# Effect of olive oil phenolic compounds on osteoblast differentiation

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## ABSTRACT

**Background:** Osteoporosis is a skeletal disorder characterized by compromised bone strength that predisposes individuals to an increased risk of fracture. Previous *in vivo* and *in vitro* studies have reported that phenolic compounds present in extra virgin olive oil have a beneficial effect on osteoblasts in terms of increase cell proliferation. The aim of this study was to determine whether phenolic compounds present in olive oil could modify the expression of cell differentiation markers on osteoblasts.

**Study Design:** An *in vitro* experimental design was peformed using MG-63 osteoblasts cell line.

**Methods:** MG63 cells were exposed to different doses of luteolin, apigenin, or p-coumaric, caffeic, or ferulic acid. Alkaline phosphatase (ALP) was evaluated by spectrophotometry and antigen expression (CD54, CD80, CD86, and HLA-DR) by flow cytometry.

**Results:** At 24 h, treated groups showed an increased ALP and modulated antigen profile, with respect to the non-treated group.

**Conclusion:** These results demonstrate that the phenolic compounds studied induce cell maturation *in vitro*, increasing ALP synthesis and reducing the expression of antigens involved in immune functions of the osteoblast which would improve bone density.

**Keywords:** phenolic compounds, *olea europaea,* osteoblastic cells, phenotype profile, alkaline phosphatase activity.

**Abbreviations:** ALP, Alkaline phosphatase; CD54, Cluster of Differentiation 54; CD80, Cluster of Differentiation 80; CD86, Cluster of Differentiation 86, HLA-DR, Human Leucocyte Antigen D-Related, DMEM, Dulbecco's Modified Eagle Medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FBS, fetal bovine serum; EDTA, ethylene diamine tetra-acetic acid; PBS, phosphate-buffered saline; mAb, monoclonal antibody; FITC, Fluorescein-Isothiocyanate; TGF-β1, Transforming Growth Factor beta 1, IL1β, Interleukin-1 beta; IFNγ, Interferon gamma; LPS, Lipopolysaccharide, IL-4,12,15,18, Interleukin 4,12,15,18; FGF, Fibroblast Growth Factor; PDGF, Platelet-Derived Growth Factor; DCs, Dendritic cells.

## 1. INTRODUCTION

Bone metabolic diseases develop when there is an imbalance between the formation and resorption of bone, that depend on the interaction between osteoblasts and osteoclasts. Osteoporosis is the most common bone metabolic disease in adults, especially among the elderly, which is characterized by compromised bone strength that predisposes individuals to an increased risk of fracture. Most current pharmacological approaches focus on inhibiting bone resorption in patients with osteoporosis or at risk of developing the disease [1,2]. However, researchers have also provided evidence on dietary components that can optimize bone mass and stimulate bone formation [3]. Thus, Habauzit & Horcajada (2008) reported that phytonutrients in our diet, especially polyphenols, can act on both osteoblasts and osteoclasts to modulate bone metabolism.

Bone formation involves a complex series of events, including osteoprogenitor cell proliferation and differentiation, which eventually result in the formation of a mineralized extracellular matrix. Numerous cytokines, hormones, and growth factors control bone formation by regulating osteoblast cell proliferation and differentiation [5]. Bone is a dynamic tissue under continuous remodeling process where osteoblasts, which derive from undifferentiated pluripotent mesenchymal cells [6], are responsible for bone development. It has been described that osteoblasts have immunological functions because the expression of markers of antigen-presenting capacity (CD54, CD80, CD86, HLA-DR) which is modulated in the presence of cytokines, growth factors, platelet-rich plasma, bacterial lipopolysaccharide, or even certain pharmaceuticals [7–12].

Olive oil is the main fat used in the Mediterranean Diet and contains numerous minor compounds, including phenolic compounds, that can be divided among phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavones [13–15].

It has been demonstrated that phenolic compounds can modulate osteoblast cell functions [16–20]. However, to our knowledge, no data are available on the ability of olive oil phenolic compounds to regulate cultured osteoblast differentiation. The objective of the present study was to determine the effect of phenolic compounds on osteoblastic (MG-63) cell differentiation by evaluating the antigenic profile and alkaline phosphatase (ALP) activities.

## 2. MATERIAL AND METHODS

### 2.1. Study Design

An *in vitro* experimental design was performed using MG-63 osteoblasts cell line.

### 2.2. Chemicals compounds

Standards of apigenin, luteolin and caffeic, p-coumaric, and ferulic acids were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of phenolic compounds were prepared in methanol and stored at -20ºC. All solvents used were of analytical or HPLC grade (Sigma-Aldrich). Water was of Milli-Q quality (Millipore Corp, Bedford, MA).

