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Antiproliferative effect of bitter melon (*momordica charantia*) seed and fruit extracts through the non-genomic oestrogen receptor signaling pathway on oestrogen-sensitive endometrium cancer cells

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Abstract

Background and Aims: *Momordica charantia* has garnered much attention as an alternative medicine due to its phytopharmaceutical content and anti-cancer properties. This study aimed to demonstrate the anti-cancer potential of *M. charantia* fruit extract (*Mc*FE) and seed extract (*Mc*SE) by investigating their effects on the migration and proliferation of human endometrial cancer (EC) cells as well as on the activation (phosphorylation) of non-genomic oestrogen receptor (ER) signaling pathway kinases and caspase-9 phosphorylation.

Methods: *Mc*FE and *Mc*SE samples were extracted with ethanol using the maceration method. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test was used to evaluate EC cell (RL 95-2) viability, Western blot was used to analyse protein phosphorylation, and cell migration was evaluated using a wound-healing assay.

Results: Cell proliferation was inhibited by McSE and McFE, with IC_{50} values of 134,8 µg/mL and 165,9 µg/mL, respectively. The phosphorylation of non-genomic ER signaling kinases, such as extracellular signal-regulated kinases (ERK1/2) (8.59-fold), P38 mitogen-activated protein kinase (P38MAPK) (13.99-fold), and protein kinase B (AKT) (4.52-fold), increased in comparison to control cells after a short (15 min.) treatment with low concentrations (1 nM) of 17 β-oestradiol. The phosphorylation of caspase-9 (Thr125 2.59 fold, Ser196 3.48 fold) was additionally elevated by oestradiol treatment. The activation of ERK1/2, P38MAPK and AKT, and the phosphorylation of caspase-9 were significantly decreased by treatment with *Mc*SE and *Mc*FE. Additionally, the relative closure of the scratch in the cell migration assay was significantly reduced after treatment with McSE and McFE.

Conclusion: *M. charantia* can induce cell death in EC cells by preventing the phosphorylation of caspase-9 and non-genomic kinases involved in the ER signaling pathway. These findings imply that *M. charantia* could be used as an alternative supportive treatment for EC.

Keywords 17 β-oestradiol · Endometrium cancer · Non-genomic oestrogen signaling pathway · Caspase-9 · *Momordica charantia*

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INTRODUCTION

The most prevalent gynaecological cancer, endometrial cancer (EC), is subdivided into two categories based on histopathology (Bokhman, 1983; Lortet-Tieulent et al., 2018). Endometrial tumours, especially Type 1, commonly referred to as low-grade endometrial tumours, comprise the majority of EC events. These tumours are thought to be hormone-driven because they often exhibit high levels of oestrogen receptor (ER) α (Carlson et al., 2014). Type II tumours, known as high-grade endometrial tumours, clear cell tumours, mixed histology endometrial tumours, serous tumours and carcinosarcomas, are characterised by their ability to express low levels of ER and poor prognosis (Shen et al., 2017). It is highlighted that both forms of EC may be affected by oestrogen signaling through the ER. After binding with the ER, oestrogen may interact in genomic and non-genomic pathways. In genomic signaling, oestrogens, after connecting with the ER, the oestrogen/ER complex binds to the genome and controls transcription by performing the standard steroid hormone receptor action (Vrtačnik et al., 2014). In non-genomic signaling, oestrogen binds directly to the target membrane-bound ER and initiates several intracellular signaling cascades (Lösel & Wehling, 2003). Genes regulating cell proliferation are regulated significantly more quickly (within minutes) by the non-genomic action of oestrogen than via the genomic pathway (Fuentes & Silveyra, 2019). Several protein kinase signaling cascades [phospholipase C (PLC)/protein kinase C, rat sarcoma (Ras)/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinases (MAPK), the phosphatidyl inositol 3 kinase (PI3K)/ protein kinase B (AKT) and cAMP/protein kinase A (PKA)] are activated within the cell by non-genomic ER-signaling (Gu & Moss, 1996; Marino et al., 1998; Dos Santos et al., 2002; Marino et al., 2003). Oestrogen-mediated G protein-coupled ER 1 activation triggers cell proliferation by inducing calcium release and PI3K-AKT pathway activation. Through the activation of downstream related effector molecules that are crucial for the cell cycle, growth, and proliferation, the PI3K/AKT signaling pathway regulates a variety of cellular physiological processes in EC cells (Shi et al., 2019). The p38MAPK pathway is known as "stress-activated kinases" because it is typically triggered by a stress reaction, stimulating inflammation and programmed cell death (Johnson & Lapadat, 2002). Oestrogen may have a nongenomic effect in the endometrium through MAPK signaling, as evidenced by its involvement in controlling p38 MAPK activity in endometrial cells (Seval et al., 2006). In particular, stimulation of the extracellular signal-regulated kinase (ERK)/MAPK signaling cascade leads to the suppression of apoptosis through the phosphorylation of caspase-9 at a specific site, threonine 125 (Thr125) (Allan et al., 2003). Simultaneously, AKT phosphorylates caspase-9 at Serine 196 (Ser196), inhibiting cellular apoptosis (Cardone et al., 1998). Suppression of caspase-9 phosphorylation, regulated by ERK and AKT and associated with the prevention of apoptosis, is a possible therapeutic target for cancer treatment.

