

# Oleocanthal Inhibits Proliferation and MIP-1 $\alpha$ Expression in Human Multiple Myeloma Cells

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**Abstract:** Multiple myeloma (MM) is a plasma cell malignancy that causes devastating bone destruction by activating osteoclasts in the bone marrow milieu. MM is the second of all hematological malignancies. Thus, the search for new pharmacological weapons is under intensive investigation being MM a critically important public health goal. Recently, it has been demonstrated that macrophage inflammatory protein 1- $\alpha$  (MIP-1 $\alpha$ ) is crucially involved in the development of osteolytic bone lesions in MM. Phenolic components of extra virgin olive oil are reported to have anti tumor activity. However, the underlying mechanisms and specific targets of extra virgin olive oil remain to be elucidated. In the present study, we investigated the effects of a recently isolated novel extra virgin olive oil polyphenol, oleocanthal, on the human multiple myeloma cell line ARH-77. Here we report that this natural compound has a remarkable *in vitro* activity by inhibiting MIP-1 $\alpha$  expression and secretion in MM cells. In addition, we also demonstrated that oleocanthal inhibits MM cells proliferation by inducing the activation of apoptosis mechanisms and by down-regulating ERK1/2 and AKT signal transduction pathways. This *in vitro* study suggests a therapeutic potential of oleocanthal in treating multiple myeloma.

**Keywords:** Apoptosis, chemokines, multiple myeloma, natural drugs, nutraceuticals, oleocanthal, proliferation.

## INTRODUCTION

Multiple myeloma (MM) is the second most frequent hematologic malignancy, with an estimated incidence of 56 new cases per million and year, and is the 14th cause of death by cancer when considering all tumors [1]. The investigation of novel treatments for this disease and the subsequent clinical approval of some of them, with demonstrated anti-myeloma activity, such as thalidomide, bortezomib, or lenalidomide, changed the outcome of MM patients in the last years [2-5]. Nevertheless, many patients relapse since they may acquire resistance to therapeutic treatment [6] and MM is still considered an incurable disease. There is an urgent need for more efficient treatment and for an identification of the pathways leading to the major pathologic events associated to myeloma.

Interactions of myeloma cells with the bone marrow microenvironment seem to be a major driving force for disease development. Through interactions with bone marrow stromal cells, myeloma cells stimulate production of survival/proliferation factors as well as osteoclast-activating

cytokines, such as interleukins (IL-6 and IL-1), and macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ). Macrophage inflammatory protein 1 $\alpha$  (also named CCL3) is an osteoclast differentiation-promoting factor. As MIP-1 $\alpha$  is detected at high concentrations in bone marrow samples obtained from patients with advanced myeloma, it is thought to play an important role in the etiology of disease. According to several reports, the effects of MIP-1 $\alpha$  action include the secretion from tumor cells of interleukin-6 (IL-6) and parathyroid hormone-related protein, which are osteoclast differentiation-promoting factors, and the activation of osteoclasts by promoting the expression of cell adhesion molecules [7, 8]. It has also been reported that MIP-1 $\alpha$  promotes the receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) expression in mouse bone marrow stromal cells and osteoblasts and directly acts on osteoclastic precursor cells inducing osteoclastogenesis and osteoclastic bone resorption [9]. Moreover, it has been demonstrated that MIP-1 $\alpha$  also directly affects cell-signaling pathways mediating growth, survival and migration in MM cells evidencing that MIP-1 $\alpha$  might play a pivotal role in the pathogenesis of MM [10]. *In vitro* and *in vivo* studies have shown that MIP-1 $\alpha$  is able to induce osteoclast formation in marrow cultures [11] and is chemotactic for rat osteoclasts [12]. It is known that MIP-1 $\alpha$  also is an osteoclast stimulating factor in human marrow cultures and is overexpressed in patients with multiple myeloma, but not in healthy individuals [13].

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All these reports suggest that a vicious circle takes place in which additional osteoclastic differentiation-promoting factors are secreted, and osteoclastic bone desorption increases further. Accordingly, it is feasible that the inhibition of MIP-1 $\alpha$  has the potential to serve as a therapeutic method for inhibiting the growth of tumor cells or preventing the destruction of bones. At present, however, there is no drug that can be used effectively to inhibit the secretion of MIP-1 $\alpha$  from tumor cells.