### 2.3. Cell Culture

The human MG-63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5 μg/mL amphotericin B (Sigma, St Louis, MO, USA), and 1% glutamine (Sigma, St Louis, MO, USA), 2% HEPES (Sigma, St Louis, MO, USA), supplemented with 10% fetal bovine

serum (FBS) (Gibco, Paisley, UK). Cultures were kept at 37ºC in a humidified atmosphere of 95% air and 5% $CO_2$. Cells were detached from culture flask with a solution of 0.05% trypsin (Sigma, St Louis, MO, USA) and 0.02% ethylene diamine tetra-acetic acid (EDTA) (Sigma, St Louis, MO, USA) and were then washed and suspended in complete culture medium with 10% FBS.

### 2.4. Antigenic phenotype by flow cytometry

Osteoblasts were previously treated with $10^{-6}$M and $10^{-8}$M of apigenin, luteolin, or p-coumaric, ferulic, or caffeic acid for 24 h at 37ºC. Cells were then detached from the cultured flask by treatment with 0.4% EDTA solution, washed and suspended in phosphate-buffered saline (PBS) at $2x10^4$cells/mL. Cells were labeled by direct staining with anti-CD54, CD80, CD86, and HLA-DR monoclonal antibodies (mAbs) (IOL1b, CD80, CD86, and OKDR, respectively; all from Invitrogen Corp, Carlsbad, CA). The fluorochrome used to label the antibody was Fluorescein-Isothiocyanate. Aliquots of 100 µl of the cell suspension were incubated with 10 µl of the appropriate mAb for 30 min at 4 ºC in the dark. Cells were washed, suspended in 1 mL of PBS and immediately analyzed in a flow cytometer with diode laser (FASC Canton II, SE Becton Dickinon, Palo Alto, CAL) at a wavelength of 488 nm to determine the percentage of fluorescent cells. Untreated cells were stained with mAbs as negative control. The percentage of antibody-positive cells was calculated from counts of 2000- 3000 cells. At least three experiments were run for each antigen in all cultures.

### 2.5. Alkaline phosphatase activity

MG-63 cells were treated at confluence with culture medium containing 10mM β-glycerophosphate and 50µg/mL ascorbic acid to promote differentiation. After 6 days, cells were incubated with $10^{-6}$M and $10^{-8}$M of apigenin, luteolin, or p-coumaric, ferulic, or caffeic

acid for 48 h at 37ºC in a humidified atmosphere of 95% air and 5% $CO_2$. Cells were lysed with 0.1% Triton X-100 at 37ºC. The samples were centrifuged at 1500 rpm and the supernatants stored at -70ºC until used. ALP activity was quantified with a colorimetric assay (Diagnostic kit 104-LL, Sigma, St. Louis, MO) using p-nitrophenylphosphate as substrate. An aliquot (50µL) of cell lysate solution was added to 50µL of ALP substrate (Sigma) and was incubated at 37ºC for 45min in darkness. The enzymatic reaction was stopped by adding 50µL of 0.1M NaOH, and the absorbance was measured at 405 nm with a spectrophotometer (Biotek ELx800). The total protein content was estimated by the Bradford method using a protein assay kit from Bio-Rad Laboratories (Nazareth-Eke, Belgium). All samples were run in triplicate, and the ALP activity was expressed as a percentage, considering enzymatic activity in the absence of phenolic compounds as 100%. All assays were compared to a control group of cells grown under the same conditions but not treated.

### 2.6. Statistical analysis

Data were expressed as means ± standard deviation (SD) and compared using the Student's *t*-test. $P < 0.05$ was considered significant in all tests. At least three experiments were performed for each culture. SPSS version 20.0 (IBM, Chicago, IL) was used for all data analyses.

## 3. RESULTS

### Antigenic phenotype by flow cytometry

Flow cytometry results showed a significant decrease in CD54 expression *versus* untreated control cells ($p \leq 0.001$) after treatment with each dose of each phenolic compound tested (Table 1). A significant decrease in CD80 expression was observed after treatment with apigenin or ferulic or p-coumaric acid at doses of $10^{-6}$M (p=0.006, p=0.025, and p=0.012, respectively) and $10^{-8}$M (p=0.007, p=0.04, and p=0.007 respectively). Ferulic acid increased the expression of

CD86 or HLA-DR at all doses ($p<0.001$) and caffeic acid increased CD86 expression at doses of $10^{-6}$ and $10^{-8}$M ($p<0.001$).

