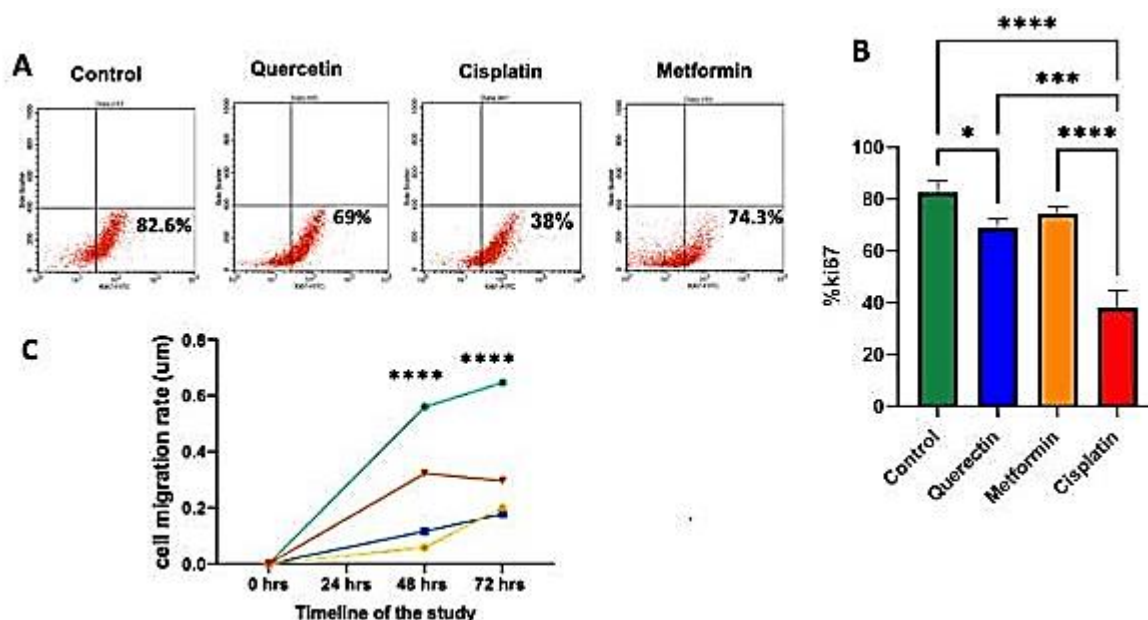


Figure 4: Effect of quercetin, metformin, and cisplatin on the proliferation assay and migration characteristics of cultured endometrial mesenchymal stromal cells (EndMSCs). **(A)** Flow cytometric scatter plots showing Ki-67 expression of EndMSCs control (untreated), treated with quercetin, cisplatin, or metformin. **(B)** One-way ANOVA statistical analysis of Ki-67 expression among treated groups, data are presented as mean $\pm$ SD for control (untreated), treated with quercetin, cisplatin, or metformin (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$). **(C)** Graph showing the average cell migration rate ($\mu m/h$), **** $P < 0.0001$; for all groups, compared to untreated controls at 48 and 72 h

EndMSCs proliferative capacity

Control EndMSCs from endometriotic lesions that were not exposed to any treatment during culture showed the highest proliferation rate, as shown by the 82.57 ± 4.4 % Ki-67 expression using flow cytometric analysis. EndMSCs treated with metformin and quercetin showed significant reductions in Ki-67 expression, with means of 74.30 ± 1.02 % ($p < 0.05$) and 69.04 ± 3.11 % ($p < 0.01$), respectively. However, cisplatin showed a mean of 37.96 ± 2.1 % ($p < 0.001$) compared to the control group (Figure 4 A and B).