Therefore, new treatments are still needed to achieve longer-lasting remissions and reach the promising objective of transforming MM into a chronic disease, and eventually to cure it. In this regard, one of the sources of drugs that gained interest in the last years is the polyphenolic fraction of extra virgin olive oil, the most important lipid of the Mediterranean Diet. The Mediterranean diet is associated with beneficial health properties, including lower incidences of cardiovascular disease, age related cognitive disease, and cancer. The incidence of cancer in the Mediterranean countries is lower than in the rest of European countries and the United States [14]. This is attributed to the dietary practices, apart from possible genetic factors [15]. The favorable effect of olive oil against cancer versus other forms of added lipids is well-documented [16]. In addition to its unsaturated fatty acids, olive oil is rich in other minor phenolic compounds with anticancer effects [17]. Olive oil polyphenolic fraction includes several factors whose potent tumoricidal effects was demonstrated in several types of tumors including breast carcinoma and soft tissue sarcoma. Oleocanthal (Fig. S1 shows its chemical structure) showed anti-inflammatory activity comparable to ibuprofen via inhibition of COX-1 and COX-2 activities [18]. It also altered the oligomeric structure or function of the neurotoxic  $\beta$ -amyloid, which contributes to the debilitating effects of Alzheimer's disease [19]. Recently, our group demonstrated that oleocanthal is able to act as anti-inflammatory drug by inhibiting nitric oxide production and NOS2 synthesis in chondrocytes [20].

In addition, we showed that oleocanthal is able to inhibit others pro-inflammatory factors, such as MIP-1  $\alpha$  and IL-6 in J774 macrophages and in ATDC5 chondrocytes, confirming the anti-inflammatory activity of this compound [21].

In the present paper, we have investigated the action of oleocanthal on human MM cells. We show that oleocanthal has a potent antimyeloma activity *in vitro*. It is noteworthy that oleocanthal exerts its very active antimyeloma function by inhibiting expression and secretion of MIP-1 $\alpha$  in human MM cells. To gain further insights into the mechanism of action exerted by oleocanthal, studies aimed to define the intracellular pathways potentially involved have been also performed.

## MATERIALS AND METHODS

### 1. Chemical and Reagents

Fetal bovine serum (FBS) and MTT dye were purchased from Sigma (St. Louis MO, USA). RPMI 1640 medium, Hepes Buffer, Sodium Pyruvate, l-glutamine and antibiotics were purchased from Lonza (Switzerland).

### 2. Cell Culture

ARH-77 cells, human myeloma-derived cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Cells were cultured in 75-cm<sup>2</sup> flasks in RPMI medium supplemented with 10% FBS, 2mM l-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, 10mM HEPES, 1 mM Na Pyruvate at 37 °C under 5% CO<sub>2</sub> humidified air.

MOPC-31C is a murine myeloma-derived cell line also obtained from ATCC. Cells were cultured in 75-cm<sup>2</sup> flasks in Leibovitz's L-15 medium supplemented with 20% FBS, 2mM l-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C under 5% CO<sub>2</sub> humidified air.

### 4. Cell Viability

Cell viability was examined using a colorimetric assay based on the MTT labeling reagent. Briefly, ARH-77 cells (25 x 10<sup>3</sup>/well) were seeded in 96-well plates. Then, cells were stimulated with oleocanthal (10-100  $\mu$ M) in 10 % FBS medium for 24 and 48 hours at 37 °C. Next, cells were incubated with 10  $\mu$ l of MTT (5 mg/ml) for 4 hours at 37 °C. Then, after dissolving the formazan salt, the spectrophotometric absorbance was measured using a microtiter enzyme-linked immunosorbent assay reader at 550 nm (Multiskan EX; Thermo LabSystems, Germany).

### 5. Proliferation Assay

Cell proliferation was analyzed with a carboxyfluorescein diacetate succinimidyl ester (CFSE) Cell Proliferation Kit (Molecular Probes, CA). ARH-77 cells (1 x 10<sup>6</sup>/well) were seeded in 6-well plates and stimulated with oleocanthal for 24 and 48 hours. Briefly, cells were suspended gently in pre-warmed (37 °C) PBS containing the CFSE with a concentration of 10  $\mu$ M and incubated for 15 min at 37 °C. Then, after centrifugation, the cells were suspended in fresh pre-warmed medium and incubated for another 30 min to ensure complete modification of the CFSE and were washed again. Next, ARH-77 cells were analyzed using a FACScan flow cytometer (Becton Dickinson, NJ, USA) with 488 nm excitation and emission filters appropriate for fluorescein (FL1). Analysis of results was done using Cell Quest Pro software (Becton Dickinson, NJ, USA).

### 6. Apoptosis Assay

We used a FITC Annexin V Apoptosis Detection Kit II (Becton Dickinson) to identifying apoptotic or necrotic processes induced by oleocanthal. Briefly, cells (1 x 10<sup>6</sup>/well) were seeded in 6-well plates and treated with OC for 3, 6, 12, 24 hours, at different concentrations (10, 25, 50  $\mu$ M). Next, cells were analyzed according to the manufacturer's instructions of kit.

### 7. Cell Cycle Analysis

After treatment with oleocanthal at different concentrations (10, 25 and 50  $\mu$ M) for 3, 6 and 24 h, the ARH-77 cells were harvested, washed twice with PBS and fixed with 70% ethanol at -20 °C for 2 hours. After centrifugation, cells were washed with PBS and resuspended in a propidium iodide

(PI)/RNase staining buffer (100 $\mu$ g/ml PI; 50  $\mu$ g/ml RNase A) and incubated for 15 minutes at room temperature in the dark. Subsequently, cells were analyzed by flow cytometry at 488 nm (FL2).

