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## Review Article

# Effects Of Ethanol Extract From Foeniculum Vulgare On Human Mesenchymal Stem Cells' Osteogenesis

Jadhav Supriya\*, Nitin patil, Madhuri poulkar, Pawar Chaitanya, Padmaja giram

Department of pharmacology channabasweshwar pharmacy college, latur.

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## ABSTRACT

**Objective:**-In the modern day, osteoporosis, often known as silent illness, is a serious bone ailment that affects older women. Osteogenesis and the avoidance of bone fractures are significantly influenced by oestrogen. The side effects of hormone replacement treatment (HRT) typically include ovarian and breast malignancies. As a result, there is a growing need for plant phytoestrogen substitution. The goal of this work is to ascertain how a Foeniculum vulgare extract affects human mesenchymal stem cell proliferation and osteogenesis. **Materials and Methods:** - Isolated human mesenchymal stem cells were subjected to several concentrations of plant extracts (0.5 to 100 µg/ml). MTT assay was used to assess the cytotoxicity of the extract. **Result:**-The MTT assay and alkaline phosphates activity results indicated that the foeniculum vulgare extract, at 5 to 50 µg/ml, may have a beneficial effect on mineralization and cell proliferation. At a dosage of 5 µg/ml, the highest levels of proliferation and enzyme activity were seen. **Conclusions:** Foeniculum vulgare has been used in Iranian folk medicine for many years. Our in vitro study showed that Foeniculum vulgare extract has osteoprotective effects.

## INTRODUCTION

According to Gronholz (2008), osteoporosis is a condition that is highly common in the twenty-first century and is characterised by the degeneration of bone microarchitecture due to a decrease in bone density. An imbalance in the function of osteoblast and osteoclast cells, which results in bone resorption during the process of bone remodelling, is the primary cause of osteoporosis (Pagani et al.,

2005). A process of continual resorption and formation, bone remodelling involves restructuring already-formed bones (Meunier, Delmas et al., 1999 •). According to Kushida Takahadhi et al. (1995), bone disease arises when this process goes out of equilibrium. According to Christian et al. (1982), oestrogen inhibits bone loss via influencing the activity of osteoblasts and osteoclasts. Special proteins and growth factors

**\*Corresponding Author:** Jadhav Supriya

**Address:** Department of pharmacology channabasweshwar pharmacy college, latur

**Email** ✉: [supriyagjadhav08@gmail.com](mailto:supriyagjadhav08@gmail.com)

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are stimulated by oestrogen activation of osteoblasts (Tielens, Wymeersch et al., 2008). Because there is less hormonal regulation of osteoblast cell activity when oestrogen levels are lower, osteoclastic activity rises (Huang, Ettinger et al., 2007). The main treatment for osteoporosis over the past few decades has been hormone replacement therapy, or HRT (New, Robins et al., 2000). This technique lowers the risk of fractures and effectively stops bone loss in postmenopausal women. Because of the potential for breast and endometrial malignancies as well as the unintended side effects linked to these chemicals, there have been worries in recent years about employing this technique. The Goal of scientific study has therefore been to discover novel natural alternatives to pharmaceutical medications. Since plants are a rich source of bioactive secondary metabolites, they may offer a viable substitute for antiosteoporotic drugs. According to Horiuchi, Onouchi, et al. (2000), they can also break down into non-toxic compounds. Plant-derived phytoestrogens are non-estradiol phenolic chemicals that are thought to offer protection against hormone-related disorders, osteoporosis, and cardiovascular diseases (Horiuchi, Onouchi et al., 2000). Phytoestrogens are easily broken down and eliminated. Asian women who consume high doses of phytoestrogens have been shown in numerous studies to have lower rates of hip fractures and osteoporosis (Knight and Eden, 1996). Fennel (*Foeniculum vulgare* Mill) is an umbelliferous plant. According to Ozbek et al. (2003), the fruit and root infusions have anti-inflammatory, analgesic, oestrogenic, and relaxing properties. Herbal treatments for stomach and respiratory tract issues include fennel, which also helps nursing moms produce more milk (Choi et al., 2004; Amjad and Jafary 2000). Research has demonstrated that fennel seeds can improve milk production, encourage menstruation, reduce the symptoms of dysmenorrhea, and aid childbirth. In

order to evaluate the possible role of fennel root ethanolic extract in human mesenchymal stem cell proliferation and alkaline phosphatase enzyme activity based on its oestrogenic activity, the current investigation was carried out. All of the procedures employed in this investigation were performed at Tarbiat Modares University's Department of Hematology and the Hematology Research Centre of Shariati Hospital in Tehran.