Momordica charantia, a plant of the Cucurbitaceae family, commonly known as bitter melon, is commonly used as a folk medicine (Grover & Yadav, 2004). The literature has shown that *M. charantia* has antiviral, antibacterial, immunomodulatory, antidiabetic, antihelminthic, anti-inflammatory, and anti-cancer properties (Grover & Yadav, 2004; Joseph & Jini, 2013; Chao et al., 2014; Salehi et al., 2018).

The main aim of this study was to investigate the antiproliferative effect of McSE and McFE on human endometrium cancer cells (RL 95-2) and the potential mechanism underlying their effects on non-genomic ER signaling kinases and caspase-9 phosphorylation.

MATERIALS AND METHODS

Reagents

17 β-Oestradiol, MTT (3-[4,5-dimethylthiazol-2-yl]–2,5 diphenyl tetrazolium bromide) reagent, phosphate-buffered saline (PBS), and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies were ordered from Elabscience (USA) and Affinity Biosciences (China). Cell culture mediums and supplements were obtained from Pan-Biotech (Aidenbach, Germany). The BCA total protein assay kit and the ECL kit were purchased from Thermo Fisher Scientific.

Plant Extraction

M. charantia fruits were grown in unheated greenhouse conditions at Erciyes University, Faculty of Agriculture, Department of Horticulture. Seeds of the same fruits were used in our study. The fruit (31.7 g) and seed (20 g) were ground up and macerated in 70% ethanol for 72 h at 42 °C in a water bath with a shaking function. After filtering the extracts, the ethanol was evaporated using a rotary evaporator. Crude extracts were obtained after lyophilisation.

Cell Culture

RL 95-2 cell lines were purchased from the American Type Cell Collection (Cat No: CRL-1671™ ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) high glucose was used to cultivate RL 95-2 cells in a 75 cm^2 culture flask in an incubator with 5% CO_2. The medium included antibiotics (1%), penicillin, streptomycin, and heat-inactivated foetal bovine serum (10%).

Cytotoxicity Assay: MTT Reduction Assay

The MTT assay was performed for cytotoxicity analysis (van Meerloo et al., 2011). RL 95-2 cells were seeded in a 96-well plate at a cell density of 15,000 cells/well. The cells were allowed to attach to the bottom of the culture plate overnight. Subsequently, the cells were exposed to 25–400 µg/mL concentrations of *Mc*SE and *Mc*FE for 24 h. After that, the extract-containing solution was discarded, and the cells were incubated for 1 h with 10 µl of MTT in 100 µl of the medium. After removing the unreduced MTT solution from the wells, DMSO was applied to dissolve the purple formazan crystals. A microplate reader functioning at 570 nm was used to record the different absorbance readings of the solution in each well.