## 8. Cell Treatments, Protein Extraction and Western Blot Analysis

ARH-77 were plated at a density of  $1 \times 10^6$  cells/well in 6-well plates and incubated with different doses of oleocanthal (10, 25, 50  $\mu$ M) during 3 hours at 37°C in 10 % FBS medium.

Proteins were extracted using a NucleoSpin kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. SDS-PAGE and blotting procedure were carried on as previously described [19]. Immunoblots were incubated with the appropriate antibody (phospho-AKT; AKT; caspase-9; RANKL; cleaved caspase-3; phospho-p38, Cell Signaling MA, USA) and visualized with an Immobilon Western Detection kit (Millipore Massachusetts, USA) using horseradish peroxidase-labeled secondary antibody. To confirm equal loading in each sample, the membranes were stripped in glycine buffer at pH 2 and re-blotted with anti- $\beta$ -actine antibody (Sigma). The images were captured and analyzed with an EC3 imaging system (UVP).

## 9. RNA Isolation and RT-PCR

RNA was isolated from cell culture by Trizol LS, according to the manufacturer's instructions (Gibco BRL Life Technologies, Grand Island, NY).

Human MIP-1 $\alpha$  levels were determined using SYBR Green-based quantitative PCR. RNA was extracted using a NucleoSpin kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. For relative quantification, we performed an RT reaction with a Thermo Scientific Verso cDNA Synthesis Kit (42°C for 30 min, followed by an incubation at 95°C for 2 min). RT-PCR was performed in a Stratagene MX3005P thermal cycler using a standard protocol (95°C for 10 min followed by 40 cycles for 15 s of denaturation at 95°C and 1 min annealing/extension at 60°C), a PCR Master Mix (SABiosciences, Maryland, USA) and specific primers (for human MIP-1 $\alpha$ , 114 bp, PPH00566E, reference position 124, GenBank accession no. NM\_002983.2; for human GAPDH, 175 bp, PPH00150E, reference position 1287-1310, GenBank accession no. NM\_002046.3). Results of comparative RT-PCR experiments were analyzed using MxPro software (Stratagene, California, USA).

## 10. Enzyme-Linked ImmunoSorbent Assay

MIP-1 $\alpha$  levels were measured using Quantikine human MIP-1 $\alpha$  immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions in cell culture supernatant of cell treated or not with oleocanthal during 3 hours.

## 11. Lactate Dehydrogenase (LDH) Assay

Lactate dehydrogenase (LDH) levels in cell supernatant were determined as marker of cell necrosis. LDH converts L-lactate and NAD to pyruvate and NADH respectively. The

rate of increase in absorbance of the reaction mixture at 340 nm, due to the formation of NADH, is proportional to the LDH activity. LDH was quantified with ADVIA<sup>®</sup> 1650 chemistry system. Thus, ARH-77 cells were cultured in 6-well plates ( $1 \times 10^6$  cells/ml) and treated or not with oleocanthal for 24 and 48 hours. Next, cell culture supernatants were harvested and quantified for LDH levels. Hydrogen Peroxide (5mM) was used as positive control.

## 12. Statistical Analysis

Data are reported as the mean  $\pm$ SEM of at least 3 independent experiments, each with at least 3 independent observations. Statistical analysis was performed using analysis of variance followed by the Student-Newman-Keuls test or Bonferroni multiple comparison test using the Prism computerized package (GraphPad Software). *P* values less than 0.05 were considered significant.

## RESULTS

### Effect of Oleocanthal on ARH-77 Human Multiple Myeloma Cell Line Viability and Proliferation

ARH-77 cells treated with OC (10-100  $\mu$ M) for 24h (Fig. 1A) and 48h (Fig. 1B) showed a significant reduction of cell viability rate evaluated by MTT colorimetric assay. This effect is dependent on the dose. To note, oleocanthal was able to decrease cell viability also in the MOPC-31C murine myeloma cell line suggesting that the effect is independent by the taxa (Fig. 1C and 1D).

ARH-77 cells treated with OC (10-100  $\mu$ M) for 24h (Fig. 1E) and 48h (Fig. 1F) showed a significant reduction of cell proliferation rate evaluated by CFSE Cell proliferation assay. This effect is also dependent on the dose.

### Oleocanthal Inhibits MIP-1 $\alpha$ mRNA and Protein Expression in Human Multiple Myeloma ARH-77 Cell Line

As shown in the (Fig. 2A), treatment of ARH-77 with oleocanthal significantly inhibits the expression of MIP-1 $\alpha$  mRNA in a dose-dependent manner (10, 25 and 50  $\mu$ M).

