RESEARCH ARTICLE

# Caffeic Acid Induces Apoptosis in MG-63 Osteosarcoma Cells via Protein Kinase C Delta (PKCδ) Translocation and Mitochondrial Membrane Potential Reduction

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## Abstract

**BACKGROUND:** Caffeic acid has been reported to activate caspases in MG-63 osteosarcoma cells, which can lead to apoptosis via both extrinsic and intrinsic apoptotic pathways. Translocation of protein kinase C delta (PKCδ), which reduces mitochondrial membrane potential (ΔΨm), is involved in apoptosis. The role of PKCδ translocation and ΔΨm alteration in caffeic acid-induced MG-63 cell apoptosis are largely unknown. Present study investigated the effect of caffeic acid on PKCδ translocation and ΔΨm in MG-63 cells.

**METHODS:** MG-63 cells were cultured and starved, followed by pretreatment with or without Z-VAD-FMK and treatment with or without 10 μg/mL caffeic acid. MG-63 cells were collected, lysed, and processed to obtain cytosolic and mitochondrial fractions. Each fraction was subjected to immunoblotting analysis by using anti-PKCδ antibody. Mitochondrial membrane potential (ΔΨm) was measured using flow cytometry.

**RESULTS:** Cytosolic PKCδ levels were higher than mitochondrial PKCδ levels in untreated and 1 h caffeic acid treatment groups. Inversely, cytosolic PKCδ levels were lower than the mitochondrial PKCδ levels after 6 and 12 h caffeic acid treatment. By Z-VAD-FMK pretreatment, cytosolic PKCδ levels were higher than mitochondrial PKCδ after 6 and 12 h caffeic acid treatment. After 6 h treatment with caffeic acid, ΔΨm was slightly shifted. More shifting occurred in MG-63 cells treated with caffeic acid for 12 h. The ΔΨm shifting was inhibited by Z-VAD-FMK pretreatment.

**CONCLUSION:** Caffeic acid could trigger apoptosis of MG-63 osteosarcoma cells by inducing PKCδ translocation to mitochondria and reducing ΔΨm, which might cause MMP.

**KEYWORDS:** caffeic acid, MG-63, osteosarcoma, PKCδ, mitochondrial membrane potential, mitochondrial membrane permeabilization, Z-VAD-FMK

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## Introduction

Osteosarcoma is one of the most frequent bone sarcomas found in humans and is characterized by immature bone matrix-producing malignant cells of mesenchymal origin. (1,2) This malignancy usually occurs in long bones of the extremities, and is uncommon in the head and neck region, including jaw.(2) Osteosarcoma of the jaw, which usually afflicts patients at the mean age of 35 years (3), constitutes only 1% among all head and neck cancer and 6-7% of all osteosarcoma cases.(4) Jaw osteosarcoma has a lower

risk of metastasis and higher survival rates.(3) However, osteosarcoma that afflicts the jaw has been reported to have diverse histological variants with their unique biological and clinical behaviors (1,5), and are likely more aggressive.(6) Since jaw osteosarcoma cells have distinct characteristics as compared to those of their other counterparts, diagnosis and treatment of this tumor are challenging.(3,5)

Jaw osteosarcoma is generally managed by surgical resection of affected mandible and/or maxilla. Because of the delicate and complex structure of the jaw, complete resection of osteosarcoma is more difficult to perform in the jaw compared to long bones.(4,6) Therefore, surgery should be combined with other treatment regimens, such as chemotherapy (7) to help treat this malignancy. Several studies have shown that combination of neoadjuvant and/or adjuvant chemotherapy and surgery give better outcomes to jaw osteosarcoma patients.(8,9) However, chemotherapy agents have been known to have cytotoxic effects towards normal cells. To avoid adverse effects caused by the administration of chemotherapy agents, natural products have been suggested to be used in cancer treatment.(10) In addition, natural products may also have an ability as co-chemotherapy agents to enhance the efficacy of other chemotherapy agents.(11)

Amongst natural products, caffeic acid (3,4-dihydroxycinnamic acids), which commonly occurs in fruits, tubers, legumes and grains, has been reported to provide protection from various diseases, such as bone resorption (12-16), diabetic kidney disease (17), Alzeheimer's disease (18) and cancers.(19,20) Studies have shown that caffeic acid triggers MG-63 osteosarcoma cell apoptosis by activating caspases (21,22) and may affect intrinsic and extrinsic apoptotic pathways.(23)

