Toxic Effects of *Elaeagnus angustifolia* Fruit Extract on Chondrogenesis and Osteogenesis in Mouse Limb Buds

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Objectives: We determined the effect of *Elaeagnus angustifolia* extract on chondrogenesis and osteogenesis in mouse embryo limb buds in vitro and in vivo.
Limb bud mesenchyme from day 12.5 embryos were used for high-density micromass cultures. Water/alcohol extract was added to culture media at 10, 100, 1000 and 10000 µg/L. Cytotoxicity was tested with neutral red. Chondogenesis was detected by alcian blue and osteogenesis was detected by alizarin red S and alkaline phosphatase activity. For in vivo experiments, 40 pregnant mice were given 0.5, 5.0 or 50.0 mg/kg of the extract between days 8 and 18 of gestation. Embryos were stained with alizarin red S and alcian blue to measure femur and ossified region lengths. Total bone mass volume was measured stereometrically. Data were compared with ANOVA and LSD.
Results: In limb bud cultures 10 µg/mL of extract reduced chondrogenesis but not osteogenesis. Higher concentrations had no effect on chondrogenesis or osteogenesis. In pregnant mice 50 mg/kg of the extract significantly increased fetal femur and ossified zone length, but significantly decreased bone and cartilage volumes.
Significance: The extract had no favorable effects on chodrification or ossification and appeared to reduce chondrogenesis. This is in apparent contradiction to its empirical effects in human adults.

Key words: *Elaeagnus angustifolia*, chondrogenesis, osteogenesis, limb bud

INTRODUCTION

Elaeagnus angustifolia (Russian olive, Russian silverberry, Oleander), a plant native to Western Asia including Iran, is used as an anti-ulcerogenic [1], muscle relaxant [2], antinocioceptive and anti-inflammatory [3, 4] in traditional medicine. Extracts of *E. angustifolia* are also used to treat rheumatoid arthritis symptoms [5]. The extract contains a number of flavonoids [6] which are believed to be responsible for the plant's therapeutic effects. Flavonoids play different roles in biological processes such as chondrification and ossification. They act as antioxidants and are also agonists or antagonists of endogenous estrogen and can interact with several cellular receptors [7]. They can also protect cells against cell death [8]. Estrogens or phytoestrogens (flavonoids) inhibited DNA synthesis, creatine kinase and reactive oxygen species formation in osteoblasts [9]. In addition, flavonoids can reduce levels of glutathione and free radicals [8].

Oxidative stress influences chondrogenesis. Free radicals inhibit proteoglycan and collagen synthesis by chondrocytes [9]. Oxidative stress may change chondrification by impairing cell proliferation or cell death [10].

In mammals, flavonoids in the diet appear later in the amniotic fluid [12, 13], where they can pass though the placenta to the embryonic circulation [14]. It has been shown that flavonoids have anabolic effects and their uptake impacts bone formation in ovariectomized rats [15]. Flavonoids also increased calcium components in the diaphyseal tissue in a rat femoral culture system [16]. We hypothesized that the flavenoids in *E. angustifolia* might account for the use of this plant as a traditional remedy for bone and joint disorders. Therefore, the aims of this study were to investigate the effects of *E. angustifolia* on chondrogenesis and osteogenesis in an in vitro and in vivo mouse embryo model.

MATERIAL & METHODS

Extract preparation

Elaeagnus angustifolia extract was obtained with the percolation method. To obtain the water/alcohol extract, fresh fruits were obtained from a tree in growing on the campus of Fasa University of Medical Sciences in Fasa, southwestern Iran, during the late spring fruiting season. The fruit sample is deposited at the Fars Province Research Center for Agriculture and Natural Resources in Shiraz, with voucher number 14785. The fruits were dried in the shade. The dried fruits were powdered, 50 g of the powder was suspend-

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ed in 500 mL of water/alcohol and the suspension was percolated for 72 h. The solvent was evaporated and then dessicated under a vacuum. The final yield was 25 g powdered extract.