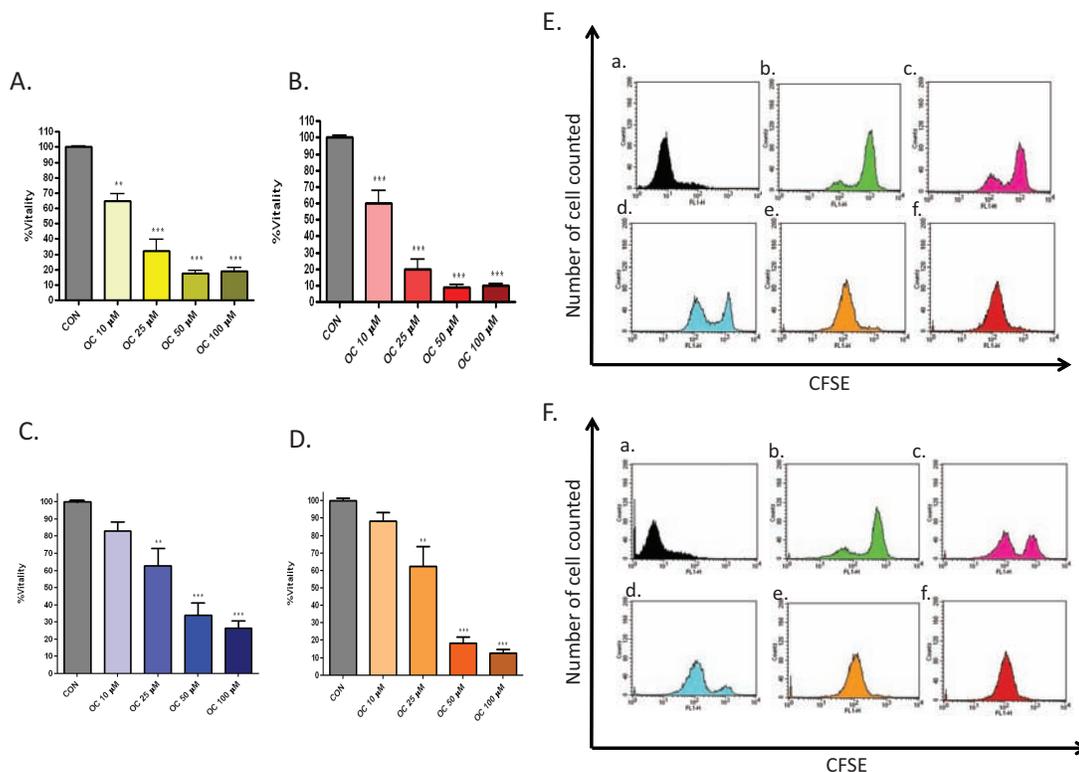
To note, oleocanthal significantly suppresses also MIP-1 $\alpha$  protein accumulation in supernatants from human myeloma cells stimulated with OC (10, 25 and 50  $\mu$ M) during 3 hours (Fig. 2B).

### Effect of Oleocanthal on LDH Levels

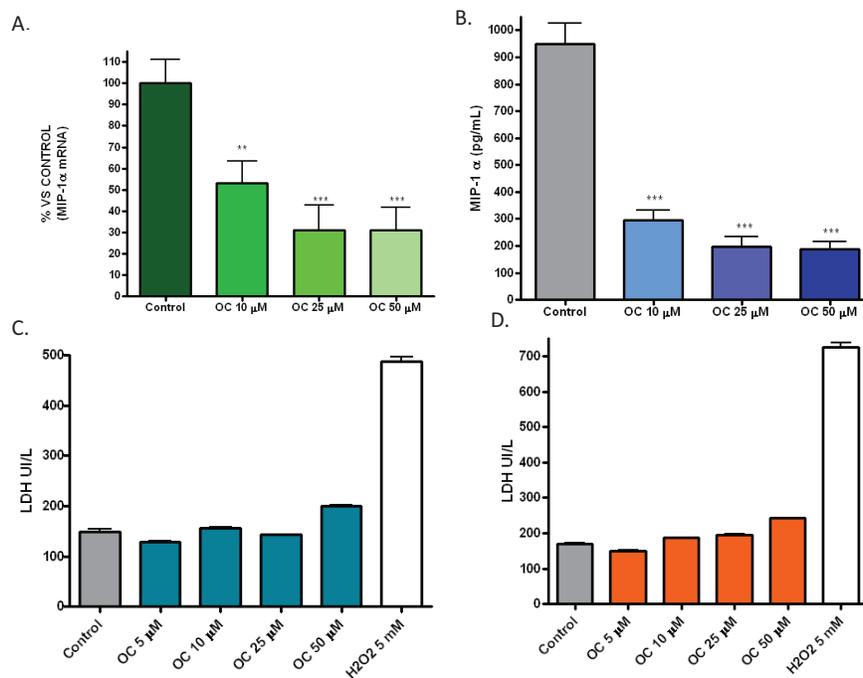
To evaluate whether oleocanthal exerts necrotic effect, we have assessed a lactate dehydrogenase (LDH) analysis. As shown in (Fig. 2), oleocanthal produced an increase of LDH's levels but only at higher doses (50, 100  $\mu$ M) after 24 (Fig. 2C) and 48 (Fig. 2D) hours of treatment indicating that secondary necrosis by oleocanthal on multiple myeloma cells may be at play [22].