Cell migration

The untreated control EndMSCs showed a high cell migration rate with a significant difference between 0 and 48 h ($p < 0.0001$) and 72 h ($p < 0.0001$). At 48 h, a substantial drop in cell migration rates was observed in quercetin, metformin, and cisplatin-treated cells compared to the controls ($p < 0.0001$). Additionally, at 48 h, metformin showed the lowest cell migration rate ($p < 0.0001$) as compared to cisplatin and quercetin (Figure 4 C). After 72 h of treatment, there were significant differences for all drugs when compared to the untreated control ($p < 0.0001$). However, quercetin exhibited the lowest migration rate, which was significantly different from that of the other drugs at 24 and 48 h ($p < 0.0001$), as shown in Figure 5.

Oxidative stress markers in EndMSCs

The levels of MDA and TAC indicate the extent of oxidative stress caused by endometriosis and the total antioxidant capacity induced by the various therapeutic drugs, respectively. The cisplatin-treated EndMSCs showed a significant reduction in the TAC and elevation of MDA levels compared to the metformin and quercetin-treated EndMSCs ($p < 0.0001$). However, metformin and quercetin-treated EndMSCs showed a significant decrease in the MDA/TAC index compared to cisplatin-treated EndMSCs ($p < 0.0001$), suggesting lower levels of oxidative stress in these cells (Figure 6).

Phosphorylation level of ERK1/2 in EndMSCs

ERK signalling pathways may cooperate with other pathways, such as the AKT pathway, to support the growth of deep endometriotic lesions. ERK1/2 showed a non-significant change between EndMSCs control group and the treated groups, either with quercetin, metformin, or cisplatin. On the other hand, pERK1/2 and its ratio with ERK1/2 were significantly reduced after the addition of quercetin, metformin, and cisplatin

compared to untreated-control EndMSCs. Metformin treatment resulted in the highest reduction in pERK1/2 and pERK/ERK ratio ($p < 0.0001$; Figure 7).

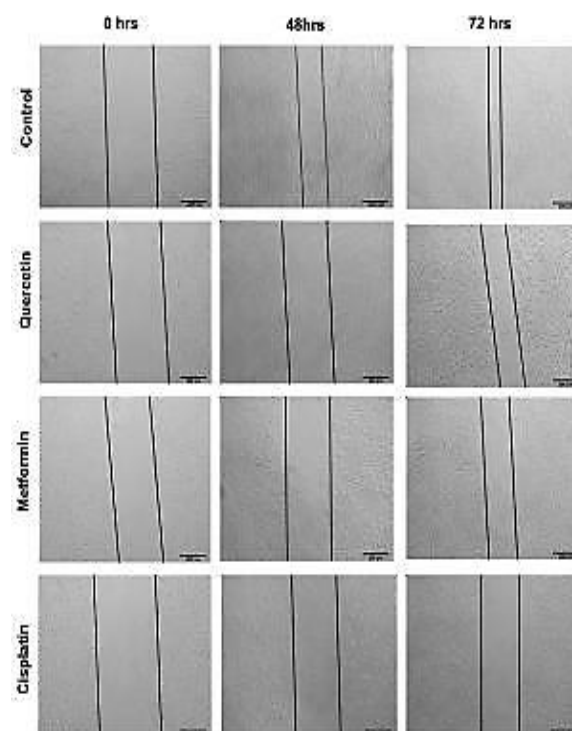


Figure 5: Representative images of the scratch analysis test done on EndMSCs groups over the 72-h scale bar 200µm. Results represent the mean of three measurements of each scratch area, and the experiment was done for each drug in duplicates (n = 6)

