UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Effect of caffeic acid and caffeic acid phenethyl ester on murine osteosarcoma cells: regulation of the NADPH oxidase complex

Efeito do ácido cafeico e do éster fenetil do ácido cafeico em células de osteossarcoma murinos: regulação do complexo NADPH oxidase

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Dissertação constituída por artigo apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Biologia Oral, Estomatologia, Radiologia e Imaginologia.

Orientador: Prof. Dr. Rodrigo Cardoso de Oliveira

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Versão Corrigida

BAURU

2021

Pagnan, Ana Lígia
Effect of caffeic acid and caffeic acid phenethyl ester on murine osteosarcoma cells: regulation of the NADPH oxidase complex / Ana Lígia Pagnan. -- Bauru, 2021.
64p. : il. ; 31 cm.

Dissertação (mestrado) -- Faculdade de Odontologia de Bauru, Universidade de São Paulo, 2021.

Orientador: Prof. Dr. Rodrigo Cardoso de Oliveira

Nota: A versão original desta dissertação encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru – FOB/USP.

ERRATA

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Universidade de São Paulo
Faculdade de Odontologia de Bauru

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Serviço de Pós-Graduação

FOLHA DE APROVAÇÃO

Dissertação apresentada e defendida por
ANA LÍGIA PAGNAN
e aprovada pela Comissão Julgadora
em 03 de maio de 2021.

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DEDICATÓRIA

A **Deus,** que está sempre presente na minha vida, por ter me dado forças e sabedoria para realizar este trabalho.

Ao meus pais, **Márcio e Rosely,** minha irmã **Maria Clara,** e meu namorado **Fábio** pelo suporte e amor incondicional. Por sempre estarem ao meu lado, por acreditarem em mim, me dando amparo, apoio e incentivo em todos os momentos de minha vida.

A todos os meus amigos do grupo pesquisa e do laboratório de Bioquímica que me receberam tão bem e me auxiliaram durante esta trajetória.

AGRADECIMENTOS

Agradeço imensamente a Deus, por tudo, principalmente por me dar sabedoria e saúde para concluir mais uma etapa em minha vida! Por me dar forças diariamente para enfrentar a vida e tudo o que ela tem a oferecer. Obrigada!

Aos meu pais e minha irmã por serem a minha base. Por estarmos em união sempre que possível, por me incentivarem a dar sempre o meu melhor e acreditar na minha capacidade. Eu não teria chegado até aqui sem vocês ao meu lado. Eu amo muito vocês

Ao meu namorado, que ao decorrer desses inúmeros anos juntos sempre me ofereceu segurança, carinho e amor. Por ter sempre apoiado as minhas decisões, acreditado nos meus sonhos, e estado ao meu lado em todas essas etapas. Eu te amo!

Aos meus amigos da cultura celular Vanessa, Adriano, Cintia, Gabi, Mari e Kelly. Por estarem sempre presentes, por me auxiliarem nos experimentos e nos momentos que eu mais precisei. Obrigada por serem parceiros em todos os momentos, pelas longas conversas e risadas que tivemos juntos, sempre vou leva-los no coração. Agradeço em especial pelo Adriano e da Vanessa, que desde o começo, estivemos juntos em todos os momentos, dentro e fora do laboratório, uma amizade que desejo levar para a minha vida toda. Obrigada a todos por todo o conhecimento obtido com cada um de vocês!

Ao meu orientador professor Rodrigo, pela orientação, atenção e incentivo desde o princípio. Agradeço por estar sempre disponível para me atender e solucionar minhas dúvidas, pela paciência e compreensão. Obrigada imensamente pela confiança depositada em mim.

Aos meus queridos amigos que o laboratório me presenteou, Aline, Vini, Talita, Even, Natara, Juliana e todos os outros que passaram pela minha vida nesses anos. Obrigada por cada momento de amizade, carinho e descontração. Vocês foram essenciais.

Agradeço à Thelma e a Larissa pelas conversas, orientações e todos os conhecimentos que pude adquirir com vocês. Obrigada por sempre estarem disponíveis e não medirem esforços para me ajudar.