Western Blotting

Cells were exposed to 1, 10, and 100 nM 17 β-oestradiol to find the optimum amount of oestrogen administration for activating non-genomic ER signaling kinases. The optimum incubation time of oestrogen was chosen based on the results of previous studies in our laboratory (Çınar & Cumaoğlu, 2023). Following a six h treatment period using different extract dosages for cells, 17 β-oestradiol was applied to the cells for 15 min. Radioimmunoprecipitation assay (RIPA) cell lysis solution and the bicinchoninic acid (BCA) reagent were used to measure the total protein concentration. Protein samples (30 µg) were loaded onto 5%–10% sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the protein samples were separated according to their molecular weights. The separated proteins were transferred to the polyvinylidene fluoride (PVDF) membrane following electrophoresis, and they were blocked using a milk powder solution (5%) for 1 h. Following this, the membranes were treated with specific primary antibodies [Phospho-AKT (Ser473) (E-AB-20802, Elabscience, Houston, Texas, USA), AKT (E-AB-81455, Elabscience, Houston, Texas, USA), Phospho-ERK1/2 (Thr202/Tyr204) (E-AB-70310, Elabscience, Houston, Texas, USA), ERK1/2 (E-AB-31374, Elabscience, Houston, Texas, USA), Phospho-P38 (Thr180/Tyr182) (E-AB-21027, Elabscience, Houston, Texas, USA), P38 (E-AB-66279, Elabscience, Houston, Texas, USA), Phospho-Caspase-9 (Ser196) (AF3870, Affinity Bioscience, Changzhou, China), Phospho-Caspase-9 (Thr125) (AF3348, Affinity Bioscience, Changzhou, China), GAPDH (AF7021, Affinity Bioscience, Changzhou, China)] for during a night. The following day, after removing the primary antibody, horseradish peroxidase (HRP)-linked secondary antibodies. were used to identify the protein bands, and protein bands were visualised using enhanced chemiluminescence (ECL) reagents.

Wound-Healing Assay

The 24-well plates were used to seed RL 95-2 cells, with $3.5x10^5$ cells in each well. Using a 10 µL pipette tip, a scratch or wound was made at the bottom of each well after the cells reached a sufficient density. After unattached cells were removed using sterile PBS, the cells were treated with different extracts and 17 β-oestradiol concentrations. Analysis of cell migration was done after 48 h. A camera fixed on an inverted microscope was used to take pictures of the cells (Grada et al., 2017).

Statistical Analysis

The SigmaPlot 12.0 program was used for statistical analyses. The *Student-T* test was employed to compare the changes between the groups. Significance has been assessed with p values less than 0.05.

RESULTS

Effect of *Mc*FE and *Mc*SE on Cell Viability

The effect of McSE and McFE on cell viability was analysed using an MTT assay. For 24 h, the cells were exposed to varying concentrations of McSE and McFE. The viability of cells treated with McFE 50 µg/mL was 89.51%. In a dose-dependent manner, increasing concentrations of McFE significantly decreased cell viability (Figure 1). Cells treated with 50 µg/mL McSE showed a decrease in viability (95.81%), but this decrease was not statistically significant. In a dose-dependent manner, increasing concentrations of McSE substantially decreased cell viability (Figure 1). The IC_{50} values of McFE and McSE were calculated as 165.9 µg/mL and 134.8 µg/mL in RL 95-2 cells, respectively. The preventive effects of McSE and McFE against 17 β-oestradiol activated non-genomic ER signaling kinases and phosphorylation of caspase-9 were investigated in subsequent studies using concentrations of 75 and 50 µg/mL McFE and 50 and 100 µg/mL McSE, based on the findings of the MTT experiment.

Effect of 17 β-Oestradiol on Non-Genomic Oestrogen Receptor Signaling Kinases in RL95-2 Cells

This investigation determined the concentration at which 17 β-oestradiol promotes the phosphorylation of caspase-9 and the activation of kinases in the non-genomic ER signaling pathway. Our study showed that incubation with 17 β-oestradiol at concentrations of 1, 10, and 100 nM (15 min.) significantly increased the phosphorylation of caspase-9 at Thr125 and Ser196 (Figure 2). Based on the findings of this experiment, 1 nM of oestradiol was determined to be the concentration needed to activate the non-genomic oestrogen

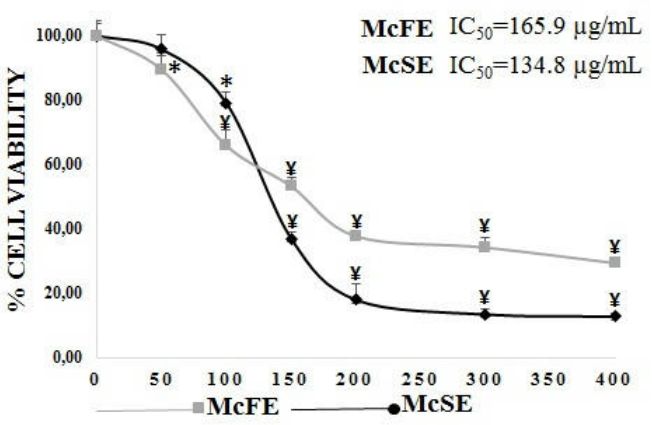

Figure 1. Effect of *Mc*FE and *Mc*SE (24 h incubation) on RL 95-2 cell viability. n = 4, *p<0.05, **p<0.01 *vs.* the control.