Protein kinase C delta (PKCδ) has been reported to be involved in apoptosis of several cancer cells, including lung (24) and prostate cancer.(25) This protein is translocated from cytosol to various organelles, including mitochondria, in the presence of apoptotic stimuli, such as anticancer agents. Translocation of PKCδ to the mitochondria reduces mitochondrial membrane potential (ΔΨm), which causes cytochrome *c* (Cyt *c*) release and caspases activation.(26) Alteration of ΔΨm is strongly related to mitochondrial membrane permeabilization (MMP), a key process that occurs in the intrinsic apoptotic pathway.(27) The role of PKCδ translocation and ΔΨm alteration in the cell death process of caffeic acid-induced MG-63 cells are largely unknown. Hence, the present study investigated the effect of caffeic acid on PKCδ translocation and ΔΨm in MG-63 osteosarcoma cells.

## Methods

### Cell Culture and Caffeic Acid Treatment

MG-63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with antibiotic-antimycotic (200 µg/mL streptomycin, 200 U/mL penicillin and 0.5 µg/mL amphotericin) (Gibco) and 10% fetal bovine serum (FBS) (BioSource, Camarillo, CA, USA). MG-63 cells were then maintained in a humidified incubator (5% $CO_2$, 37°C). At 80% confluency, MG-63 cells were sub-cultured and maintained in starving condition for 12 h in DMEM containing antibiotic-antimycotic. Starved MG-63 cells were pretreated with or without 100 µM Z-VAD-FMK (R&D Systems, Minneapolis, MN, USA) for 2 h and incubated with 10 µg/mL caffeic acid (Wako, Osaka, Japan) for 1, 6, or 12 h.

### Preparation of Cytosolic and Mitochondrial Fraction

Preparation of cytosolic and mitochondrial fraction was carried out as described in previous study.(28) Briefly, $5\times10^6$ caffeic acid-treated MG-63 cells were added with 200 µL of ice-cold solution containing 0.3 M sucrose, 10 mM Tris-HCl (pH 7.5), and protease inhibitors cocktail and then homogenized. MG-63 cells were centrifuged for 60 min at 4°C, 10,000 *g*. Supernatant was collected as a cytosolic fraction. Meanwhile, the pellet was resuspended in 200 µL ice-cold solution containing 1% triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and protease inhibitors. Ultrasonication of the precipitate, followed by centrifugation for 30 min at 10,000 g at 4°C was performed and the supernatant was collected as mitochondrial fraction.

### Immunoblotting

Caffeic acid-treated MG-63 cells were incubated with lysis buffer containing 10 mM sodium pyrophosphate, 5 mM EDTA, 20 mM Tris buffer (pH 7.4), 2 mM sodium orthovanadate, 1% Triton X, 1 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride, 50 mM sodium fluoride, and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Cytosolic and mitochondrial fractions were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane (BioRad, Richmond, CA, USA). The membrane was probed with rabbit polyclonal anti-PKCδ antibody (C-20) (Santa Cruz, Dallas, TX, USA) diluted 1:1000 in PBS after the membrane was blocked with 5% skim milk in PBS (pH 7.4). Then, donkey anti-

rabbit IgG horseradish peroxidase-conjugated (Amersham, Buckinghamshire, UK) diluted 1:1000 in PBS was added. The reactive proteins on the blot were then visualized using the ECL system (Amersham), documented with Alliance 4.7 (UVItech, Cambridge, UK), and semi-quantified with UVIband software (UVItech).

### ΔΨm Measurement

ΔΨm analysis was carried out as previously described.(29) Caffeic acid-treated MG-63 cells were suspended in 250 mL of 20 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) in phosphate-buffered saline (PBS) and incubated for 15 min. ΔΨm measurement was performed using FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Flow cytometry histograms of experimental groups were superimposed with histogram from the untreated group. Non-overlapping histogram area was measured using ImageJ software (National Institute of Health, Bethesda, MD, USA) and expressed as an arbitrary value.