Limb bud assay

All experiments were performed in accordance with our center's guidelines for the ethical handling of animals. Pregnant mice were killed on day 12.5 of gestation. The embryos were removed and washed in PBS containing penicillin/streptomycin. The forelimbs and hindlimbs were cut away from the rest of the body and collected in PBS. The ectoderm was dissociated from the mesenchyme with 1 U/mL dispase (Sigma, St. Louis, MI, USA). The mesenchymal parts of the limb buds were suspended in 0.1% trypsin (Sigma). The cells were then washed to remove the trypsin and transferred to the culture media (DMEM/F12 (Gibco, Carlsbad, CA, USA) containing 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin). The mesenchymal cells were cultured at a density of 2.5×10^7cells/mL. Twenty microliters of the cell suspension was placed in the center of each well in 24-well plates. The cells were incubated at 37°C with 5% CO_2 for 90 min and then culture media containing 10, 100, 1000 or 10000 µg/mL of the extract was added. Osmolarity was checked in the solution with the highest concentration of extract before it was added to the cultures. The cells were cultured for 7 and 14 days.

Viability assay

On the 5th day of culture the media were discarded and micromasses were washed with PBS. One milliliter of 0.05% neutral red (wt/vol) in PBS was added to each well and cultures were left at 37°C for 2 h. The cells were fixed with calcium formol for 1 min and washed with PBS. One milliliter of acid alcohol (0.5% glacial acetic acid [vol/vol] in 50% ethanol) was added to each well and cultures were incubated for 2 more h. The acid alcohol was collected and the optic density of the elute was measured at a wavelength of 550 nm. The absorbance measured with this method was directly proportional to the surviving cells in each micromass culture [8].

Cartilage differentiation assay

The micromasses were washed with PBS and fixed by Kahle fixative for 20 min. The cells were washed with PBS and stained with 5% alcian blue, pH 2, overnight. The cultured cells were washed in ethanol to remove unbound dye and then washed four times with PBS. The stained cells were photographed by stereomicroscopy. Maturation and the presence of functional chondrocytes were verified by alcian blue staining. The alcian blue-stained foci were evidence of chondrogenesis. The foci in each micromass were counted with a dissecting microscope and the number of micromasses was used as an index of cellular differentiation [8].

Mineralization assay

The micromasses were also stained with alizarin red S. The cells were washed and fixed in 70% ethanol and stained with 5% alizarin red S in PBS overnight. The cells were washed with PBS and photographed by stereomicroscopy.

Alkaline phosphatase activity is involved in the initiation of inorganic phosphate generation for matrix mineralization [17]. For alkaline phosphatase staining, cells were fixed with citric acid/formaldehyde/acetone fixative, washed in PBS, and stained with alkaline phosphatase solution (Sodium Nitrite Solution, FRV-Alkaline Solution and Naphthol AS-BI Alkaline Solution (Sigma)) in the dark at room temperature for 15 min.

The intensity of the reaction, the number of nodules and the area of each were calculated with Java Image Analyses software (http://mac.softpedia.com/get/Graphics/ImageJ.shtml) after calibration to detect chondrogenic activity. All experiments were performed in triplicate.

In vivo study

Forty female balb/C mice aged 4–8 weeks were mated, and the observation of a vaginal plug was considered day zero of gestation. They were divided into four groups of 10 animals each: three experimental groups and one control group. Pregnant mice were fed a diet containing 0.5, 5.0 or 50.0 mg/kg of the *E. angustifolia* extract per day between days 8 and 18 of gestation. The control group was fed a diet containing the vehicle only. On day 18 the mice were killed under deep anesthesia and the embryos were removed. The embryos in each group were divided into two subgroups, one for ethanol fixation the other for formaldehyde fixation. All embryos were examined for congenital malformations by stereomicroscopy.

Whole-mount alcian blue/alizarin red staining

The embryos were fixed in absolute ethanol. The skin and viscera were removed and the embryos were then fixed in acetone and stained in 0.1% alcian blue/0.1% alizarin red in 70% ethanol. They were cleared by immersion in 20% glycerol in 1% KOH for 3 days and then in increasing proportions of absolute glycerol. The total length of the femurs and their ossified zone was measured under a stereomicroscope equipped with a ruled eye piece.