### **Materials and Methods**

Gibco, BRL, Grand Island, NY, USA, supplied the foetal bovine serum (FBS), pen/strep, and Dulbecco's Modified Eagle's Medium (DMEM). Sigma-Aldrich, MO, USA, supplied the L-ascorbic acid,  $\beta$ -glycerophosphate, dexamethasone, and 17 $\beta$ -Estradiol (E2).

### **Preparation of plant extract**

After soaking dried and powdered fennel roots in 95% ethanol for 48 hours, FE was repeatedly extracted at room temperature using 70% ethanol (in water, v/v). The extract was filtered and concentrated using a revolving evaporator. The extract was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 20 mg/ml, and then diluted in culture medium to the working solution before use. The extract was stored at 4 °C after filtering.

### **Cell viability and proliferation assay**

hMSCs were cultivated at a density of  $5 \times 10^3$  per well on a 96-well flat-bottom plate. Cells were treated to 0.5, 1, 5, 10, 50, and 100  $\mu$ g/ml of fennel extract during a 24-hour incubation period. After that, the cells were cultured for one, two, or three days at 37 °C. Following the addition of 20  $\mu$ l of MTT (5  $\mu$ g/ml), the samples were incubated for four hours. After discarding the medium, the formazan salts were dissolved in 100  $\mu$ l of DMSO and left at 37 °C for 30 minutes. A reference wavelength of 630 nm was used to measure absorbance at 540 nm in order to analyse the final product's colour. The information is shown as a



percentage of cell viability relative to a control culture.

### **Osteogenic differentiation and treatment with fennel extract**

A density of  $1 \times 10^5$  hMSCs per well was seeded onto 12-well plates. The  $\alpha$ -MEM medium with osteogenic supplement, which contained DMED+10% FBS supplemented with 0.2 mM L-ascorbic acid-2-phosphate, 10nM dexamethasone, 10mM  $\beta$ -glycerophosphate, and varying concentrations of fennel extract, was used to stimulate osteogenesis once above 80% confluency was achieved. Every three days, a new medium was installed. Cells were taken on days 7 and 14 in order to measure ALP activity. In every experiment, tests were performed in triplicate.

### **Measurement of alkaline phosphates (ALP) activity**

As previously mentioned, hMSCs were cultivated in 35 mm culture dishes for 24 hours before being exposed to 0.5, 1, 5, and 10  $\mu\text{g/ml}$  of fennel extract for 15 days. As a control group, synthetic oestrogen 17 $\beta$ -estradiol (Sigma-aldrich, MO, USA) was used at a final concentration of  $10^{-8}$  M. 200  $\mu\text{l}$  of RIPA buffer was used to extract the total cell protein in order to measure the amount of alkaline phosphatase activity. After that, the lysate was centrifuged for 15 minutes at 4 °C and 14,000  $\times g$ . The ALP assay kit (Parsazmun, Tehran, Iran) was used to measure the ALP activity after the supernatant was collected. The kit's alkaline phosphatase was used as a standard, and p-nitrophenyl phosphate (p-NPP) was used as the substrate. The total protein content of the cell lysate was used to normalise the enzyme activity (IU). The ALP activity in this study is expressed as nmol (P-nitrophenyl)/mg protein.

### **Statistical analysis**

Data are expressed as mean  $\pm$  standard deviation (SD). Statistical significances were analyzed using the ANOVA test.  $p < 0.05$  was considered significant.

## **RESULTS**

The isolation of human mesenchymal stem cells was processed as previously mentioned. Figure 1 depicts the shape of human mesenchymal stem cells. Flow cytometry analysis was used to assess the phenotypic of hMSCs. The surface-positive markers of MSCs, CD44, CD73, and CD105, were measured following four cell passages. The cells were kept at 37°C in a humidified incubator that was 5% CO<sub>2</sub>-equilibrated. Alizarin red staining and differentiation to osteoblast were carried out for additional approval (Figure 2). Figure 3 illustrates how fennel extract affects the viability of hMSCs. The effects of extract were investigated at concentrations ranging from 1 to 100  $\mu\text{g/ml}$  during 24, 48, and 72-hour periods. All of the applied concentrations were hazardous for hMSCs, despite the fact that cell viability decreased at 100 $\mu\text{g/ml}$ . The proliferative activity of the treated samples and the control sample showed significant statistical differences ( $p < 0.05$ ).