Aos funcionários do Centro Integrado de Pesquisa (CIP), Márcia, Marcelo e Rafaela, por todo o auxílio e tempo dedicado durante a realização dos experimentos.

Aos professores da disciplina de Bioquímica, Ana Carola Magalhães, Marília Rabelo Buzalaf e Rodrigo Cardoso de Oliveira. Obrigada por toda a atenção e pelos ensinamentos.

Ao professor Valdecir Farias Ximenes, Universidade Estadual, Paulista Júlio de Mesquita Filho - UNESP, por aceitar ser meu coorientador neste projeto, pela colaboração e auxilio durante esses anos.

A secretária do departamento Dalva Ribeiro de Oliveira, por ser sempre tão educada e solicita em todos os momentos que eu necessitei, agradeço muito toda a sua ajuda.

Ao professor Danilo Antonini, UNISAGRADO, por ter me encorajado a tomar a decisão de realizar o mestrado, por todos os conselhos e auxílios. Agradeço também por ter se tornado um amigo tão querido.

A todas as pessoas que de forma direta ou indireta contribuíram para a realização deste trabalho, deixo os meus sinceros agradecimentos.

Agradeço a Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP pela bolsa de pesquisa concedida, processo n° 2018/10321-2, indispensável para que a pesquisa alcançasse os resultados obtidos.

O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Agradeço à FAPESP processo nº 2015/12894-1 pelo equipamento multiusuários utilizados na execução deste projeto, BD FACSAria™ Fusion - Citometria de Fluxo e Separação celular (cell sorting).

Agradeço também a Faculdade de Odontologia de Bauru-FOB/USP por ter utilizado as dependências da instituição e todos os equipamentos para a realização deste projeto de mestrado.

“Each dream you leave behind is a part of your future thar will no longer exist”.

Steve Jobs

ABSTRACT

Effect of caffeic acid and caffeic acid phenethyl ester on murine osteosarcoma cells: regulation of the NADPH oxidase complex

Osteosarcoma is the most common type of bone cancer among children and adolescents. Metastasis for this cancer happens around 10-25% of the cases, increasing the mortality rate. The search for new therapeutic strategies has increased for phytochemicals due to their potential as antioxidants and anticancer properties. Studies have reported these properties on caffeic acid phenethyl ester (CAPE) and caffeic acid (CA). In this way, the present study aimed to analyze CAPE and CA's anticancer properties on UMR-106 murine osteosarcoma cells after 72 h of treatment. Cell viability was assessed using the MTT and violet crystal reduction assay, with inhibitory concentrations corresponding to 25 and 50% (IC25 and IC50) of 1.3 and 2.7 µM for CAPE and 91.0 and 120.0 µM for AC, respectively. In addition, a control cell line (MC3T3-E1) was also used for the viabilities assay. The number of apoptotic cells and proliferation rate were quantified by flow cytometry with ANEXIN V-FITC/DRAQ7 and CFSE, respectively and the cell migration behavior was evaluated by the Wound Healing assay. The quantification of reactive oxygen species (ROS) was performed by DCFH-DA fluorescence. NOX-2 and NOX-4 genes were analyzed by RT-qPCR. Data were analyzed by one-way ANOVA. Significant differences between groups were determined by Tukey's post-hoc test at $P<0.05$. Thus, the present study shows the potential anticancer properties of CAPE and highlights how a simple chemical modification can improve the pharmacological potency of a phytochemical in relation to its precursor CA. Our results showed that CAPE was more efficient and selective in reducing the viability of tumor cells, with significant differences, when compared to the control ($P<0.05$) and it was 44-fold (IC_{25}) and 70-fold (IC_{50}) more cytotoxic than CA. CAPE also induced apoptosis and decreased ROS generation, in addition to limiting cell migration. In summary, CAPE was more selective for tumor cells, preserving normal ones, suggesting its potential role as an anticancer drug.

Keywords: Caffeic ester phenethyl ester. Caffeic acid. Cancer. Phenolic compounds. Osteosarcoma.