Stereological technique

We used the Cavalieri method to estimate bone and cartilage volume [18]. The formalin-fixed embryos were prepared histologically, sectioned with a microtome (Lieca, Wetzlar, Germany) and stained with hematoxylin and eosin. Ten to twelve sections were selected using a systematic sampling design with a random start method for stereological estimates (Howard and Reed, 1998). Each sample section was analyzed with a video-microscopy system comprising a microscope (E-200, Nikon, Tokyo, Japan) linked to a video camera (SONY SSC Dc 18P, Minato, Tokyo, Japan,), a P4 PC computer, and an LG monitor (795 FT Plus, Seoul, GuroGu, South Korea). We used stereology software designed ad hoc at our lab. Stereological points were superimposed upon the images of tissue sections and viewed on the monitor. Bone mass and cartilage mass volumes were estimated with the following formula:

$V(\text{total}) = \Sigma P.(a/p).t$

where V (total) is the bone mass and cartilage vol-

ume, Σ P is the sum of the points falling on the section profile, a/p is the area associated with each point, and t is the distance between section samples. a/p was calculated with the formula:

$(a/p) = (\Delta x \,.\, \Delta y)/m^2$

where Δx and Δy are the distance between two adjacent points of the grid on the x-axis or y-axis, respectively, and m is the final linear magnification of the microscopic images [18].

Statistical analyses

The data for each group were compared with analysis of variance (ANOVA) and the least significant difference method (LSD). All statistical analyses were done with SPSS v. 11.5 software for Windows and the data were graphed with Excel.

RESULTS

Osteogenesis and chondrogenesis in mouse fetuses

Fetuses from pregnant mice fed a diet containing 0.5, 5.0 or 50.0 mg/mL of the *E. angustifolia* extract showed no congenital malformations or limb deformities. Daily dietary supplementation with 50 mg/kg of the extract significantly increased embryo femur length ($P = 0.045$) and ossified zone length ($P = 0.049$) (Fig . 1).

Stereological studies

Total cartilage volume in the embryonic limb buds was reduced by feeding with 0.5 and 50.0 mg/kg of the extract ($P = 0.001$). Compared to the control group, total bone tissue volume increased in all experimental groups; however, statistical analyses showed that the increase was significant only for the 50 mg/kg concentration ($P = 0.042$) (Fig. 2).

Osteogenesis and chondrogenesis in limb bud mesenchymal cell cultures

Cell viability was assessed with neutral red staining. The extract at a concentration of 10 000 µg/mL significantly decreased cell viability ($P = 0.000$) (Fig. 3), and cells exposed to this concentration did not differentiate into chondrocytes or / and osteoblasts (Fig. 4). The plant extract at a concentration of 1000 µg/mL decreased viability significantly; however, the remaining viable cells were able to differentiate into cartilage and bone.

Cells exposed to the other doses survived and formed cartilage nodules. Alcian blue staining revealed the presence of proteoglycans secreted by chondrocytes into the matrix. Cells exposed to 10 and 1000 µg/mL of the extract formed significantly fewer nodules ($P = 0.000$, Fig. 5). At 10 µg/mL, the extract reduced nodule area (Table 1). Alcian blue staining showed that proteoglycan secretion by differentiated chondrocytes did not change in response to exposure to the extract (Fig. 5, Table 1).

Mineralization assays showed that cultured cells were able to calcify the matrix; however, we found no significant differences in calcium content between cells cultured in the presence of the extract and control cultures (Fig. 6). Alkaline phosphatase activity confirmed that mesenchymal cells differentiated toward osteoblasts (Fig. 7). Alkaline phosphatase activity was weaker in cells cultured with the lowest concentration of *E. angustifolia* fruit extract (10 µg/m); however, the difference in staining intensity compared to the higher doses of extract was not significant.

DISCUSSION

Elaeagnus angustifolia fruit extract has been used in traditional folk medicine as a remedy to treat numerous diseases. It has been shown that the administration of the extract improves arthritis symptoms including joint pain, inflammation and limited movement [5]. However, its mechanism of action is unknown. *Elaeagnus angustifolia* fruits have anti-nociceptive and anti-inflammatory effects [4]. However, its use in folk medicine suggests that it may affect condrification and ossification processes. Our in vitro results showed that 1000 µg/mL of the extract reduced cell survival. High cell density can induce the mesenchymal cells to differentiate into the chondroblasts [19]. At 1000 µg/mL, cell density decreased; therefore the number of differentiated nodules was reduced. Lower concentrations of the extract led to a decrease in the number and the area of the nodules. Stereological study confirmed that bone and cartilage volumes were reduced by feeding the animals with a high dose of the extract. The difference in the effective concentrations in vitro and in vivo may be attributed to differences in the bioavailability of the extract components.

The highest dose (50 mg/kg) of extract in the diet of pregnant mice increased total femur length and ossified zone length, but decreased cartilage and bone volume. These data indicated that feeding with the extract may have led to enlargement of the marrow spaces compared to the control group.