### Statistical Analysis

The normality of the data was tested using Shapiro-Wilk test. To compare cytosolic and mitochondrial PKCδ levels, Mann-Whitney test or independent sample t-test was performed. IBM SPSS Statistics version 20 (SPSS IBM, Armonk, NY, USA) was used to analyze the data and $p<0.05$ was considered as statistically significant.

## Results

### Caffeic Acid Induced Mitochondrial Translocation of PKCδ in MG-63 Cells

Cytosolic PKCδ level following 1 h caffeic acid treatment was not significantly different to the untreated MG-63 cells (Mann-Whitney test, $p=0.426$). However, cytosolic PKCδ levels after 6 and 12 h caffeic acid treatment were significantly lower than those of the untreated group (independent sample t-test; $p=0.000$). Cytosolic PKCδ levels of caffeic acid-treated MG-63 cells with Z-VAD-FMK pretreatment for 6 (independent sample t-test; $p=0.000$) and 12 h (Mann-Whitney test, $p=0.000$) were significantly higher than those without Z-VAD-FMK pretreatment (Figure 1). On the contrary, mitochondrial PKCδ levels after 1 h (independent sample t-test, $p=0.037$), 6 h (Mann-Whitney test, $p=0.000$), and 12 h (Mann-Whitney test, $p=0.000$) caffeic acid treatment were significantly higher compared to those of the untreated group in a time-dependent manner. Mitochondrial PKCδ levels after 6 and 12 h caffeic acid

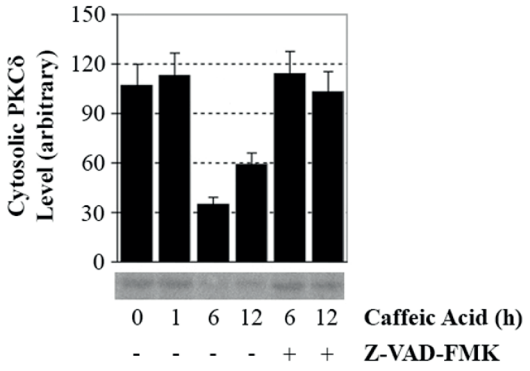


**Figure 1. Cytosolic PKCδ levels after caffeic acid treatment.** MG-63 cells were starved for 12 h, pretreated with/without 100 μM Z-VAD FMK for 2 h, and incubated with/without 10 μg/mL caffeic acid for 1, 6, or 12 h. Cells were collected, lysed, and processed to obtain cytosolic fractions. Each fraction was subjected to immunoblotting analysis by using anti-PKCδ antibody as described in Methods. These experiments were repeated 3 times.

treatment in MG-63 cells with Z-VAD-FMK pretreatment were significantly lower than those without Z-VAD-FMK pretreatment (Mann-Whitney test, $p=0.000$) (Figure 2). Cytosolic PKCδ levels were higher than mitochondrial PKCδ levels in untreated and 1 h caffeic acid treatment groups. Inversely, cytosolic PKCδ levels were lower than mitochondrial PKCδ levels after 6 and 12 h caffeic acid treatment. By Z-VAD-FMK pretreatment, cytosolic PKCδ levels were higher than mitochondrial PKCδ levels after 6 and 12 h caffeic acid treatment (Figure 1, Figure 2).

### Caffeic Acid Reduced ΔΨm of MG-63 Cells

Untreated MG-63 cells showed a ΔΨm curve with a single peak located between $10^1$ and $10^2$ in x-axis (FL1-H) of the

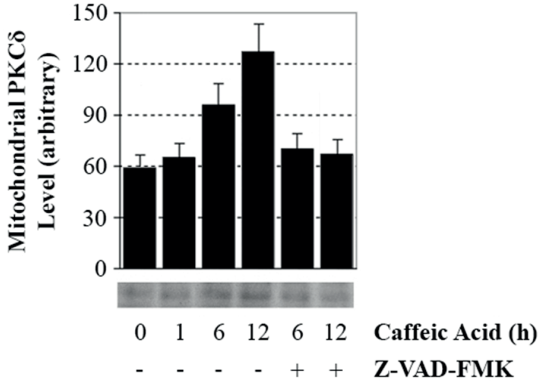


**Figure 2. Mitochondrial PKCδ levels after caffeic acid treatment.** MG-63 cells were starved for 12 h, pretreated with/without 100 μM Z-VAD FMK for 2 h, and incubated with/without 10 μg/mL caffeic acid for 1, 6, or 12 h. Cells were collected, lysed, and processed to obtain mitochondria fractions. Each fraction was subjected to immunoblotting analysis by using anti-PKCδ antibody as described in Methods. These experiments were repeated 3 times.