**Effect of phenolic compound on alkaline phosphatase activity**

The presence in the osteogenic medium of apigenin, ferulic acid, p-coumaric acid, luteolin, and caffeic acid had a positive effect ($p=0.084$) on the ALP activity of the MG63 cells at all doses except for the lowest doses of luteolin and caffeic acid (Table 2).

## 4. DISCUSSION

The present results demonstrate that phenolic compounds found in extra virgin olive oil exert an action on various cell parameters. With regard to cell differentiation, doses of $10^{-6}$M and $10^{-8}$M of each compound produced a major and significant increase in ALP activity, suggesting a favorable effect on the maturation process. The strongest effect on ALP was obtained with luteolin at higher doses.

It has been demonstrated that different phenolic compounds in vegetable species (e.g., resveratrol in wine or quercetin in grape or onion) can modulate osteoblastic functions, such as their proliferative capacity or cell maturation, by increasing ALP activity and calcium ion deposition in the extracellular matrix [16,18–20]. In that sense, previous studies have demonstrated that exposure of osteoblasts to phenolic compounds in olive oil, such as, hydroxytyrosol, luteolin, apigenin, p-coumaric, caffeic, and ferulic acids resulted in an increase in the number of cells in 24 h. Other researches have studied different phenolic extracts which stimulated MG-63 cell growth, and they induced higher cell proliferation rates than individual compounds [17,21,22].

Osteoblast lineage differentiation is a complex process characterized by the sequential and defined expression of tissue-specific genes that permit the identification of three phases: proliferation, maturation, and extracellular matrix synthesis/mineralization. Specific genes for the differentiated osteoblast phenotype are expressed in the second phase, including genes that encode for ALP and osteocalcin, producing a high level of ALP synthesis [23].

With regard to the effect of polyphenols on bone cell function, it is known, for example, that resveratrol can modify osteoblast function by modulating transcription factors such as Cbfa1/Runx2 and bone morphogenetic proteins such as osterix and osteocalcin, all essential molecules in the induction of osteoblastic differentiation that can in turn activate different genes involved in these processes [16,24,25]. It has been described that the consumption of olives, olive oil, and oleuropein can prevent the loss of bone mass in animal and cell models [26] . In this sense, Santiago-Mora *et al* (2011) found that oleuropein, a secoiridoid polyphenol, increases the expression of Runx2 and osterix, indicating that oleuropein enhances osteoblast formation in both initial and later phases of differentiation. The role of oleuropein in osteoblast differentiation was supported by the higher expression levels of other osteoblast markers, such as ALP gene expression/activity or collagen type I gene expression after exposure to this secoiridoid. In line with these findings, we have observed that other polyphenols, i.e., apigenin, luteolin, and ferulic, p-coumaric and caffeic acid, which are found in olive oil, also produce a significant increase in ALP synthesis, favoring osteoblastic differentiation.

Treatment with luteolin or apigenin or caffeic, ferulic, or p-coumaric acid produces a major decrease in the expression of CD54 and CD80 antigens and modulates the expression of CD86 and HLA-DR. Previous studies have described that human osteoblasts obtained by primary culture from bone samples showed a significantly reduced expression of CD54 and CD86 after *in vitro* Transforming Growth Factor beta 1 (TGF-β1) treatment but a significantly increased

expression of these co-stimulatory molecules in response to Interleukin-1 beta (IL-1β), Interferon gamma (IFNγ), or Lipopolysaccharide (LPS) [7]. In another study, osteoblastic expression of Interleukin 4,12,15,18 (IL-4, IL-12, IL-15, IL-18), and IFNγ cytokines was similarly modified by different growth factors (Fibroblast Growth Factor (FGF), TGF-β1, and Platelet-Derived Growth Factor (PDGF)) and cytokines (IL-1 and IFNγ) [11]. This accumulated evidence led the authors to propose that the functional capacity of osteoblasts is modulated during their differentiation and maturation, with a gain in their bone-forming function at the expense of their immunological function [11]. Schett (2011) further corroborates this hypothesis by showing that proinflammatory cytokines (such as IL-1, TNFα and IL-17) are involved in bone loss. The present results also support this hypothesis, with observations of an increase in ALP activity and a parallel decrease in the expression of CD54 and CD80. The presence of any tested dose of ferulic or caffeic acid produced a major increase in the expression of CD86 and HLA-DR, which may be associated with a state of cell activation, given that their expression has been related to the degree of differentiation and/or cellular activation in dendritic cells (DCs) [29]. Osteoblasts and DCs share features in common, including cytokine synthesis, phagocytic capacity, antigen presentation to T lymphocytes, and the expression of certain antigens, such as CD54, CD80, CD86, and HLA-DR [11,12,29]. For this reason, the two cell populations have been described as antigenically, morphologically, and functionally related [12]. Both the increase in ALP synthesis and the modulated expression of the co-stimulatory molecules may be related to a greater synthesis of TGF-β1 [30,31].