RESUMO

O osteossarcoma é o tipo de câncer ósseo mais comum entre crianças e adolescentes. A metástase para esse câncer ocorre em torno de 10 a 25% dos casos, diminuindo a taxa de sobrevivência. A busca por novas estratégias terapêuticas tem aumentado para os fitoquímicos devido ao seu potencial como antioxidantes e propriedades anticâncer. Estudos relataram essas propriedades no éster fenetil do ácido cafeico (CAPE) e no ácido cafeico (AC). Deste modo, o presente trabalho teve como objetivo analisar as propriedades anticâncer de CAPE e AC em células de osteossarcoma murino UMR-106 após 72 horas de tratamento. A viabilidade celular foi avaliada por meio do ensaio de redução do MTT e cristal violeta, sendo as concentrações inibitórias correspondentes a 25 e 50% (IC_{25} e IC_{50}) de 1.3 e 2.7 µM para CAPE e 91.0 e 120.0 µM para AC, respectivamente. Adicionalmente, uma linhagem controle (MC3T3-E1) também foi usada para os ensaios de viabilidade. O número de células apoptóticas e a taxa de proliferação foram quantificados por citometria de fluxo com ANEXIN V-FITC/DRAQ7 e CFSE, respectivamente, e o comportamento de migração celular, pelo ensaio de “wound healing”. A quantificação das espécies reativas de oxigênio (ROS) foi realizada por fluorescência DCFH-DA. Os genes NOX-2 e NOX-4 foram analisados por RT-qPCR. Os dados foram analisados por ANOVA de um fator e diferenças significativas entre os grupos foram determinadas pelo teste post-hoc de Tukey em $P<0.05$. Sendo assim, o presente trabalho demonstra as potenciais propriedades anticâncer do CAPE e destaca como uma modificação química simples pode melhorar a potência farmacológica de um fitoquímico em relação ao seu precursor AC. Nossos resultados mostraram que o CAPE foi mais eficiente e seletivo na redução da viabilidade das células tumorais, com diferenças significativas, quando comparado ao controle ($P<0.05$) e foi 44 vezes (IC_{25}) e 70 vezes (IC_{50}) mais citotóxico do que o AC. O CAPE também induziu a apoptose e diminuiu da geração de ROS, além de limitar a migração celular. Em resumo, o CAPE foi mais seletivo para a células tumorais, preservando as normais, sugerindo um papel potencial deste como uma droga anticâncer.

Palavras-chave: Éster fenetil do ácido cafeico. Ácido cafeico. Câncer. Compostos fenólicos. Osteosarcoma.

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

According to the World Health Organization (WHO), in 2018 there were about 9.6 million deaths due to cancer. Globally, cancer is the second leading cause of death, killing about 1 in 6 people (WHO, 2018). The global cancer observatory (GCO) reports that in 2020 breast and lung cancer accounted for 11.7 and 11.4%, respectively, of the number of new cases for all ages in the world (GCO, 2020).

Tumor formation is a multi-stage process that involves a series of events such as the accumulation of genetic and epigenetic changes. Modifications can be initiated by external agents and hereditary genetic factors. The transformation and progression of cancer cells involves complex events, including downregulation of several genes that are fundamental to the processes of differentiation, proliferation, angiogenesis, invasion, metastasis and programmed cell death (CARMONA-FONTAINE et al., 2017). Despite the development of new treatments, cancer is still the second leading cause of death in Western countries. One of the causes is the fact that several types of tumor develop mechanisms of resistance to the drugs used in conventional treatments (KHAN; MAALIK; MURTAZA, 2016; SAINZ; LOMBO; MAYO, 2012).

Osteosarcoma, for example, is a type of primary bone cancer responsible for 3-5% of the new cases worldwide, representing 0.2% of all malignant neoplasms. They are a group of rare neoplasms composed by chondrosarcoma, chordoma and osteosarcoma, in which the last one is the most frequent in children and teenagers and the third most frequent in adults. Worldwide, its incidence represents 3.4 per million per year (CZARNECKA et al., 2020; KUO et al., 2015).