histogram (Figure 3A). After 6 h treatment with 10 µg/mL caffeic acid, another smaller peak appeared on $10^1$, indicating that ΔΨm was slightly shifted to the left side (Figure 3B). More shifting was seen in the MG-63 cells treated with caffeic acid for 12 h, as shown by the peak that appeared on $10^1$ was larger compared to one that appeared between $10^1$ and $10^2$ (Figure 3C). The shifting of ΔΨm curve was inhibited by pretreatment of Z-VAD-FMK (Figure 3D).

Superimposition results of ΔΨm curve of untreated MG-63 cells with ΔΨm curves of the experimental groups showed non-overlapping histogram peaks. The non-overlapping area resulted from the superimposition of ΔΨm curve of untreated group and 6 h caffeic acid treatment was 202 (Figure 4A). The non-overlapping area resulted from the superimposition of ΔΨm curve of untreated group and 12 h caffeic acid treatment was 908 (Figure 4B). Meanwhile, the non-overlapping area resulted from the superimposition of ΔΨm curve of untreated group and Z-VAD-FMK pretreatment followed by 12 h caffeic acid treatment was 102 (Figure 4C).

## Discussion

In the current study, cytosolic PKCδ levels after 6 and 12 h caffeic acid treatment were lower than those of untreated MG-63 cells. In addition, the PKCδ level in mitochondria was upregulated within 6 h of caffeic acid treatment and was getting higher within 12 h of caffeic acid treatment. The results showed that caffeic acid induced mitochondrial translocation of PKCδ. Starting from 6 h of caffeic acid treatment, PKCδ was translocated to mitochondria, which later increased in 12 h treatment. Caffeic acid-induced mitochondrial translocation of PKCδ was inhibited by Z-VAD-FMK, a pan caspase inhibitor. The involvement of PKCδ activity in cancer cell apoptosis is also observed in previous studies using several compounds purified from natural sources and their derivatives. Lanatoside C extracted from *Digitalis lanata* has been reported to activate PKCδ in human hepatocellular carcinoma cells by inducing phosphorylation of the protein at Thr505 residue, augmenting its translocation to the cell membrane, and triggering PKCδ cleavage, hence stimulates apoptosis. (30) Furthermore, it has been reported that 7α-acetoxy-6β-benzoyloxy-12-obenzoylroyleanone (Roy-Bz), a derivative of 7α-acetoxy-6β-hydroxyroyleanone obtained from *Plectranthus grandidentatus* (31) shows antitumor activity against colon cancer cells by increasing the generation of PKCδ-catalytic fragment (CF) in a time-dependent manner, as well as stimulating PKCδ translocation to the cell membrane and perinuclear region.(32) Ellagic acid has also been reported to activate PKCδ in lymphoma-bearing mice.(33) Activation and mitochondrial translocation of PKCδ play a critical role in the intrinsic apoptosis pathway. During apoptosis, PKCδ is proteolytically cleaved by caspase-3 generating PKCδ-CF. PKCδ-CF translocates to mitochondria and mediates ΔΨm reduction, Cyt *c* release, and caspases activation.(26)

Results of the current study also demonstrated that caffeic acid reduced ΔΨm of MG-63 cells, which might indicate the occurrence of MMP. This caffeic acid's effect on ΔΨm was also inhibited by pretreatment of Z-VAD-FMK. This is in accordance with previous studies demonstrating that caffeic acid induces apoptosis by causing mitochondrial membrane depolarization in several cancer cell lines.(34,35) Several other natural compounds and their derivatives have