Based on the present findings, we conclude that the phenolic compounds studied induce cell maturation *in vitro,* favoring formation of the extracellular matrix, as indicated by the increase in ALP synthesis and loss of the expression of antigens involved in osteoblastic immune functions. Further research is warranted to confirm the mechanism by which these phenolic compounds favor cell maturation.

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## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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## TABLES

**Table 1.** Expression of different antigens in MG63 cell line after 24 hours of incubation with different doses of phenolic compounds by flow cytometry.

| | CD54 | | CD80 | | CD86 | | HLA-DR | |
|---|---|---|---|---|---|---|---|---|
| | Mean sd[a] | P value | Mean sd[a] | p value | Mean sd[a] | P value | Mean sd[a] | P value |
| **Control** | 75.13 (4.5) | - | 21.86 (2.81) | - | 13.33 (0.25) | - | 4.60 (0.81) | - |
| **Luteolin$10^{-6}$ M** | 2.06 (0.70) | 0.0001*** | 17.80 (3.20) | 0.17 | 12.40 (0.45) | 0.037* | 5.23 (0.90) | 0.42 |
| **Luteolin $10^{-8}$ M** | 2.36 (0.45) | 0.001*** | 22.46 (0.76) | 0.74 | 13.96 (0.68) | 0.20 | 4.96 (0.60) | 0.56 |
| **Apigenin $10^{-6}$M** | 1.60 (0.26) | 0.001*** | 10.13 (1.96) | 0.006** | 14.33 (1.27) | 0.25 | 7.60 (0.96) | 0.016* |
| **Apigenin $10^{-8}$M** | 0.86 (0.57) | 0.001*** | 10.30 (1.66) | 0.007** | 11.50 (1.21) | 0.063 | 5.96 (1.74) | 0.31 |
| **Cafeic acid $10^{-6}$ M** | 3.16 (0.41) | 0.001*** | 16.43 (1.61) | 0.058 | 17.63 (0.90) | 0.010* | 5.46 (0.56) | 0.21 |
| **Cafeic acid $10^{-8}$ M** | 3.20 (1.12) | 0.001*** | 18.90 (1.15) | 0.20 | 19.00 (1.64) | 0.004** | 5.23 (0.51) | 0.33 |
| **Ferulic acid $10^{-6}$M** | 0.93 (0.25) | 0.001*** | 15.90 (0.88) | 0.025* | 29.13 (1.95) | 0.005** | 37.50 (3.67) | 0.0001*** |
| **Ferulic acid $10^{-8}$ M** | 1.06 (0.15) | 0.001*** | 15.60 (1.93) | 0.04* | 33.16 (0.72) | 0.0001*** | 36.30 (1.21) | 0.0001*** |
| **Cumaric acid $10^{-6}$ M** | 1.76 (0.25) | 0.001*** | 12.26 (1.62) | 0.012* | 13.96 (1.04) | 0.40 | 7.00 (1.57) | 0.10 |
| **Cumaric acid $10^{-8}$M** | 0.90 (0.10) | 0.001*** | 7.33 (1.02) | 0.007** | 12.53 (0.49) | 0.08 | 5.23 (0.11) | 0.31 |

[a] standar deviation. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$

**Table 2.** Alkaline phosphatase (ALP) activity in MG63 cell line after 48 h of treatment with different doses of phenolic compounds. Data are expressed as U/mg protein.

| | % of expression | sd[a] | P value |
|---|---|---|---|
| **Control** | 100 | | - |
| **Luteolin$10^{-6}$ M** | 209.09 | 8.69 | 0.008** |
| **Luteolin $10^{-8}$ M** | 81.81 | 11.11 | 0.08 |
| **Apigenin $10^{-6}$M** | 209.08 | 8.51 | 0.014* |
| **Apigenin $10^{-8}$M** | 145.45 | 18.75 | 0.05* |
| **Cafeic acid $10^{-6}$ M** | 145.00 | 6.24 | 0.006** |
| **Cafeic acid $10^{-8}$ M** | 127.27 | 7.14 | 0.016* |
| **Ferulic acid $10^{-6}$M** | 209.90 | 9.37 | 0.007** |
| **Ferulic acid $10^{-8}$ M** | 209.09 | 8.69 | 0.015* |
| **Cumaric acid $10^{-6}$ M** | 145.45 | 18.75 | 0.045* |
| **Cumaric acid $10^{-8}$M** | 100 | 9.09 | 0.68 |

[a]standar deviation, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$