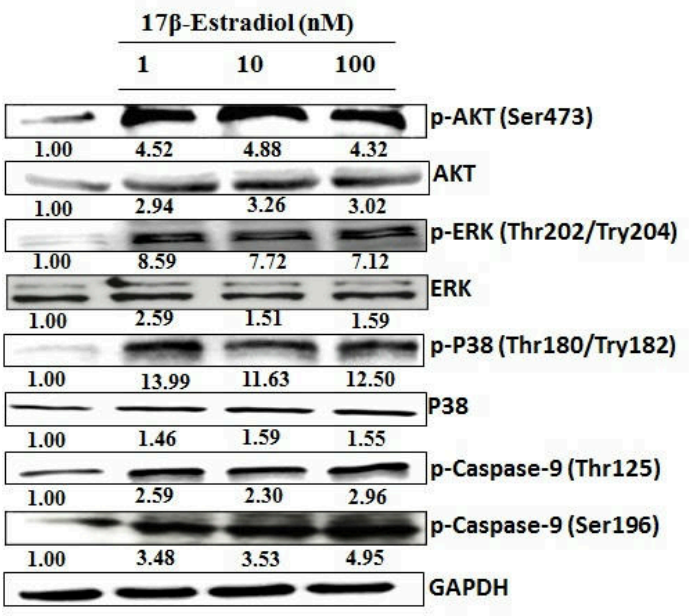

Figure 2. Western blot analysis of the phosphorylation of non-genomic oestrogen receptor signaling protein kinases and caspase-9 in RL 95-2 cells treated with different concentrations of 17 β-oestradiol.

signaling pathway. This concentration of 17 β-oestradiol was then used in other research.

Effects of *Mc*SE and *Mc*FE on the Non-Genomic Oestrogen Receptor Signaling Kinases ERK/MAPK and P38/MAPK Stimulated by 17 β-oestradiol

The phosphorylation of ERK/MAPK and P38/MAPK was significantly increased after 1nM 17 β-oestradiol treatment for 15 min in RL 95-2 cells. Both concentrations of McFE caused a decrease in p38 and ERK phosphorylation. McSE caused a decrease in p38 and ERK phosphorylation at the administered concentrations. McSE 50 µg/mL administration decreased ERK phosphorylation. However, this difference (Figure 3) was not deemed statistically significant.

Effects of *Mc*SE and *Mc*FE on the Non-Genomic Oestrogen Receptor Signaling Kinase AKT, Stimulated by 17 β-oestradiol

The phosphorylation of AKT was significantly increased after 1 nM 17 β-oestradiol treatment for 15 min in RL 95-2 cells. AKT is a significant kinase that phosphorylates caspase-9 at Ser 196. *Mc*SE at concentrations of 50 and 100 µg/mL and *Mc*FE at 75 and 150 µg/mL significantly suppressed the phosphorylation of AKT (Figure 4).

Effects of *Mc*SE and *Mc*FE on Caspase-9 Phosphorylation, Stimulated by 17 β-oestradiol

This study showed for the first time that 17 β-oestradiol stimulates caspase-9 phosphorylation in RL 95-2 cells. 50 and 100 µg/mL *Mc*SE and 75 and 150 µg/mL *Mc*FE significantly suppressed the phosphorylation of caspase-9 at Ser196 and Thr125 (Figure 5).

The Effect of *Mc*SE and *Mc*FE on 17 β-oestradiol-induced Cellular Migration in RL 95-2 Cells

17 β-oestradiolestradiol-induced cell migration and the effect of *Mc*SE and *Mc*FE on scratch closure were investigated using a wound-healing assay in RL 95-2 cells. Incubation with