### Oleocanthal Induces Apoptosis in ARH-77 Cells

To further verify whether oleocanthal may exert also apoptotic effects on ARH-77 cells, flow cytometry analysis was conducted using dual staining with annexin V and propidium iodide (PI), which was used to distinguish between viable, early apoptotic, late apoptotic or necrotic cells.



**Fig. (1).** Oleocanthal decreases vitality and proliferation of myeloma cells. ARH-77 cells treated with OA (10-100 μM) for 24h (A) and 48h (B) were analyzed using a MTT colorimetric assay. MOPC-31C cells treated with OA (10-100 μM) for 24h (C) and 48h (D) were analyzed using a MTT colorimetric assay. Results represent mean ± SEM of at least 4 independent experiments, each with at least 8 independent observations. After treatment with oleocanthal for 24h (E) and 48h (F), ARH-77 cells were examined using CFSE cell proliferation assay.



**Fig. (2).** Oleocanthal inhibits MIP1-α mRNA expression and protein secretion in ARH-77 cells. (A). Human MIP-1α mRNA expression in ARH77 cells after treatment with OC (10, 25 and 50 μM) for 3 hours (OC 10 μM \*\* p<0,01 vs control, OC 25 μM \*\*\* p<0,001 vs control, OC 50 μM \*\*\* p<0,001 vs control) (B). ARH-77 cells were treated with OC (10, 25 and 50 μM) for 3 hours. Supernatants were collected and analyzed for MIP-1α by ELISA assay. Results are represented as mean ± SEM of at least 3 independent experiments. **Effect of oleocanthal on LDH levels.** ARH-77 cells were treated with oleocanthal (10-100 μM) for 24h (C) and 48h (D) and supernatants were collected and analyzed by LDH assay.

As shown in (Fig. 3), the population of early and late apoptotic cells increased after treatment with oleocanthal at different concentrations (early apoptotic cells: 2,15%; 2,24%; 4,36%; 3,71% after 3 h and late apoptotic cells: 9.93%; 12.57%; 18.11%; 18.79% in the panel A, for 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M oleocanthal, respectively). Panel B showed the effect of OC at 6h (early apoptotic cells: 3,55%; 5,04%; 10,34%; 17,61% and late apoptotic cells: 11.29%; 13.63%; 19.20%; 23.57% for 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M oleocanthal, respectively). To note, treatment of OC 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M during 12 hours only induced late apoptotic death cells (Figs. S2). Finally, the data summarized in (Fig. S3) confirm the secondary necrotic effect of oleocanthal (25 and 50  $\mu$ M) after 24 hours previously showed in (Fig. 3) (panel A and B).

### Induction of Cell Cycle Arrest in Multiple Myeloma Cells by Oleocanthal

To confirm that oleocanthal inhibited cellular proliferation of ARH-77 cells by affecting cell cycle, we performed a cell cycle analysis after treatment with various concentrations of OC (10, 25 and 50  $\mu$ M) using flow cytometry and PI staining. As shown in (Fig. 3) panel C and supplemental (Fig. S4), compared to vehicle-only treated cells, oleocanthal treatment resulted in a clear arrest of cell cycle in G0/G1 phase after 3, 6 and 24 h accompanied by a concomitant decrease in the percentage of cells in G2/M phase. These results suggested that OC arrested MM cells at Go/G1 phase and suppressed cellular proliferation. Treatment with oleocanthal led to a dose-dependent accumulation of sub-G1 cells indicating that oleocanthal induces MM cell death.

### Induction of Apoptosis Signaling by Oleocanthal in Multiple Myeloma Cells

To gain further insights into the molecular mechanism exerted by oleocanthal, lysates from ARH-77 cells, treated with 10, 25 or 50  $\mu$ M of oleocanthal for 3 hours were assessed for cleaved caspase cleavage by western blotting with antibodies to caspase-3 or -9.

As shown in the (Fig. 4), panel A, oleocanthal is able to cleave caspase-3 (32 to 33 kDa) and active caspase-3 (17 to 19 kDa) at doses of 25 and 50  $\mu$ M after 3 hours of treatment. Oleocanthal also is able to cleave caspase-9 (47 kDa) as well as active caspase-9 (35 to 37 kDa) at doses of 25 and 50  $\mu$ M after 3 hours of treatment (panel B).

The effect of oleocanthal on caspase-3 and 9 after 6 and 12 hours is also shown (Fig. S5).

### Inhibition of AKT and Map Kinase Signaling Pathways by Oleocanthal

To evaluate the effect of oleocanthal on the inhibition of cell proliferation, we have studied the involvement of the some important pathways that mediate cell survival and proliferation.

ARH-77 cells were treated 3 hours with oleocanthal 10, 25, 50  $\mu$ M. Cell lysates underwent western blotting analysis using antiphospho-p38, antiphospho-AKT (Ser473), antiphospho-ERK1/2 or anti-RANKL antibody.

### -Oleocanthal Induces P38 Phosphorylation

Western blot experiments showed a phosphorylation of P38 after 3 hours of treatment with OC at 10, 25 y 50  $\mu$ M in myeloma cells (Fig. 5A). P38 antibody served as a loading control.