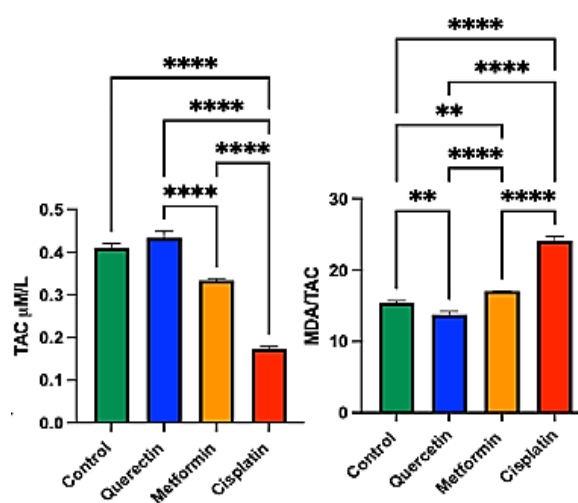


Figure 6: Oxidative stress (malondialdehyde, MDA nmol/ml) and antioxidant evaluation (Total antioxidant capacity (TAC) µM/L) of culture media of cultured endometrial stromal cells (EndMSC) in all studied groups (* $p < 0.05$, ** $p < 0.001$, **** $p < 0.0001$)

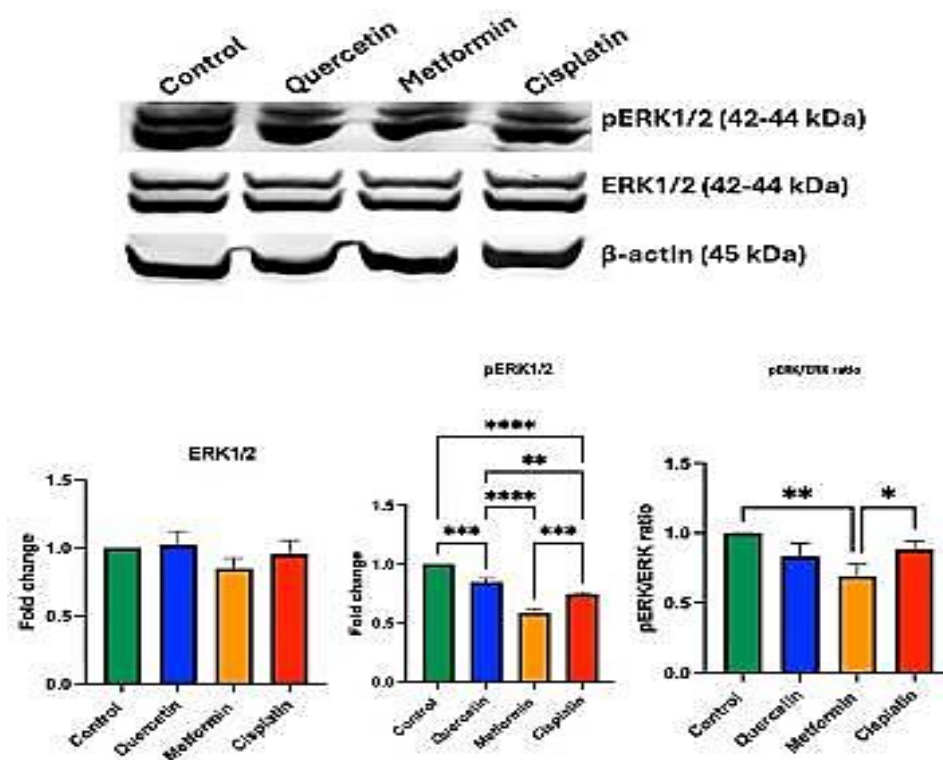


Figure 7: Protein expression level of ERK1/2, pERK1/2, and their ratio. One-way ANOVA statistical analysis and Tukey's multiple comparisons test were used to study the expression among the treated groups (* $p < 0.05$, ** $p < 0.001$, **** $p < 0.0001$). Data are presented as mean $\pm$ SD ($n = 3$)

DISCUSSION

Treatment for endometriosis is a complex clinical dilemma that requires innovative treatments that minimize lesions and preserve or rescue fertility. The definitive treatment has not yet been established due to the uncertainty surrounding the disease pathogenesis. The hormonal treatments neither improve the patient's fertility outcomes nor affect the disease course. Over-activation of kinase signalling pathways in endometriotic tissue and EndMSCs may arise due to the significantly altered peritoneal environment in women with endometriosis. The EndMSCs possess tissue regenerative and remodelling characteristics as well as immunomodulatory effects. These cells are part of the basal and functional layers of the endometrium. EndMSCs in endometriosis exhibit shorter doubling times, higher proliferation rates, reduced decidual response, and an imbalanced proliferation/differentiation ratio compared to normal eutopic EndMSCs [15]. Furthermore, EndMSCs are recognized as a potential therapeutic target in both endometriosis-related ovarian cancer and endometrial adenocarcinoma.