Elaeagnus angustifolia contains antioxidants [20] that may impact chondrification and ossification [11]. However, our in vitro findings showed that cell viability did not improve after incubation of the cells with the extract. As the neutral red assay showed, the highest dose of the extract was even toxic. Therefore, it seems that the effects of the *E. angustifolia* extract on improvements in chondrification are not due to its antioxidant contents.

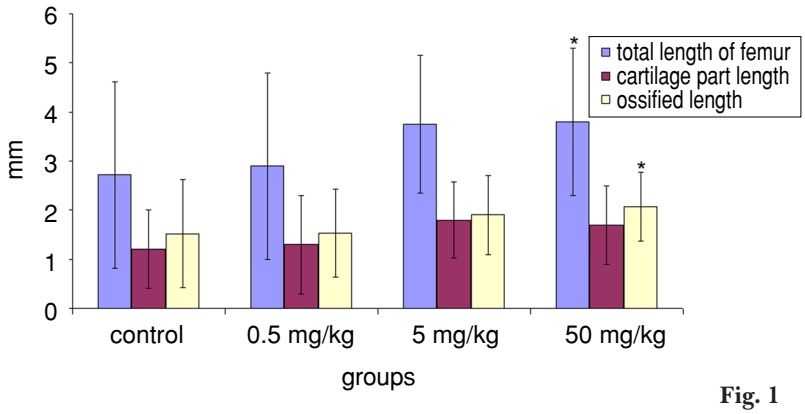
Earlier work found that phytoestrogens, known as natural estrogens [21], are able to stimulate the transcriptional activity of the human estrogen receptor expressed in cultured cells by transient transfection. It has been shown that the *E. angustifolia* extract is rich in phytoestrogens such as flavonoids and tannins [2, 20]. Estrogens can stimulate bone formation [22]. Flavonoids have anabolic effects on bone components; however, not all flavonoids have the same effects on bone calcium content: some reduce calcium whereas others have no effect [16]. Our in vivo findings showed that the extract decreased bone and cartilage volume. Our in vitro data showed that the extract was more effective at lower doses. However, the extract did not modify osteoblast differentiation or calcium resorption.

Cartilage is a sex hormone-responsive tissue. In vitro differentiation of human mesenchymal stem cell into chondrocyte can be inhibited via a non-classic estrogen signaling pathway, G protein receptor 30 (GPR 30) [23]. Phytoestrogens have been also demonstrated that act through GPR30. Isoflovonoids such as genistein

Table 1 Nodule area, nodule number and alcian blue intensity after exposure to different doses of Elaeagnus angustifolia extract

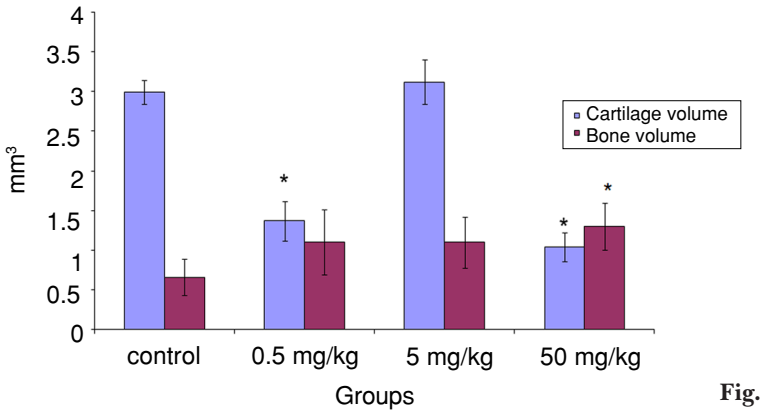
Groups	Mean of the area of the nodules (μm² ± SD)	Mean of the number of the nodules ± SD	Mean of the intensity of the reaction (Micropixel ± SD)
Control	9.64 ± 2.84	419.66 ± 12.09	98.87 ± 3.6
10 μg/mL	6.59 ± 2.16*	183.65 ± 11.56*	83.92 ± 16.03
100 μg/mL	10.28 ± 3.01	457.66 ± 20.79	81.17 ± 6.5
1000 μg/mL	9.35 ± 3.21	363.83 ± 47.18*	88.92 ± 14.34

*Significantly different from control
Data for 10000 μg/mL are not shown because this concentration of the extract was toxic and no nodules formed.