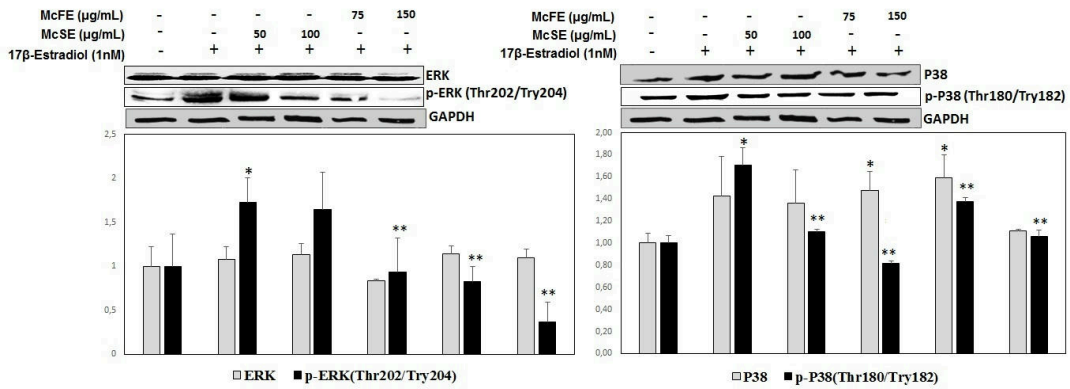

Figure 3. 17 β-oestradiol-mediated P38 and ERK1/2 activation: Regulatory effect of *Mc*FE and *Mc*SE on P38 and ERK1/2 activation. n = 3, *p<0.05 compared to the control group, **p<0.05 compared to the 17 β-oestradiol-treated group.

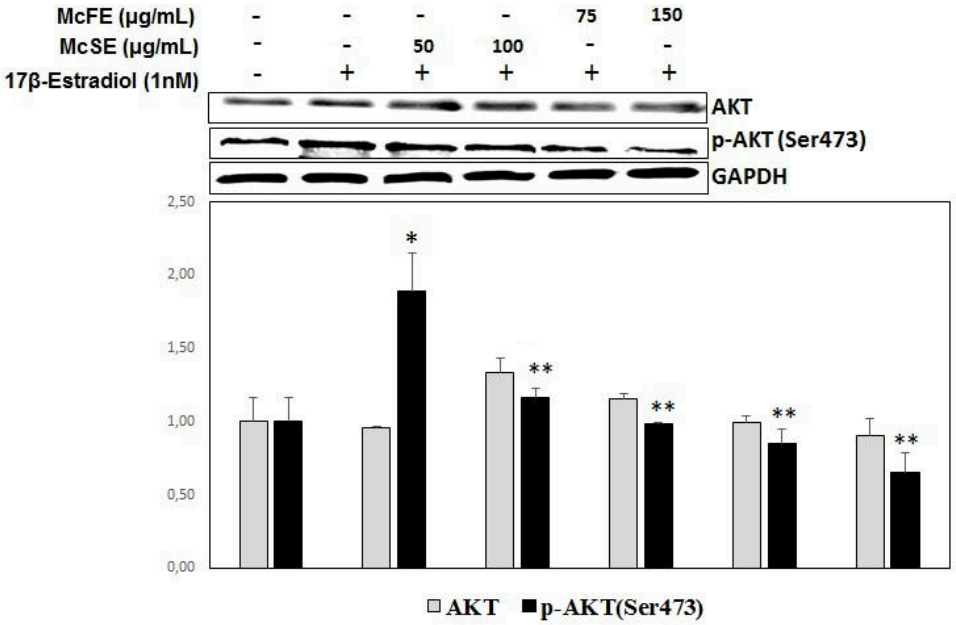

Figure 4. 17 β-oestradiol-mediated AKT activation: Regulatory effect of *Mc*FE and *Mc*SE on AKT activation. n = 3, *p<0.05 compared to the control group, **p<0.05 compared to the 17 β-oestradiol-treated group.

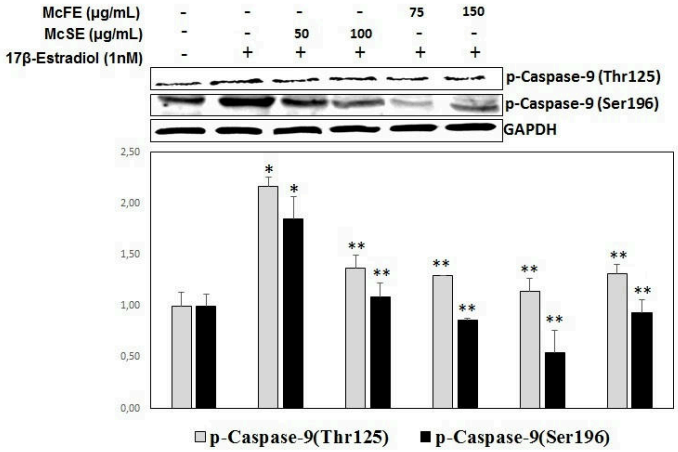

Figure 5. 17 β-oestradiol-mediated caspase-9 phosphorylation: Regulatory effect of *Mc*SE and *Mc*FE on caspase-9 phosphorylation. n = 3, *p<0.05 compared to the control group, **p<0.05 compared to the 17 β-oestradiol-treated group.