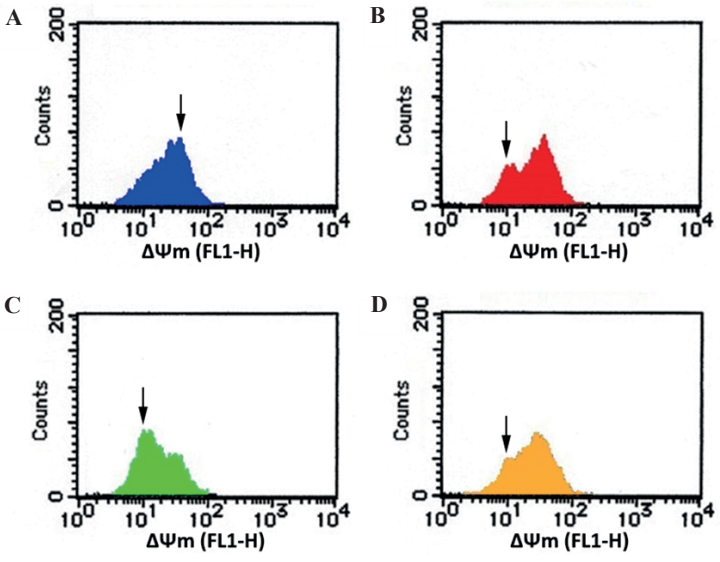


**Figure 3. Caffeic acid reduced ΔΨm of MG-63 cells.** MG63 cells were starved for 12 h, pretreated with/without 100 µM Z-VAD FMK for 2 h, and incubated with/without 10 µg/mL caffeic acid for 6 or 12 h. MG-63 cells were collected, rinsed, incubated in $DiOC_6(3)$ and analyzed with FACSCanto II as described in Methods. These experiments were repeated 3 times. A: Untreated; B: Caffeic acid treatment for 6 h; C: Caffeic acid treatment for 12 h; D: Pretreatment of Z-VAD-FMK followed by caffeic acid treatment for 12 h.

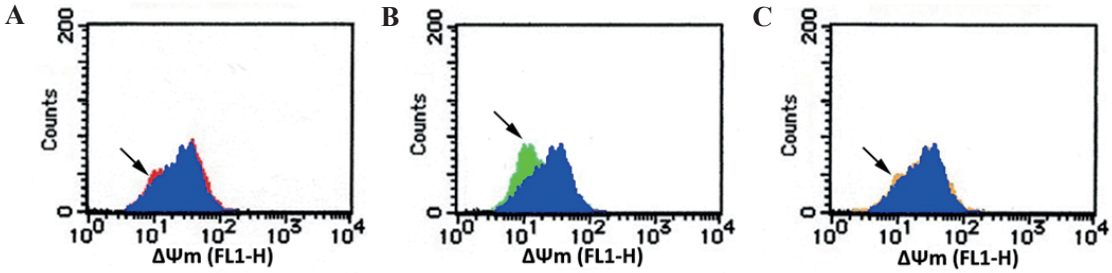


**Figure 4. MG-63 cells ΔΨm curves superimposition results.** ΔΨm curves obtained from flow cytometry were superimposed and non-overlapping histogram area (black arrow) was measured with ImageJ as described in Methods. A: Untreated + Caffeic acid treatment for 6 h; B: Untreated + Caffeic acid treatment for 12 h; C: Untreated + Pretreatment of Z-VAD-FMK followed by caffeic acid treatment for 12 h. Blue: Untreated; Red: Caffeic acid treatment for 6 h; Green: Caffeic acid treatment for 12 h; Yellow: Pretreatment of Z-VAD-FMK followed by caffeic acid treatment for 12 h.

also been reported to trigger cancer apoptosis by reducing ΔΨm, including Roy-Bz (32) and lanatoside C.(30) MMP is one of the apoptosis hallmarks. This process is characterized by ΔΨm reduction and accompanied by the release of apoptogenic factors, including Cyt *c*.(27)

Previous studies using MG-63 cells have demonstrated that several pathways involved in intrinsic apoptotic pathway, such as Bid truncation, Cyt *c* release (23) and activation of caspase-3, -8 and -9 (21,22) are stimulated by the presence of caffeic acid. This study provides additional and more detailed evidence on caffeic acid-induced apoptotic pathway in MG-63 cells. This compound appears to activate caspase-8, which cleaves caspase-3 directly or promotes BH3-interacting death (Bid) truncation to produce truncated Bid (t-Bid). Upon truncation by caspase-3, t-Bid translocates to the mitochondria and stimulates Cyt *c* release to cytosol. Cyt *c* then forms a complex consisting of Cyt *c*, pro-caspase-9 and apoptotic protease activating factor-1 (Apaf-1), known as apoptosome, to activate pro-caspase-9 to active caspase-9. Both caspase-8 and -9 activates caspase-3, which in turn triggers proteolytic cleavage of PKCδ, causing the generation of PKCδ-CF. PKCδ-CF translocates to the mitochondria, causing ΔΨm reduction and MMP, which is followed by Cyt *c* release to cytosol. The released Cyt *c* activates caspase-3 through apoptosome formation, creating a positive feedback loop that amplifies the activation of caspase-3 (Figure 5). t-Bid might also cause ΔΨm reduction and MMP in caffeic acid-induced MG-63 cells, since it has been reported that t-Bid reduces ΔΨm and induces MMP.(27,36)