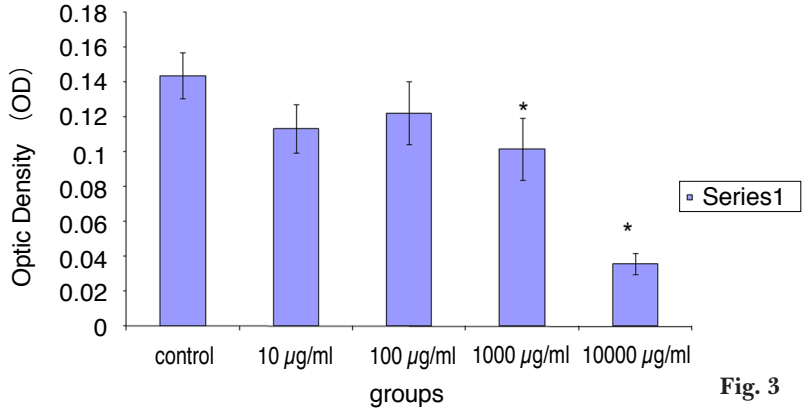
*Significant difference with control ($p < 0.05$)

Fig. 1 Total length of the femur and cartilagenous and ossified parts of the femur in the the control group and the different dose groups



*Significant difference with control ($p < 0.05$)

Fig. 2 Cartilage and bone volume in embryos from pregnant mice treated with different doses of *Elaeagnus angustifolia* extract.



*Significant difference with control ($p < 0.05$)

Fig. 3 Viability of mesenchymal cells cultured with different doses of *Elaeagnus angustifolia* extract.

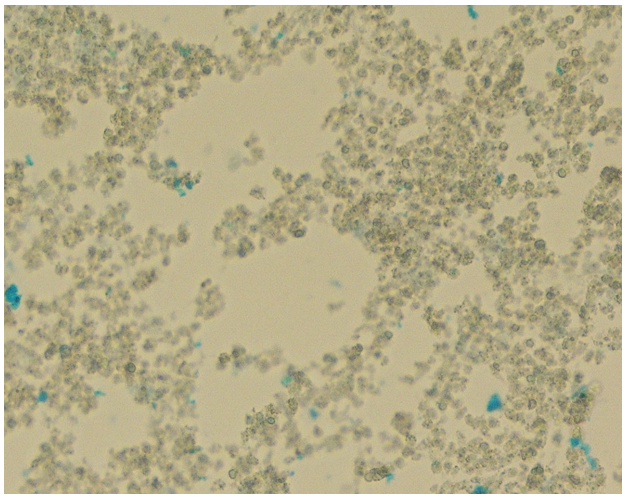


Fig. 4 Mesenchymal cells exposed to 10000 µg/mL of the extract. No cartilage was formed.