Osteosarcoma is characterized by two incidence ranges according to the age group, the first peak is in the young, aged between 15 and 19, and in the elderly from 75 to 79 years old. The incidence in the early ages is due to intense linear bone growth, especially in the long bones which have areas of high degree of dividing and multiplying cells like femur, tibia, humerus and fibula, being rare in short or flat bones (MARINA, 2004). In older adults, the second peak seems to be related to their higher risk for Paget's disease of bone, increase of bone resorption by osteoclasts as well as to

environmental exposures. Also, male patients have a slight prevalence over female in most countries (MIRABELLO et al., 2015; RECH et al., 2004).

The mechanisms involved in the pathogenesis and progression of osteosarcoma include defects in the differentiation of mesenchymal stem cells, abnormal expression of oncogenes and tumor suppressors and dysregulation of various signaling pathways (DENDULURI et al., 2016). Osteosarcoma cells can present a high capacity to form osteoid tissue and induce mineralization of the extracellular matrix due to expression of osteoblastic markers as alkaline phosphatase, osteocalcin or bone sialoprotein (BROWN et al., 2018).

The main signs and symptoms of osteosarcoma are pain, sensitivity change, redness, infiltration of soft tissues, signs of inflammation, collateral circulation, associated or not to palpable tumor or movement limitation (BRITO et al., 2005). The mortality rate of osteosarcoma patients depends on how far along the cancer is progressed, patients with localized disease have a survival rate around 65% after 5 years. However, 10-25% of osteosarcoma patients show metastasis, 90% of the cases they present in the lungs, decreasing the survival rate by 35% (FERRARI et al., 2002; TIAN; GUAN; LI, 2018).

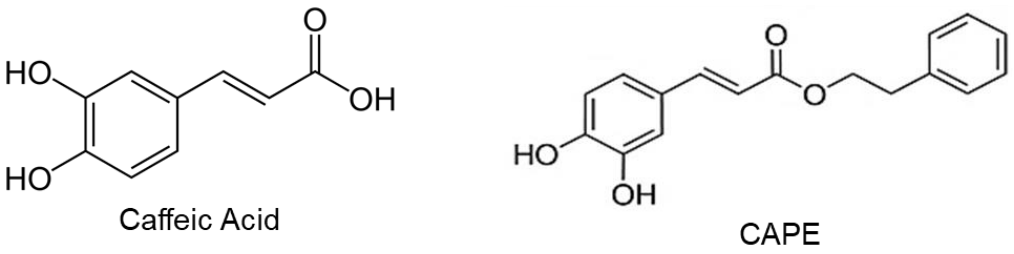
The treatment of osteosarcoma has been refined over the decades, resulting in increased patient survival (ANDERSON, 2016).The current treatment results in a set of strategies composed of surgery to remove the tumor, when possible, radiotherapy and chemotherapy and most commonly chemotherapy with associations (BIELACK; CARRLE; CASALI, 2009). New strategies have been tested, especially regarding new therapeutic targets, using monoclonal antibodies, immunomodulators, drugs that inhibit tumor vascularization, among other mechanisms (MISAGHI et al., 2018; ROTH et al., 2014).

The search for new therapeutic strategies for cancer treatment has increased immensely, justifying the pursuit for the use of phytochemicals due to their vast structural diversity and different action mechanisms (DALLAVALLE et al., 2020). The phenolic compounds represent the major phytochemical group (SANTOS et al., 2018). Phenolic compounds or polyphenols are substances that originated from the secondary metabolism of plants, being essential for their growth and reproduction.

They are a powerful antioxidants class, protecting the biological system against reactive oxygens species (ROS) (ANGELO; JORGE, 2007).

Among the polyphenols, there is the caffeic acid (3,4-dihydroxycinnamic acid, CA), a hydroxycinnamic acid found in several fruits and vegetables like berries, kiwi, apple, carrots, cabbage, and coffee. Besides fruits and vegetables, caffeic acid can also be present in propolis, which is a resinous substance, composed by plants exudates, secreted by bees. It has demonstrated anticancer, antimicrobial, antiviral and antioxidants activities (LI et al., 2020; STAGOS et al., 2012).

Derivatives of CA are naturally found and are also reported for their biological activities. One of its derivatives, the caffeic acid phenethyl ester (CAPE), has been widely studied, because with the substitution of 3,4 dihydroxyl in the aromatic ring, CAPE has proved to be 40 thousand times more fat-soluble than CA, increasing its antioxidant capacity (PARACATU et al., 2014).