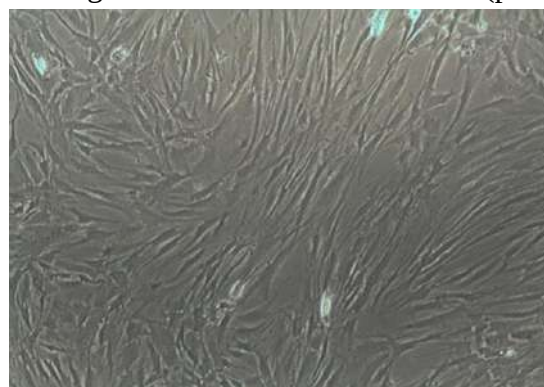


**Figure1 Morphology of human mesenchymal bone marrow stem cells before inducing with osteogenesis media**

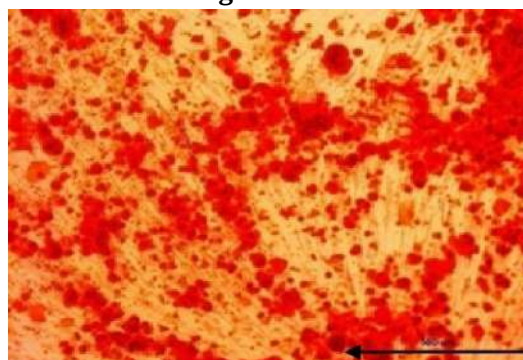


**Figure2**

Mesenchymal bone marrow stem cells after inducing with osteogenesis media. The Alizarin red staining was performed in day 21 of osteogenesis progress. Calcium nodule formation confirmed differentiation ability of the cells.

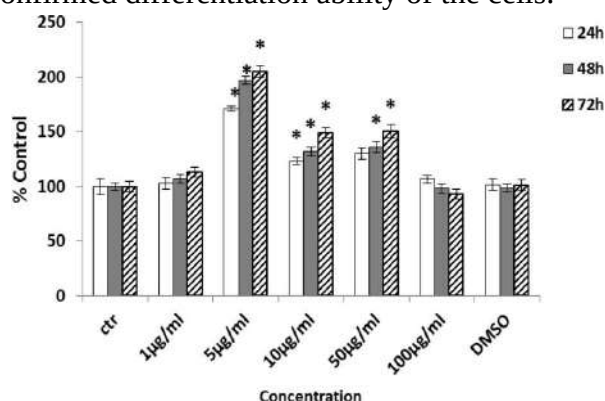


**Figure 3**

The extract dose-response increased the proliferation of hMSCs. hMSCs were cultured with medium and various concentrations extract (0.5 to 100 µg/ml) for three days. Each point represents the mean±SD. of four determinations. \*  $p < 0.05$ , compared with the control (Medium).

At 48 hours following treatment, the highest cell growth was noted at a dose of 5 µg/ml. Thus, we can conclude that the extract might promote the growth of human mesenchymal stem cells. Various concentrations of fennel extract or a carrier were used to cultivate hMSCs. During osteogenesis induction, E2 and ALP activity—a sign of osteoblast differentiation—were assessed. According to the results, fennel extract significantly raised the ALP activity of hMSCs in a dose-dependent manner when compared to the control (Figure 4). On day 14, greater activity was seen at concentration 1 µg/ml ( $p < 0.05$ ), which was equivalent with the E2 group.

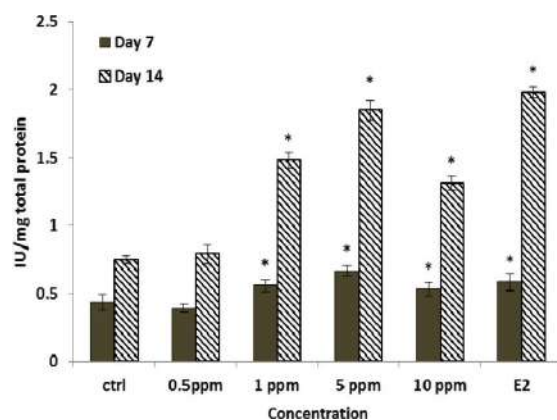


**Figure 4**

Effects of extract on ALP activities of hMSC cells. Cells were cultured in medium, ethanol extract (0.5 to 10 µg/ml), and E2 ( $10^{-8}$  M) for 15 days in presence of osteogenesis media, Results are expressed as means±SD (\*  $p < 0.05$ ), compared with the control (DMEM+10% FBS).