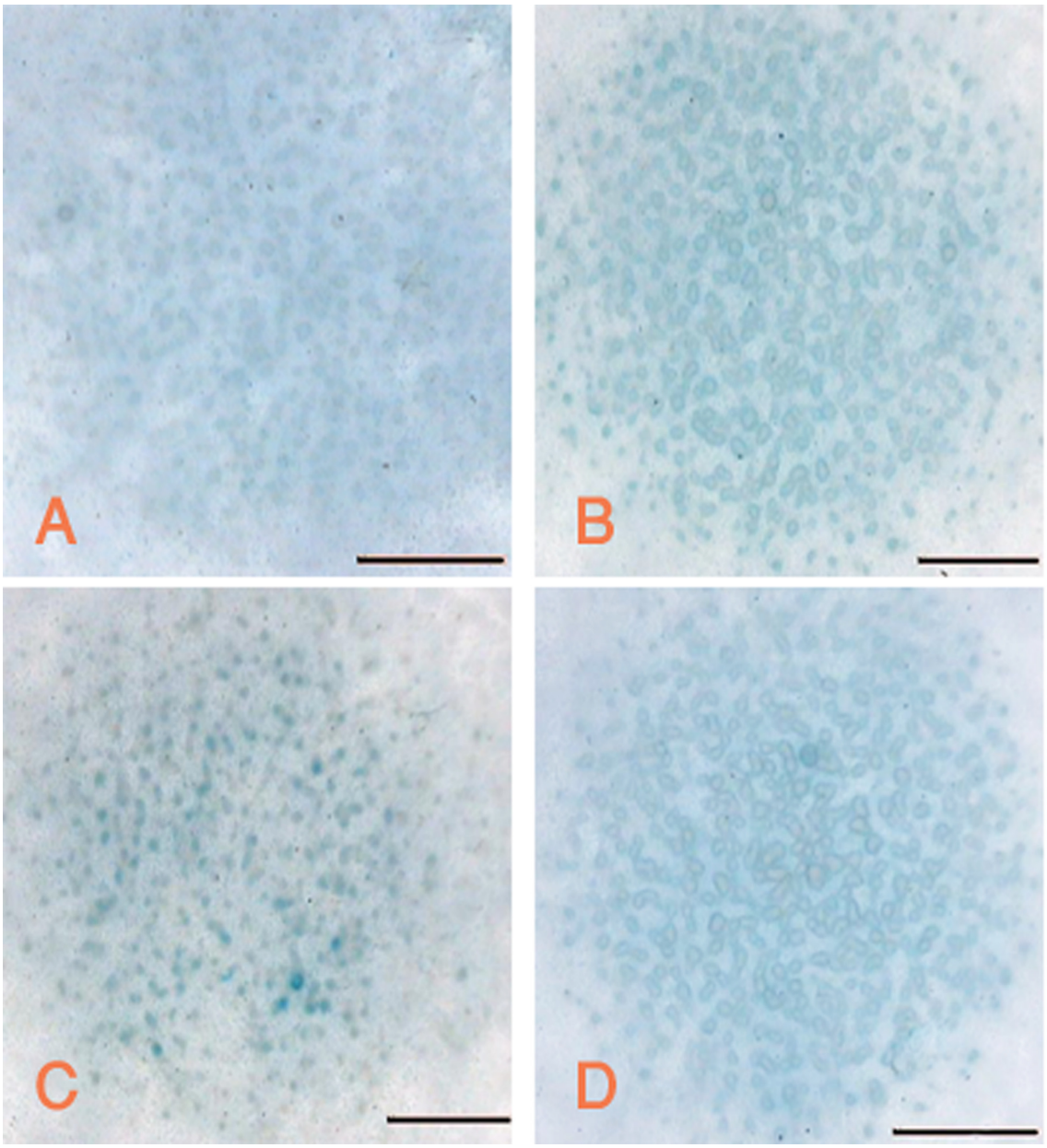


Fig. 5 Mesenchymal cells exposed to 10 µg/mL (A), 100 µg/mL (B) or 1000 µg/mL (C) of the extract, and control cells (D), stained with alcian blue. Cells exposed to 10 µg/mL of the extract had fewer nodules and less proteoglycan secretion. Scale bar = 1 mm.