Source: Monteiro Espíndola et al., (2019); Murtaza et al., (2014).

Figure 1 – Molecular structure of caffeic acid and caffeic acid phenethyl ester

Recent studies compared their activities in aggressive breast cancer cells and found that the CAPE presented more significant toxicity, apoptotic profile, and cell cycle arrest than CA in the same dosages (KABAŁA-DZIK et al., 2017). Besides, CAPE has also been characterized by its antioxidant properties showing a dose-dependent effect against free radicals, inhibition of xanthine oxidase activity, and blocking lipoperoxidation (RUSSO; LONGO; VANELLA, 2002). It was also reported a cytoprotective effect against induced oxidative stress in human endothelial cells (WANG et al., 2006).

The oxidative stress is the imbalance between the production of intracellular pro-oxidants and their elimination by antioxidants. This oxide balance is provided by and endogenous enzymatic mechanism, but is also influenced by exogenous factors as lifestyle, medications and diet (MIYATA et al., 2017).

Reactive oxygen species (ROS) are formed from oxidation and reduction processes and represent molecules that are present in both physiological and pathological conditions. ROS has been associated, over the years, with the development of cancer, as different types of tumor cells have shown an increase in their levels compared to non-cancer cells. Thus, it is believed that the high levels of ROS are oncogenic, causing damage to DNA, proteins, and lipids, developing genetic instability and the appearance of tumors. In cancer, ROS act as signaling molecules contributing to abnormal cell growth, metastasis, angiogenesis, and resistance to apoptosis. In this way, increased levels of ROS are considered to contribute to tumor growth, resulting in the stimulation of pro-survival signaling pathways, loss of tumor suppressor gene function, elevated glucose metabolism, adaptations to hypoxia and generation of oncogenic mutations (MOLONEY; COTTER, 2017).

The endogenous sources of ROS are the mitochondrial electron transport chain, xanthine oxidase (XO), lipoxygenase (LOX), the cytochrome P450 system, uncoupled nitric oxide synthase, myeloperoxidase and NADPH oxidases, when in oxidant stress they can lead to tumor initiation or progress (ROY et al., 2015).

On the other hand, NADPH oxidases (NOX) are multi-enzyme complexes that catalyze the reduction of an electron from molecular oxygen to the anionic superoxide radical (O_2^-) and are expressed in a variety of cell types. The NOX family is composed by five NOX enzymes (NOX 1 - 5) and two DUOX enzymes (DUOX 1 - 2) that generates ROS (O^{2-} and/ or H_2O_2) as their main product of enzymatic activity. These complexes are widely distributed, being localized in the plasma membrane, intracellular membranes of endoplasmic reticulum, mitochondria, nuclei, etc., depending on the cell type (LITTLE et al., 2017).

NOX enzymes have been shown to increase in association with ROS production and tumorgenicity in various cancer cells as tumor promotion and progression, inflammation and activation of angiogenesis factors (YOUSEFIAN et al., 2019). In this

sense, NOX inhibitors have been shown as an alternative for treating tumors and chronic inflammatory diseases. Chemoprotective strategies using phytochemicals have gained considerable attention for presenting these potential inhibitors (PARACATU et al., 2014).

Considering previous studies with CA and CAPE, the efficacy of the last as a NADPH oxidase inhibitor, and the fact that there are no reports in the literature about their effects on osteosarcoma cells. The purpose of the present study was to explore the potential of CAPE and CA treatment in murine osteosarcoma cells UMR-106 by evaluating their growth-inhibitory effect and elucidating their mechanisms of action.

2 ARTICLE

2 ARTICLE

The article presented in this Dissertation was written according to the instructions and guidelines of the journal Molecules for article submission.