## DISCUSSION

As an in vitro model, well-known human bone marrow mesenchymal stem cells were used to examine the effects of *Foeniculum vulgare* ethanol extract on osteogenesis. We investigated hMSC cell growth and proliferation in vitro in this work. The findings demonstrated that ethanol extract at low concentrations could increase alkaline phosphatase enzyme activity and cell growth. Between 5 and 50 µg/ml, the proliferation of hMSCs was considerably enhanced, with the highest proliferation observed at 5 µg/ml of extract. Numerous previous studies have investigated the effects of plant extracts on cell growth. The effects of green tea catechin, for instance, were investigated on the MC3T3-E1 cell line at concentrations ranging from 1 to 100 µg/ml; the highest proliferation was noted at 1 µg/ml. These findings align with the current study's findings (Wei et al., 2011). Hormones and cytokines regulate the dynamic balance between bone creation and bone resorption, which is necessary for the skeleton's integrity (Kushida et al., 1995). The physiological regeneration of bones is significantly influenced by oestrogen, and

osteoporosis in older women following menopause is linked to a decline in blood oestrogen levels (Tielinse et al., 2008). Numerous studies have demonstrated estrogen's antiosteoporotic effects. According to Jung et al. (2010), oestrogen binds to the intracellular oestrogen receptor and modifies the synthesis of target proteins to affect osteoblasts and osteoclasts. Similar in structure to 17 $\beta$ -estradiol (E2), phytoestrogens are known to protect postmenopausal women against osteoporosis. According to News et al. (2000), these substances may have both oestrogenic (increasing uterine growth and preventing bone loss) and anti-estrogenic (increasing breast cancer cell proliferation) properties. The phytochemicals found in fennel extract are abundant, and many of these substances are good for human health. Because phytoestrogen and oestradiol work similarly, we looked at the possibility that phytochemicals from fennel extract would have oestrogenic effects in a cell culture system. By widely proliferating, differentiating, and secreting growth factors in the local microenvironment at the site of injury, stem cells can aid in bone regeneration. In this investigation, we showed that fennel extract (5–50  $\mu$ g/ml) can promote human MSC proliferation in a way that is dependent on both time and dose. exhibiting inhibitory effects at high concentrations (100 $\mu$ g/ml) and peaking at 72 hours. When cells were exposed to fennel extract, their proliferation pattern was biphasic. At low doses, the fennel extract promoted MSC growth; at comparatively high quantities, it became cytotoxic. Other phytoestrogens including genistein in soy beans, glabridin, and glabrene extracted from liquorice root have also been shown to have a similar biphasic impact (Zava et al., 1997; Somjen et al., 2004; Choi, 2005). The current study looked at how fennel extract affected the amount of ALP in human bone marrow mesenchymal stem cells. One osteoblastic

marker that plays a significant role in mineralisation is ALP activity. Our findings showed that, in comparison to the control group, the fennel extract considerably increased the ALP activity in the range of 1–10  $\mu$ g/ml, with the highest enzyme activity being seen at 5  $\mu$ g/ml. The greatest amount of ALP activity was seen at 1 nM, and as extract concentration increased, its inducing effects diminished. This observation aligns with a study conducted on the impact of plant extract from *Ulmus davidiana*. The MC3T3\_E1 preosteoblastic cell line was used to test the study, and the concentration ranged from 1 to 50  $\mu$ g/ml. The highest level of ALP activity was noted.

The presence of fennel extract markedly boosted ALP activity. It is well known that ALP plays a crucial role in the start of mineralisation during the development of bones. Extracellular matrices are made favourable for mineral deposition by its action in conjunction with that of other specialised bone proteins (Perets et al., 1996). After 4–14 days of fennel extract treatments, there was an up-regulation of ALP activities, with the highest activity occurring on day 14. The essential oil of *Foeniculum vulgare* has been the subject of numerous investigations (Bilia et al., 2002; Yamini et al., 2002). Trans-Anatole makes up almost 80% of the essential oil components of fennel. (Maurya Singh et al., 2006). Trans-Anatole seems to exhibit oestrogenic activity, as was previously described. Although more research is required to elucidate the role of other components of fennel extract in osteogenesis, it appears that trans-anatole may play a significant role in the antiosteoporotic effects of the extract (Nakagawa and Suzuki 2003; Tognolini, Ballabeni et al., 2007). This study demonstrated that fennel extract can promote the proliferation of human BMSCs and their differentiation into the osteoblast lineage by applying varying doses of the extract. It appears that more research is required to determine its

positive impacts and other factors that are pertinent to extrapolation to human exposure.

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