### -Oleocanthal Inhibits AKT Pathway

In our study, oleocanthal is able to inhibit AKT-phosphorylation at doses of 25 and 50  $\mu$ M in ARH-77 cells (Fig. 5B) after 3 hours of treatment. AKT antibody served as a loading control.

### -Oleocanthal Induces pERK1/2 Dephosphorylation

We observed that oleocanthal also is able to dephosphorylate pERK 1/2 in a dose dependent manner (Fig. 5C). ERK1/2 antibody served as a loading control.

### -Oleocanthal Inhibits RANKL Expression

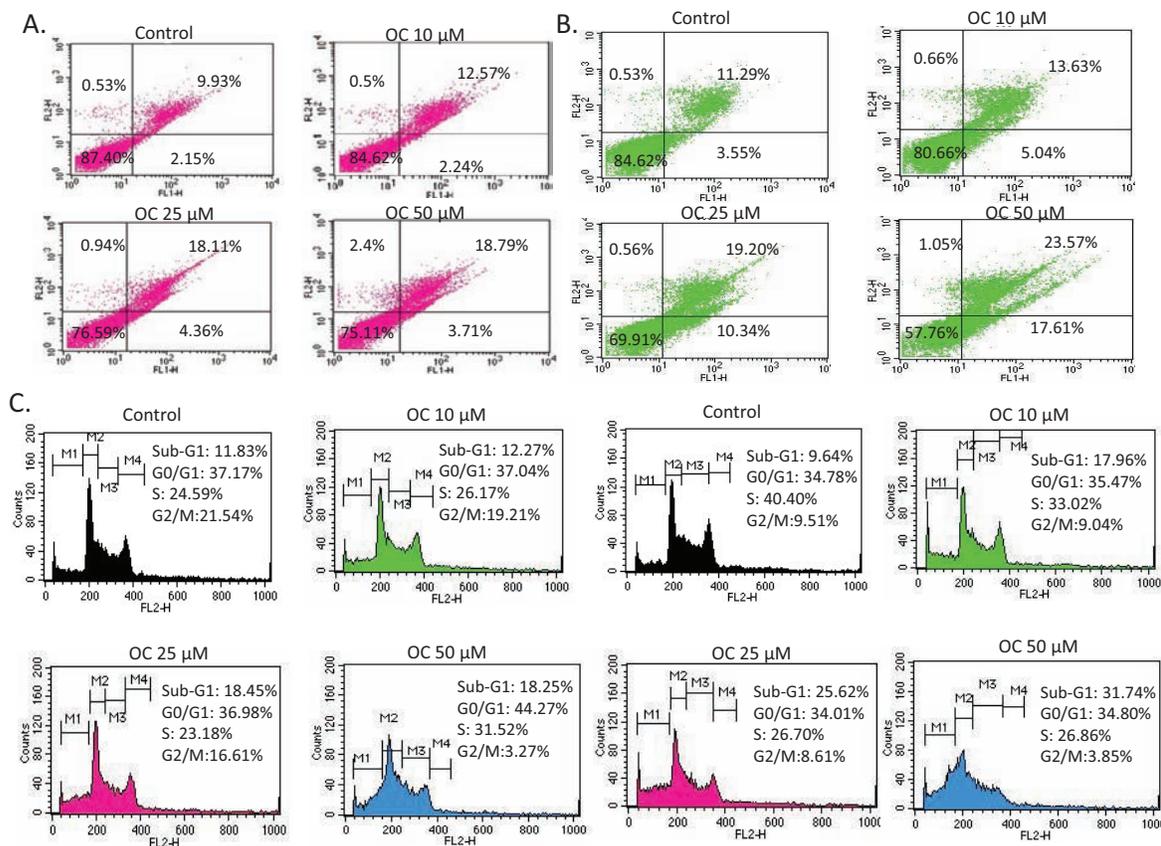
In the (Fig. 5D), we have evidenced the inhibition of RANKL in a dose dependent manner after 3 hours of treatment with oleocanthal.