Anti-tumor potential and selectivity of Caffeic acid phenethyl ester in osteosarcoma cells

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Abstract

Osteosarcoma is the most common type of bone cancer, and metastasis is widespread decreasing the survival rate. The search for new therapeutic strategies has increased for phytochemicals due to their potential as antioxidants and anticancer properties. Thus, we evaluated the caffeic acid phenethyl ester (CAPE) and caffeic acid's (CA) anticancer properties on UMR-106 murine osteosarcoma cells. The IC_{25} and IC_{50} were 1.3 and 2.7 µM for CAPE and 91.0 and 120.0 µM for CA, respectively. This study shows the potential anticancer properties of CAPE and highlights how a simple chemical modification can improve the pharmacological potency in relation to its precursor CA. Our results showed that CAPE was more efficient and selective in reducing the viability of tumour cells compared to the control osteoblasts (MC3T3-E1) ($P<0.05$). In addition, CAPE was 44-fold (IC_{25}) and 70-fold (IC_{50}) more cytotoxic than CA. CAPE also induced apoptosis, decreased ROS generation and cell migration. In summary, CAPE was more selective for tumour cells, pre-serving normal ones, suggesting its potential role as an anticancer drug.

Keywords: Antioxidants; Cancer; Cytotoxicity; Phenolic Compounds; Phytochemicals.

1. Introduction

Osteosarcoma is a type of primary bone cancer responsible for 3-5% of new cases worldwide [1]. The incidence is mainly in the young between 15 and 19 and in the elderly from 75 to 79 years old [2]. Metastasis is widespread in this type of cancer, decreasing the survival rate by 35% [3]. The treatment of osteosarcoma has been refined over the decades, resulting in increased patient survival [4]. However, there is still a need for new therapeutic strategies for cancer treatment, justifying phytochemicals' pursuit due to their vast structural diversity and different action mechanisms [5].

Phenolic compounds or polyphenols are substances that originated from the secondary metabolism of plants. They are a powerful antioxidant class, protecting the biological system against reactive oxygen species (ROS) [6]. Among the polyphenols, caffeic acid (CA), a hydroxycinnamic acid found in several fruits and vegetables, and its derivative caffeic acid phenethyl ester (CAPE) have been widely studied due to their antimicrobial, antiviral, anti-inflammatory, and anticancer activities (7, 8). CAPE is among the main active compound in propolis, a resinous substance composed of plant exudates secreted by bees. Recent studies compared their effects in aggressive breast cancer cells and found that the CAPE presented more significant toxicity, apoptotic profile, and cell cycle arrest than CA in the same dosages [9]. Besides, CAPE has also been characterized by its antioxidant properties, showing a dose-dependent effect against free radicals, inhibition of xanthine oxidase activity, and blocking lipoperoxidation [10]. It was also reported a cytoprotective effect against induced oxidative stress in human endothelial cells [11].

The imbalance between the production of intracellular oxidants and their elimination is known as oxidative stress [12]. Oxidative stress has been associated over the years with cancer development by acting as signaling molecules that contribute to abnormal cell growth, metastasis, angiogenesis, and resistance to apoptosis [13]. An endogenous source of ROS is the multienzymatic complex NADPH oxidases (NOXs). The association between NOXs and tumorgenicity in various cancer cells, via tumor promotion and progression, inflammation, and activation of angiogenesis factors has been demonstrated [14]. In this sense, NOX inhibitors have been shown as an alternative for treating tumors and chronic inflammatory diseases.

Chemoprotective strategies using phytochemicals have gained considerable attention for presenting these potential inhibitors [15].

Considering the previous studies with CA and CAPE, the efficacy of the last as a NOX-2 inhibitor [15], and the fact that there are no reports in the literature about their effects on murine osteosarcoma cells, the purpose of this study was to evaluate the cytotoxic and anticancer effects of these compounds on the osteosarcoma murine cell line UMR-106.