1 nM 17 β-oestradiol for 15 min increased cell migration and proliferation. Treatment with *Mc*SE at concentrations of 50 and 100 µg/mL and *Mc*FE at 75 and 150 µg/mL for six h suppressed cell migration and proliferation in RL 95-2 cells at the end of the 48-h experimental period (Figure 6).

DISCUSSION

The sixth most prevalent cancer in women worldwide is EC, and its frequency is rising (Sung et al., 2021). Among the primary risk factors for the formation of EC is exposure to endogenous or exogenous oestrogen (Kaaks et al., 2002; Allen et al., 2010). Oestrogens play a mitogenic role in the endometrium through the ER. Unopposed oestrogen can ini-

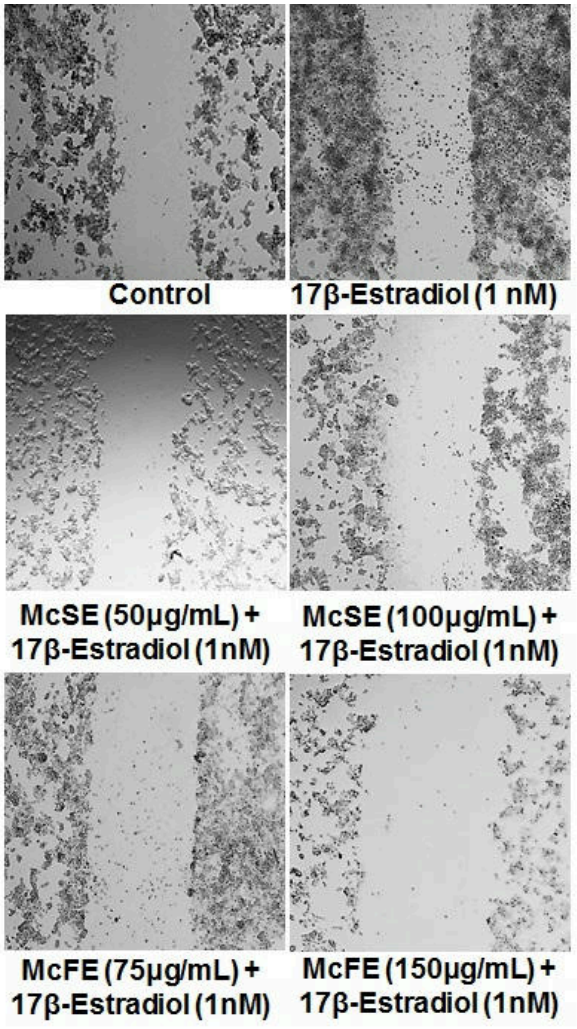

Figure 6. Effect of 17 β-oestradiol and *Mc*SE and *Mc*FE on RL 95-2 cell migration.