## DISCUSSION

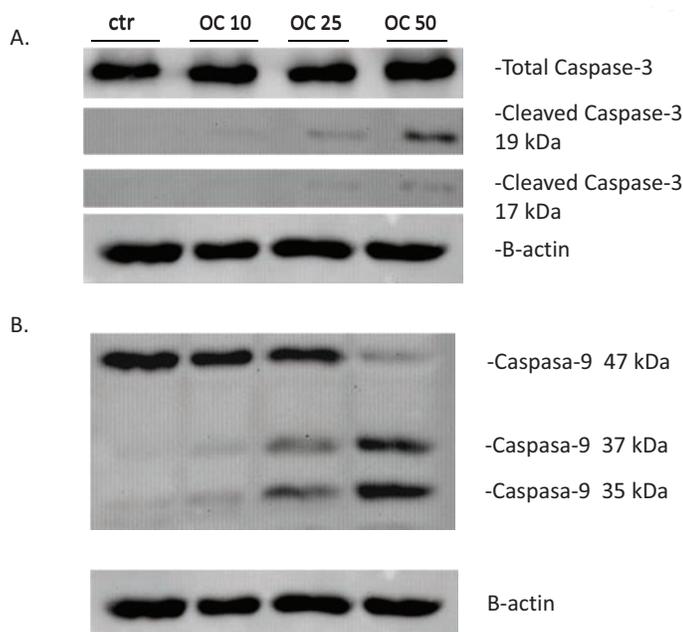
In multiple myeloma, the accumulation of malignant plasma cells in the bone marrow causes profound changes in this microenvironment. Bone destruction is a common manifestation of the disease and results from increased osteoclastic bone resorption and decreased bone formation. Furthermore, bone resorption promotes myeloma cell growth. The overall consequence is a vicious cycle that accelerated bone loss and progression of myeloma. The aggressive bone destruction has significantly contributed to its poor prognosis, which has barely been improved since the introduction of the melphalan and prednisolone therapy in the 1960s despite the development of potent chemotherapeutic regimens.

Cytokines with potent bone-resorbing activity such as MIP-1 $\alpha$  have been implicated as potential mediators of osteoclastic bone resorption in MM. MIP-1 $\alpha$  is produced by MM cell lines including ARH-77 as well as a majority of primary MM cells from patients. This chemokine stimulated formation of osteoclast-like cells (OCLs) and their resorbing activity in rabbit bone cell cultures as potentially as MM cell-conditioned media. Thus, MIP-1 $\alpha$  may be among leading candidates for being suggested as positive regulator of osteoclast differentiation and chemotaxis in MM patients. Very recently, Raje's group provided further evidence for the important pathogenic role MIP-1 $\alpha$  had and its receptors in MM by showing that MIP-1 $\alpha$ , in addition to its known catabolic activity, regulates osteoclast (OC) differentiation and reduces bone formation by inhibiting osteoblast (OB) function, and therefore contributes to OB/OC uncoupling in MM [23].

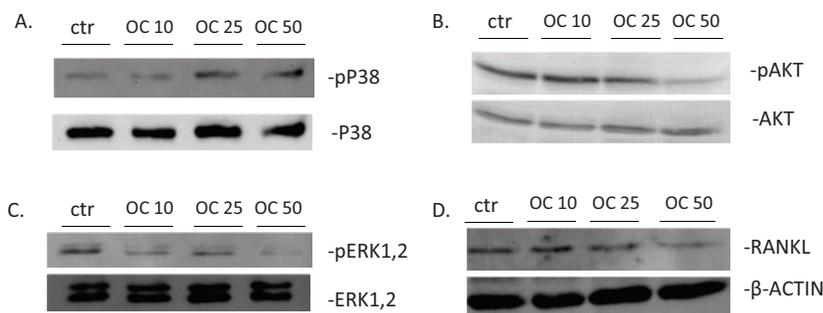
Therefore, drugs that inhibit the production of MIP-1 $\alpha$  are expected to inhibit the destruction of bone and the growth of tumors in MM cells, and have the potential to serve as very effective therapeutic agents. Thus, the overall goal of our work was to evaluate if MIP-1 $\alpha$ , released from cultured myeloma cells, can be inhibited by oleocanthal, though it would be possible to inhibit the growth of myeloma



**Fig. (3). Induction of apoptosis in human myeloma cells by oleocanthal.** (A) Cells were treated with increasing doses of oleocanthal (10, 25 and 50 μM) for 3h, apoptotic cell death was detected by staining cells with the Annexin V and PI and analyzed by flow cytometry. (B) Cells were treated with increasing doses of oleocanthal (10, 25 and 50 μM) for 6h, apoptotic cell death was detected by staining cells with the Annexin V (FL-1) and PI (FL-2) and analyzed by flow cytometry. The effect of OC on Annexin V after 12 and 24 hours is reported in (Fig. S2 and S3). (C) Cell cycle analysis of multiple myeloma cells treated with different doses of oleocanthal during 3 and 6 hours using flow cytometry with PI staining and the DNA histograms. The effect of OC on cell cycle analysis after 24 hours is reported in (Fig. S4).



**Fig. (4). Induction of apoptosis signaling molecules by oleocanthal.** ARH-77 cells were treated with oleocanthal for 3h. Cell lysates underwent Western blotting analysis using cleaved and total caspase 3 (panel A) and caspase 9 antibodies (panel B). β-actin was used as a loading control. Effect of oleocanthal on Caspase 3 and 9 after 6 and 12 hours is reported in (Fig. S5).



**Fig. (5). Oleocanthal inhibits AKT and Map Kinase signaling pathways.** Expression of p-p38 (A), p-AKT (B), p-ERK1/2 (C) and RANKL (D) in ARH-77 cells treated with 10, 25 and 50  $\mu$ M oleocanthal during 3h.