This study reports the isolation and expansion of EndMSCs from endometriosis patients as a reproducible tool for personalized drug testing *in vitro*. Our isolation protocol is a modified version of a previous endometrial isolation protocol [11].

The isolated EndMSCs retained heterogeneous stromal characteristics such as plastic adherence and spindle-shaped morphology, and surprisingly showed colony formation ability through clonal expansion. This colony formation ability suggests a stem cell population within the primary cell line generated. This has not been investigated further. Cultured EndMSCs showed positive expression of the stromal cell marker CD10. CD10 is commonly used in clinical practice as a reliable marker for endometrial stromal differentiation. CD10 is uniformly expressed in normal endometrial stromal cells. Estrogen receptor expression was also maintained in cultured EndMSCs. EndMSCs were free from the smooth muscle cell marker desmin, indicating the accuracy of the retrieved biopsy and the purity of the isolated cell population.

After validation of the cultured EndMSCs, the proliferative capacity and cell migration rates of EndMSCs derived from endometriotic lesions without any treatment was assessed. In culture, untreated EndMSCs derived from endometriotic lesions showed the highest proliferation capacity and cell migration rates, indicating that the pathophysiology of endometriosis is inherently transmitted to these cells in culture. Furthermore, the increased proliferative activity and persistent presence of estrogen receptors in cultured EndMSCs from endometriosis lesions emphasize

the stromal compartment's key role in endometriosis development. This also indicates that proliferation is regulated differently in these cells compared to eutopic endometrium.

Furthermore, the effect of metformin, quercetin, and cisplatin on cell proliferation and migration of EndMSCs has been tested in this study. Cisplatin is an anticancer drug that has been previously tested on endometrial malignant cells. In the current study, cisplatin is used as a reference drug with known reported outcomes rather than a suggested treatment option, as endometriotic lesions share some features with malignant tissues, such as increased proliferation, migration, and responsiveness to hormones. The beneficial impacts of cisplatin administration on the endometriosis rat model were reported to be through the downregulation of VEGF, extracellular matrix-disrupting protein MMP-2, and TGF- β expression, as well as an aromatase inhibitory effect [7]. An aberrant PI3K/AKT/mTOR pathway was highlighted both in the pathogenesis of endometriosis stages and endometriosis carcinogenicity potential. Nevertheless, the side effects of cisplatin are troublesome, including nausea, nephrotoxicity, cardiotoxicity, hepatotoxicity, neurotoxicity, and an additional burden of reproductive toxicity.

As it is crucial to introduce safe drugs for the treatment of endometriosis, metformin and quercetin represent non-hormonal therapeutic options with widely studied safety and promising efficacy on endometriosis. In the current study, both metformin and quercetin demonstrated statistically significant inhibition of EndMSCs proliferation and migration as compared to cisplatin. However, quercetin showed higher potency than metformin due to its significantly lower IC₅₀.

It was reported that metformin can inhibit endometrial cell migration in both endometriosis and endometrial cancer cell studies. The proposed mechanisms involved the reduction of vascular adhesion molecule (VCAM-1) expression, metalloproteinase-2,9, as well as E-cadherin and Vimentin, which are epithelial-mesenchymal transition-related proteins. Metformin also promotes the PI3K/AKT/MDM2 signalling pathway and modulates the stroma–epithelium interaction via Wnt2/ β -catenin [13,16,17]. The combination of metformin and progesterone exhibits a synergistic inhibitory effect on the proliferation of endometrial cancer cells, emphasizing the potential involvement of the LKB1-AMPK-mTOR signaling pathway [18-19]. Due to the benefits of metformin use in endometriosis, metformin enhanced the

expression of dysregulated EndMSCs' stemness-related genes, miRNAs, and restored skewed self-renewal/differentiation balance [15].