tiate endometrial hyperplasia, leading to the development of EC (Yang et al., 2011). ERα and ERβ are two subtypes of ERs that are members of the superfamily of nuclear receptors. As transcription factors, ERs are involved in the control of numerous physiological processes (Jia et al., 2015; Arnal et al., 2017). Oestrogens may exert their effects by activating the non-genomic pathways of ERs forming complexes with G proteins and growth factor receptors such as Insulinlike growth factor 1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR), which activate signaling cascades within seconds or minutes, and the MAPK/ERK and PI3K/AKT signaling pathways that play a role in preventing the apoptotic cascade (Marino & Ascenzi, 2008; Arnal et al., 2017). G protein-coupled oestrogen receptor 1 (GPER) can quickly and non-genomically change the expression of genes and regulate the rapid activation of PI3K/AKT and MAPK signaling pathways (Arnal et al., 2017). Hence, the activation of GPER results in the regulation of cell growth, cell migration and apoptotic cell death (Feldman & Limbird, 2017). GPER has been demonstrated to have significant functions in RL 95-2 cell carcinogenesis and in influencing the effects of oestrogen on proliferation and invasion (He et al., 2012). Caspase-9 phosphorylation (Thr125), a member of the cysteine proteases, is a cytoplasmic protein that initiates apoptosis (Shi, 2002). Phosphorylation of caspase-9 at Thr125 by ERK1/2 blocks caspase-9 activation, and ERK-mediated caspase-9 inhibition can suppress apoptosis by promoting cell survival (Allan et al., 2003). Caspase-9 has been reported to be inactivated by phosphorylation by AKT at Ser196 (Cardone et al., 1998). These results have encouraged in vivo and in vitro investigation of the therapeutic potential of inhibition of the MAPK/ERK and PI3K/AKT pathways to block cancer cell cycle progression and induce apoptosis. Using natural products to supplement traditional cancer treatment may effectively drive cancer cells to apoptosis by indirectly affecting caspases through the modulation of ER signaling. In this study, *Mc*FE and *Mc*SE inhibited the 17 β-oestradiol-induced migration and proliferation of ER-positive EC cells. *Mc*SE and *Mc*FE showed cytotoxic effects by inhibiting 17 β-oestradiol-activated non-genomic ER signaling kinases and caspase-9 phosphorylation. The anti-cancer and therapeutic activities of *M. charantia* extracts against various types of cancer have been evaluated in laboratory cancer cell line-based models and preclinical animal models. A naturally produced component of *M. charantia*, MAP30, has the potential to suppress the growth of liver cancer cells in both cell culture and xenograft-bearing nude mice (Fang et al., 2012). Another study demonstrated the anti-cancer properties of crude seed oil derived from Matale green bitter melon against experimentally generated hepatocellular carcinoma in Wistar rats (Ranasinghe et al., 2021). It has also been demonstrated that the pericarp and seed sections of *M. charantia* exhibit anti-cancer properties against colon cancer. (Venkatraman et al., 2021). Extracts from *M. charantia* similarly inhibited the migration of RL 95-2 cells in our investigation. In previous studies testing the effect of *M. charantia* on cell migration, extracts from various parts of the plant have been shown to suppress the migratory abilities of CL1 lung adenocarcinoma and U87 glioblastoma cells. (Hsu et al., 2012; Erdogan & Eroglu, 2022). The AKT pathway is the most commonly activated pathway in human cancers. In a study on Ishikawa H cells, treatment with *M. charantia* protein (MCP30) reduced cell proliferation in a time- and concentration-dependent manner and induced apoptosis through the AKT signaling pathway (Gu et al., 2017). The MAPK cascade is another signaling pathway critical for the survival of human cancer cells. In a previous study, *M. charantia* was shown to regulate the phosphorylation of p38 in ovarian cancer cell lines C13* and OV2008 (Yung et al., 2016). According to our previous study, *M. charantia* seed and aryl extracts induced apoptosis by upregulating the levels of caspase-9 and caspase-3 and inhibited the growth of breast cancer cells by blocking the phosphorylation of PI3K/AKT, p38MAPK and ERK1/2 pathways as downstream targets of EGFR signaling (Basaran et al., 2020). In another study, the IC_{50} value of the *M. charantia* seed extract was determined as 36.3 µg/mL in prostate cancer LNCaP cells. It was found that *M. charantia* seed ethanol extract significantly reduced dihydrotestosterone-induced non-genomic androgen receptor kinase activation and caspase-9 phosphorylation and suppressed cell proliferation and migration in androgen-sensitive LnCAP cell lines (Asta et al., 2023). In our previous study, *M. charantia* seed extract suppressed cell growth and migration by decreasing 17 β-oestradiol-induced non-genomic oestrogen signaling kinase activation and caspase-9 phosphorylation in ER-positive MCF-7 cells (Çınar & Cumaoğlu, 2023).

CONCLUSION

This study is the first to show that 17 β-oestradiol induces apoptosis in RL 95-2 cells by promoting caspase-9 phosphorylation and activation of non-genomic ER signaling kinases. *Mc*SE and *Mc*FE extracts were effective in reducing cell viability and the ability of migration by inhibiting the activation of non-genomic ER signaling kinases and phosphorylation of caspase-9 induced by 17 β-oestradiol in RL 95-2 cells. Based on the antiproliferative effect of *McSE and McFE* in EC cells, it was concluded that *M. charantia* may have a therapeutic role in the prevention of EC caused by high oestrogen exposure, and it is thought that further studies in this field may be helpful.

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