Apoptosis evasion is one of the hallmarks of cancers. (37,38) It has been reported that malignant cells have higher ΔΨm as compared to normal cells (39) causing induction of MMP more difficult, which leads to resistance to cell death.(40) Since caffeic acid may induce PKCδ translocation to mitochondria and reduce ΔΨm of MG-63 osteosarcoma cells, this compound is a potential candidate for an anti-osteosarcoma agent. Further research using other osteosarcoma cell lines is needed to elucidate the involvement of PKCδ translocation and ΔΨm reduction in caffeic acid-induced apoptosis. More investigations are also required to investigate the involvement of other

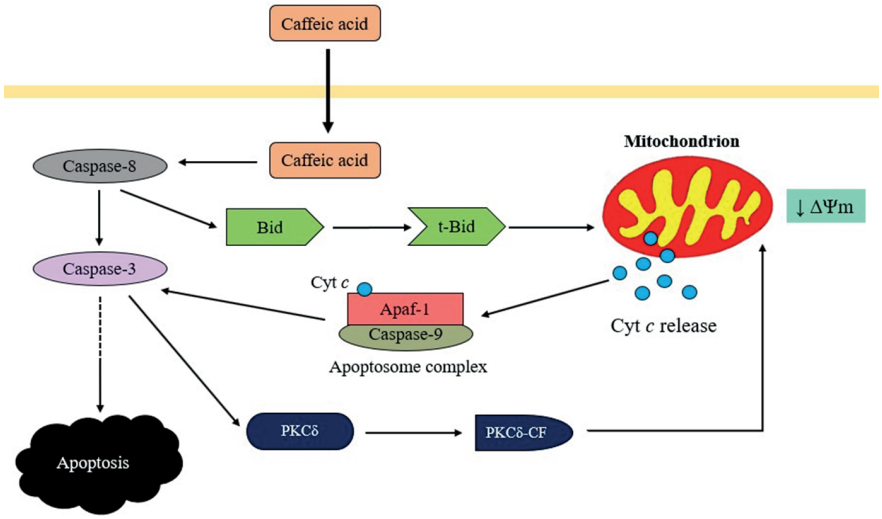


**Figure 5. Involvement of PKCδ translocation and ΔΨm reduction in caffeic acid-induced apoptosis in MG-63 osteosarcoma cells.** Caffeic acid may activate caspase-8, which in turn activates caspase-3 through direct cleavage or apoptosome formation that is preceded by t-Bid-induced Cyt *c* release. Activated caspase-3 cleaves PKCδ to PKCδ-CF. PKCδ-CF then translocates to mitochondria and induces ΔΨm reduction, which leads to Cyt *c* release, thus creating a positive feedback loop that amplifies the activation of caspase-3. Activation of caspase-3 leads to apoptosis of MG-63 cells.

apoptogenic factors, such as endonuclease G, second mitochondria derived activator of caspase (Smac)/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (DIABLO), apoptosis inducing factor (AIF), and Omi/high temperature requirement A2 (HtrA2), in caffeic acid-induced apoptosis of MG-63 cells.

## Conclusion

Taken together, caffeic acid could trigger apoptosis of MG-63 osteosarcoma cells by inducing PKCδ translocation to mitochondria and reducing ΔΨm, which might cause MMP.

## Authors Contribution

FS and MIR prepared study concept and design. FS, CCA and JCH performed processing and acquisition of data. FS, CCA, JCH and MC performed analysis and interpretation of results. CCA, JCH and MC prepared the draft of the manuscript. FS, MIR and MC made critical revisions of the manuscript. MIR, MC assisted in administrative, technical, and material support. FS and MIR performed supervision of the study.

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