Treating EndMSCs with quercetin in the current study significantly decreased the cell migration rate when the proliferation capacity was controlled under serum-free conditions. Quercetin has been proven to inhibit cell-to-cell migration through several mechanisms, such as a reduction in ROS. Quercetin is a potent antioxidant that can effectively scavenge ROS. This effect has been previously proven on cancer cell lines such as glioma and neuroblastoma cells. Although quercetin has been shown to effectively decrease cell migration *in vitro*, further evaluation of the epithelial-mesenchymal transition is required to assess the impact of quercetin on preventing endometriotic cell invasion of the basal lamina. Previous work has also shown this effect through multiple mechanisms, such as regulating Toll-like Receptor 4/Nuclear Factor-kappa B Pathway and suppressing inflammation. For this reason, current therapies for endometriosis include anti-inflammatory agents. Previous studies that investigated quercetin's effects on endometriosis reported anti-proliferative and anti-inflammatory effects. Quercetin can impede the growth of EndMSCs in endometriosis and diminish their invasiveness by downregulation of MMP-2, MMP-9, Fascin, and Erzin proteins [20-22]. Interestingly, the simultaneous administration of quercetin and metformin markedly decreased the size of endometrial implants, as well as mTOR gene expression and autophagy markers, in the ectopic endometrium of the *in vivo* rat model [23].

The results of the present study elucidated that metformin and quercetin-treated EndMSCs showed a significant decrease in the MDA/TAC index, indicating lower levels of oxidative stress in these cells compared to untreated EndMSCs. Endometriotic cells exhibit elevated endogenous oxidative stress, disrupt ROS detoxification pathways correlated to enhanced cellular proliferation, and activate the MAP kinase ERK1/2 [24]. Metformin inhibits ROS by activating AMPK. In addition, it attenuates ROS production by activating FOXO3 in Immune Cells. Quercetin was reported to prevent oxidative stress by modulating NOX2/ROS/NF-kB.

ERK signaling pathways promote endometriotic stromal cell survival and proliferation *in vitro* and support the formation of deep endometriotic lesions [12]. ERK downregulation may serve as a viable target for endometriosis treatment. Finally, treating EndMSCs with quercetin and metformin prohibited the phosphorylation of ERK and significantly decreased the pERK/ERK ratio, with

the highest reduction in metformin-treated EndMSCs. In line with our results, metformin was proven to inhibit the phosphorylation of ERK in lung tissue, maybe as a result of inhibition of an upstream receptor tyrosine kinase. Quercetin was reported to abrogate the phosphorylation of the MAP kinases, including ERK, by suppressing tyrosine phosphorylation, which is necessary for MAP kinase activation.

Quercetin and metformin effectively inhibited the proliferation and migration of isolated EndMSCs in the current study. Interestingly, their reproductive safety has been addressed in previous studies; quercetin has multiple beneficial effects on the reproductive system. It enhances ovarian response and oocyte maturation, and protects the female reproductive system from chemotherapy-induced adverse effects through antioxidant and anti-inflammatory effects [25,26]. Furthermore, it has been reported that metformin also has reproductive benefits with a positive influence on oocyte quality and hormonal profile adjustment [27].

To the best of our knowledge, there are no published studies that compare the *in vitro* effects of metformin and quercetin to those of cisplatin on EndMSCs proliferation and migration. The present findings suggest that both metformin and quercetin are promising and safe therapeutic options for reducing the proliferation and migration of endometriotic lesions.