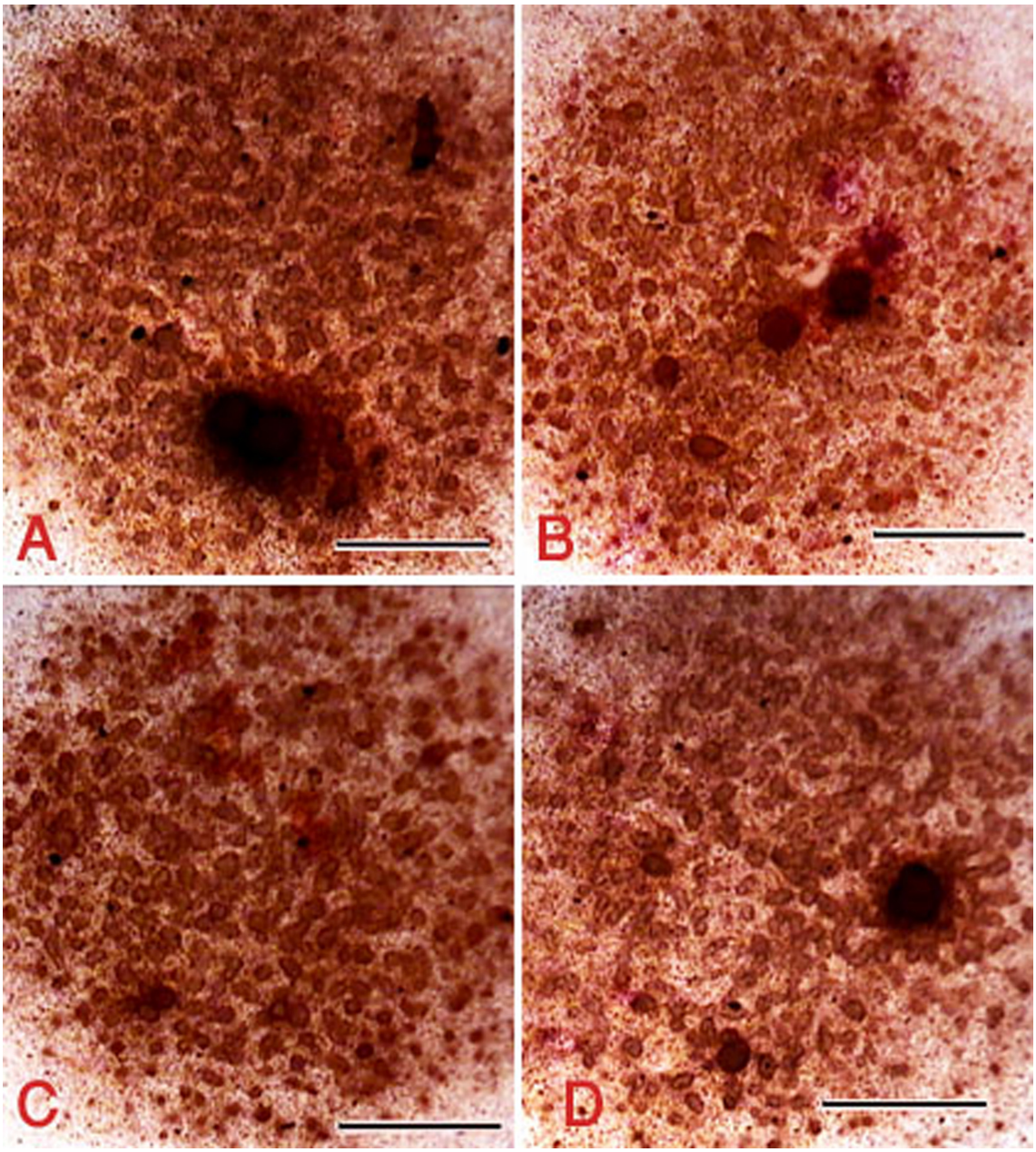


Fig. 6 Mesenchymal cells exposed to 10 µg/mL (A), 100 µg/mL (B) or 1000 µg/mL (C) of the extract, and control cells (D) stained with alizarin red /S after 14 days. No difference was observed in the amount of calcification in matrix. Scale bar = 1 mm.

act as agonist or antagonist of estrogen in a dose-dependent manner [24]. Our in vitro data showed that the extract reduced chondrocyte differentiation but did not influence osteoblast differentiation. Although, previous reports showed that GPR30 is expressed in human osteoblasts, osteoclasts, and osteocytes [25], GPR30 seems not to be a functional estrogen receptor in bone [26]. However, it can affect chondrocyte differentiation via GPR30 [23] in a dose dependent manner. Our data suggest that the extract may exert an effect on the chondrogenic potential of mesenchymal cells through GPR30.

Various flavonoids in foods affect bone calcium content and osteoclastogenesis. Some flavonoids were found to inhibit osteoclastogenesis and bone resorption instead of stimulating bone formation in vitro [16]. Our results show that the extract had detrimental effects on chondrogenesis at low doses. The alizarin red S test showed that calcium content did not change after cells were exposed to the extract, and the alkaline phosphatase test showed that calcification was not influenced by the extract.

Flavonoids have different effects on cell proliferation. Some inhibit cell growth in a dose-dependent manner, whereas others have no effect [27]. Flavonoids can also act as agonists or antagonists of estrogens [28]. Therefore, they can exert various effects on bone formation. Cell proliferation may increase cell density, which in turn influences the chondrification process. The synergistic effects and interactions of the components present in the extract may partly explain the different biological effects of lower doses compared to higher doses.