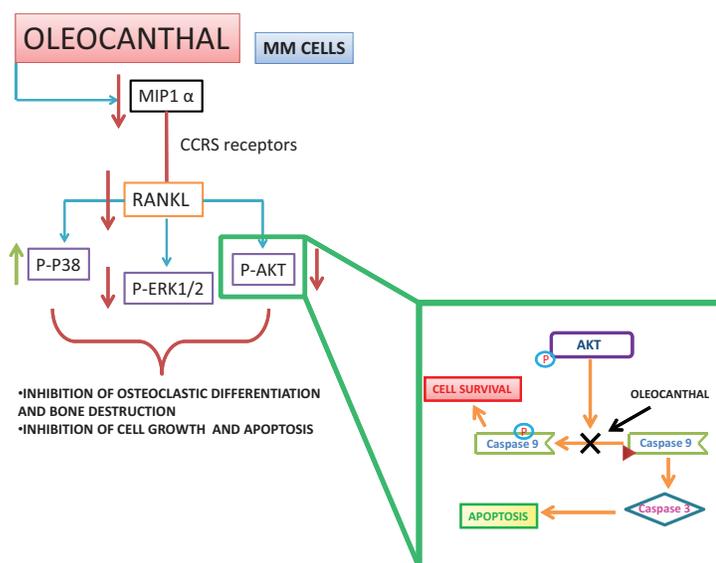
cells and prevent osteolysis. Therefore, according to our results, the use of such drugs such as oleocanthal might prolong lives, alleviate pain from osteolysis, or contribute to an improved quality of life of patients. Thus, we first investigated whether or not oleocanthal was active on MM cell proliferation rate and next whether there was any inhibitory effect on the MIP-1 $\alpha$  secreted from myeloma cells and the mechanism of any such inhibition.

The ideal treatment of multiple myeloma patients should thus aim at preventing myeloma cell growth and inhibiting bone resorption. Our study shows that oleocanthal exhibits precisely this set of properties: it prevents growth of myeloma cells, and indirectly inhibits osteoclast differentiation and bone resorption, by directly blocking the expression and secretion of MIP-1 $\alpha$ , one of the most important factors involved in MM associated bone lesions.

The proliferation and the survival of MM cell lines has been shown to be related to the activation of several pathways such as phosphatidylinositol-3 kinase (PI-3K)/Akt, Janus kinase (JAK)/signal transducer and activator of transduction 3 (STAT3), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and nuclear factor kappa-B (NF- $\kappa$ B) [24-28]. In addition, it is known that MIP-1 $\alpha$  promotes the activation of PI3K/Akt and ERK1/2 pathway and inhibits p38MAPK pathway through RANKL expression [9]. For this reason we have investigate

the implication of these pathways in the MIP-1 $\alpha$  inhibition driven by oleocanthal. In this study, we have showed also that oleocanthal exhibited an inhibitory activity on Akt and ERK1/2 phosphorylation whereas induced a phosphorylation of p38 (Fig. 6). Moreover, we have also evidenced the inhibition of RANKL expression by oleocanthal. These results suggested a mechanism that mediates MIP-1 $\alpha$  inhibition through these pathways. We have also demonstrated that oleocanthal, from extra virgin olive oil, inhibits MM cell proliferation activating apoptotic pathway. In particular our results suggested that OC is able to inhibit AKT phosphorylation leading to activation of apoptotic pathways mediated by caspase 9 and caspase 3 (Fig. 6).

In conclusion, our *in vitro* study clearly demonstrated that OC from extra virgin olive oil is a potential drug for the treatment of MM, suggesting a therapeutic scenario in which OC could work alone or in combination with other established anti-myeloma drugs. However, one should also remember the limitations that *in vitro* drug assessments, such as these presented here, impose. In any case, here we provide the first line of evidence that oleocanthal may be considered a novel anti-myeloma drug opening novel routes for the treatment of MM. However, further experiments and results are needed to facilitate the translation of our evidence into the clinic to improve patient outcome.



**Fig. (6). Schematic representation of the mechanism of action of Oleocanthal in MM cells.**

**CONFLICT OF INTEREST**

There is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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**SUPPLEMENTARY MATERIALS**

Supplementary material is available on the publisher’s web site along with the published article.

**ABBREVIATIONS**

MM	=	Multiple Myeloma
MIP-1 $\alpha$	=	Macrophage Inflammatory Protein 1-Alpha Receptor Activator of Nuclear Factor $\kappa$ B Ligand
FBS	=	Fetal Bovine Serum
CFSE	=	Carboxyfluorescein Diacetate Succinimidyl Ester
LDH	=	Lactate Dehydrogenase
PI	=	Propidium Iodide
OCLs	=	Osteoclast-Like Cells
OC	=	Osteoclast
OB	=	Osteoblast
PI-3K	=	Phosphatidylinositol-3 Kinase
STAT3	=	Signal Transducer and Activator of Transduction 3
MAPK	=	Mitogen-Activated Protein Kinase
ERK	=	Extracellular Signal-Regulated Kinase
NF- $\kappa$ B	=	Nuclear Factor Kappa-B

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