CONCLUSION

Metformin and quercetin significantly reduce the proliferative and migratory potentials of EndMSCs. Both agents decrease oxidative stress and modulate the ERK signalling pathway. However, quercetin is a more potent antiproliferative and antioxidant than metformin. *In vivo* studies are necessary to ascertain their effectiveness in treating endometriosis.

DECLARATIONS

Acknowledgement/Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability

All data generated or analysed during the study are included in this article.

Ethical issues

The procedures used adhered to national ethical standards and the international guidelines of the Declaration of Helsinki, and were approved by the Alexandria University ethical authorities on 15 November 2018 (protocol no. 0304146). Study participants (endometriosis patients) provided written informed consent.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. E.I.A.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing-original draft, writing-review & editing. P.M.M.: data curation, investigation, and resources. S.E.: data curation, investigation, resources, writing- original draft. Y.A.I.: data curation, investigation, resources, methodology. L.M.M.A.: data curation, investigation, formal analysis, software, resources, writing-original draft, writing-review, and editing. R.W.A.: data curation, investigation, methodology, formal analysis, resources, writing- original draft. E.A.H.: data curation, investigation, methodology, formal analysis, resources, writing- original draft. S.R.S.: formal analysis, investigation, methodology, writing- original draft. E.H.T.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, supervision, writing-original draft, writing-review and editing.

REFERENCES

1. Altayyeb A, Othman E, Khashbah M, Esmaeel A, El-Mokhtar M, Lambalk C, Mijatovic V, Abdelgawad M. Characterization of the mechanical signature of eutopic endometrial stromal cells of endometriosis patients. *Reprod Sci*. 2020; 27: 364-374.
2. Mai H, Wei Y, Yin Y, Huang S, Lin H, Liao Y, Liu X, Chen X, Shi H, Liu C. LINC01541 overexpression attenuates the 17 β -Estradiol-induced migration and invasion capabilities of endometrial stromal cells. *Syst Biol Reprod Med*. 2019; 65(3): 214-222.
3. Baranov V, Malysheva O, Yarmolinskaya M. Pathogenomics of endometriosis development. *Int J Mol Sci*. 2018; 19(7): 1852.
4. Vannuccini S, Clemenza S, Rossi M, Petraglia F. Hormonal treatments for endometriosis: The endocrine background. *Rev Endocr Metab Disord*. 2022; 23(3): 333-355.
5. Zakhari A, Delpero E, McKeown S, Tomlinson G, Bougie O, Murji A. Endometriosis recurrence following post-Trop J Pharm Res, September 2025; 24(9): 1124