In conclusion, our results suggest that *E. angustifolia*

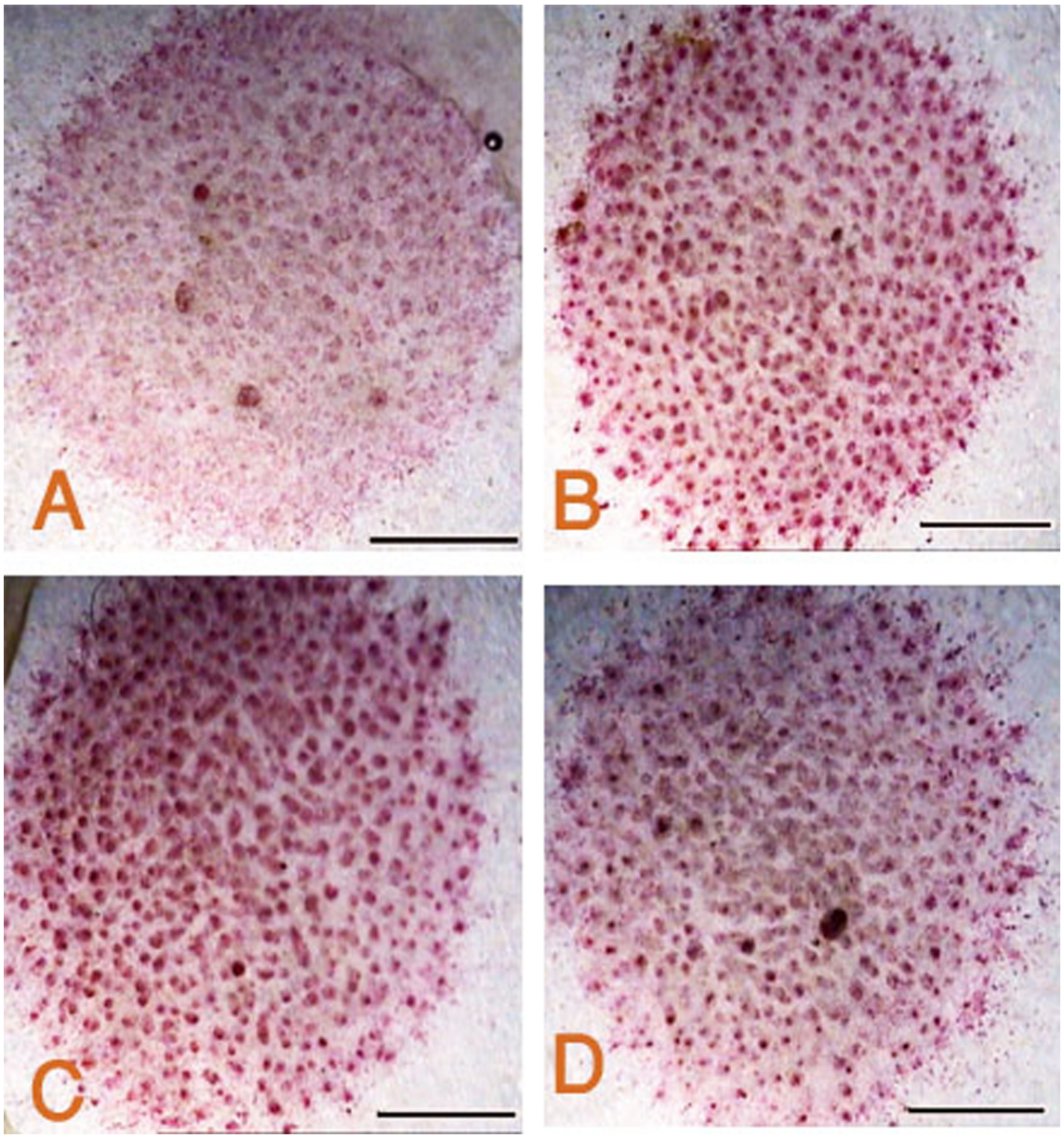


Fig. 7 Mesenchymal cells exposed to 10 µg/mL (A), 100 µg/mL (B) or 1000 µg/mL (C) of the extract, and control cells (D) stained with alkaline phosphatase after 14 days. Cells exposed to 10 µg/mL of the extract had less alkaline phosphatase activity. Scale bar = 1 mm.

extract improved osteogenesis and chondrogenesis , but also reduced bone mass in mouse embryos. However, it had no effect on calcium content. These findings are inconsistent with the use of this plant in traditional medicine. Our results indicate that pregnant mothers should avoid using traditional remedies that contain *E. angustifolia* to treat bone and joint ailments.

Regarding the benefit to biodiversity resources of COP10, avoiding the use of this plant means indirect attempt to prevent its wide use which could lead to its extinction in long-term.

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