- operative hormonal suppression: a systematic review and meta-analysis. *Hum Reprod Update*. 2021; 27(1): 96-107.
6. Troia L, Luisi S. Estro-progestins and pain relief in endometriosis. *Endocrines*. 2022; 3(2): 349-366.
 7. Li Z, Liu H, Lang J, Zhang G, He Z. Effects of cisplatin on surgically induced endometriosis in a rat model. *Oncol Lett*. 2018; 16(4): 5282-5290.
 8. Kimber-Trojnar Ż, Dłuski DF, Wierchowska-Opoka M, Ruszala M, Leszczyńska-Gorzelak B. Metformin as a potential treatment option for endometriosis. *Cancers*. 2022; 14(3): 577.
 9. Cantrell LA, Zhou C, Mendivil A, Malloy KM, Gehrig PA, Bae-Jump VL. Metformin is a potent inhibitor of endometrial cancer cell proliferation—implications for a novel treatment strategy. *Gynecol Oncol*. 2010; 116(1): 92-98.
 10. Chaichian S, Nikfar B, Bidgoli SA, Moazzami B. The Role of Quercetin for the Treatment of Endometriosis and Endometrial Cancer: A Comprehensive Review. *Curr Med Chem*. 2024; 32(1): 74-86.
 11. Park S, Lim W, Bazer FW, Whang K-Y, Song G. Quercetin inhibits proliferation of endometriosis, regulating cyclin D1 and its target microRNAs in vitro and in vivo. *J Nutr Biochem*. 2019; 63: 87-100.
 12. Jamali N, Zal F, Mostafavi-Pour Z, Samare-Najaf M, Poordast T, Dehghanian A. Ameliorative effects of quercetin and metformin and their combination against experimental endometriosis in rats. *Reprod Sci*. 2021; 28: 683-692.
 13. Bora G, Yaba A. The role of mitogen-activated protein kinase signaling pathway in endometriosis. *J Obstet Gynaecol Res*. 2021; 47(5): 1610-1623.
 14. Franken NA, Rodermond HM, Stap J, Haveman J, Van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc*. 2006; 1(5): 2315-2319.
 15. Bae-Jump VL, Zhou C, Boggess JF, Gehrig PA. Synergistic effect of rapamycin and cisplatin in endometrial cancer cells. *Cancer*. 2009; 115(17): 3887-3896.
 16. Liu Y, Liang S, Yang F, Sun Y, Niu L, Ren Y, Wang H, He Y, Du J, Yang J. Biological characteristics of endometriotic mesenchymal stem cells isolated from ectopic lesions of patients with endometriosis. *Stem Cell Res Ther*. 2020; 11: 1-17.
 17. Hufnagel D, Li F, Cosar E, Krikun G, Taylor HS. The role of stem cells in the etiology and pathophysiology of endometriosis. *Semin Reprod Med*. 2015; 33(05): 333-340.
 18. Mashayekhi P, Noruzinia M, Khodaverdi S. Metformin as a potential agent for modulating the faulty endometriotic mesenchymal stem cells: A case-control study. *Int J Reprod Biomed*. 2022; 20(10): 861-872.
 19. Sourial S, Tempest N, Hapangama DK. Theories on the pathogenesis of endometriosis. *Int J Reprod Med*. 2014; 2014(1): 179515.
 20. Arnold JT, Kaufman DG, Seppala M, Lessey BA. Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. *Hum Reprod*. 2001; 16(5): 836-845.
 21. Tan BK, Adya R, Chen J, Lehnert H, Cassia LJS, Randeva HS. Metformin treatment exerts anti-invasive and antimetastatic effects in human endometrial carcinoma cells. *J Clin Endocrinol Metab*. 2011; 96(3): 808-816.
 22. Qiang P, Shao Y, Sun Y-P, Zhang J, Chen L-J. Metformin inhibits proliferation and migration of endometrial cancer cells through regulating PI3K/AKT/MDM2 pathway. *Eur Rev Med Pharmacol Sci*. 2019; 23(4): 1778-1785.
 23. Mu N, Xu T, Gao M, Dong M, Tang Q, Hao L, Wang G, Li Z, Wang W, Yang Y. Therapeutic effect of metformin in the treatment of endometrial cancer. *Oncol Lett*. 2020; 20(5): 156.
 24. Pabona JMP, Burnett AF, Brown DM, Quick CM, Simmen FA, Montales MTE, Liu SJ, Rose T, Alhallak I, Siegel ER. Metformin promotes anti-tumor biomarkers in human endometrial cancer cells. *Reprod Sci*. 2020; 27: 267-277.
 25. Xu W, Song Y, Li K, Zhang B, Zhu X. Quercetin inhibits adenomyosis by attenuating cell proliferation, migration and invasion of ectopic endometrial stromal cells. *Drug Des Devel Ther*. 2020; 14: 3815-3826.
 26. Ngô C, Chéreau C, Nicco C, Weill B, Chapron C, Batteux F. Reactive oxygen species controls endometriosis progression. *Am J Pathol*. 2009; 175(1): 225-234.
 27. Faure M, Bertoldo MJ, Khoeiry R, Bongrani A, Brion F, Giulivi C, Dupont J, Froment P. Metformin in reproductive biology. *Front Endocrinol*. 2018; 9: 675.