2. Materials and Methods

2.1 Cell lines and reagents

2.1.1 Caffeic Acid and Caffeic Acid Phenethyl Ester

Caffeic acid (CAS number 331-39-5) and caffeic acid phenethyl ester (CAS number 104594-70-9) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.1.2 Cell lines and cell culture

The mouse pre-osteoblastic MC3T3-E1 cells, acquired commercially by the American Type Culture Collection (ATCC® - CRL-2593, subclone 4) and rat osteoblast-like osteosarcoma UMR-106 (ATCC® - CRL-1661) were maintained in Minimum Essential Medium Eagle - Alpha Modification (α-MEM) (Gibco, Thermo-Scientific) and Dulbecco's Modified Eagle Medium – high glucose (DMEM) (Sigma-Aldrich), respectively, and supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin 10.000 UI/ streptomycin 0.060 g/L (Gibco) [16]. Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. For cell expansion, the strains after reaching sub-confluence were trypsinized with Trypsin-EDTA 0.1% (Cat: 59429C, Sigma Aldrich, St. Louis, MO, US), incubated for 3 minutes at 37°C with 5% CO2, followed by inactivation of trypsin with medium containing 10% SFB. After centrifugation at 1200 rpm for 5 minutes, the pellet was resuspended in the respective media and the cells were cultivated in T75cm2 (TPP®) flasks. All cell culture plasticware was obtained from Greiner Bio-One (Frickenhausen Germany) [17].

2.1.3 MC3T3-E1 Differentiation

The pre-osteoblast control cell line MC3T3-E1 was subjected to differentiation with osteogenic medium (α-MEM + 10% SBF culture medium supplemented with 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid) for 4 days to differentiate the cells into mature osteoblasts [18].

2.2 Cytotoxicity analysis – MTT reduction

Cell viability data were obtained through the analysis of mitochondrial activity, carried out with the MTT reduction method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich). Briefly, $5x10^2$ cells from MC3T3-E1 and $3x10^3$ cells from UMR-106 were seeded into a 96-well plate in a culture medium with 10% FBS. After the adhesion period of 24 h for the MC3T3-E1 cell line, the culture media was removed, and it was added osteogenic medium for 4 days [19]. For the UMR-106 cell line the adhesion period was 48 h. After the adhesion and differentiation period, the culture medium was replaced by the medium containing different concentration of CAPE (0.25, 1.0, 4.0, 16.0, 64.0 and 128.0 µM) and Caffeic Acid (15.0, 100.0, 150.0, 200.0, 400.0, 800.0, 1600.0 and 3200.0 µM), in addition to the control group (culture medium with 10% FBS and 0.1% DMSO). After each treatment period (24, 48, and 72 h), the culture medium was completely removed, and 110 µL of MTT solution (0.5 mg/mL in culture medium) was added to each well and incubated at 37°C for 4 h. Then, the MTT solution was replaced with dimethyl sulfoxide (DMSO); Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) for 30 min [20]. The absorbance was determined at 550 nm using a microplate reader (Synergy™ Mx monochromator multimode microplate reader, Biotek Instruments Inc, Winooski, Vermont, USA).

2.3 Cytotoxicity analysis – Crystal violet

The crystal violet stains the nucleic acids of viable cells. Cells were seeded at a density of $5x10^2$ cells from MC3T3-E1 and $3x10^3$ cells from UMR-106 into a 96-well plate in culture medium with 10% FBS. After the adhesion period of 24 h for the MC3T3-E1 cell line, the culture media was removed, and it was added osteogenic medium for 4 days. For the UMR-106 cell line, the adhesion period was 48 h. After the adhesion and differentiation period, the culture medium was replaced by the medium containing different concentration of CAPE (0.25, 1.0, 4.0, 16.0, 64.0, and 128.0 µM) and Caffeic Acid (15.0, 100.0, 150.0, 200.0, 400.0, 800.0, 1600.0 and 3200 µM), in addition to the control group (culture medium with 10% FBS and 0.1% DMSO). After 72 h, the culture medium was removed entirely, 50 µL of 0.5% crystal violet staining solution was added to each well and incubated for 20 min at room temperature on a microplate shaker. Then the plate was washed under a stream of tap water. After completely removing the stain, the plate was set to air dry for at least 24 h at room temperature. After completely dry, it was added 200 µL of methanol to each well, and the plate was incubated for 20 min at room temperature on a microplate shaker to dissolve the crystals [19]. The absorbance was determined at 570 nm using a microplate reader (Synergy™ Mx monochromator multimode microplate reader, Biotek Instruments Inc, Winooski, Vermont, USA.

The absorbance of each reaction was converted to cell viability (%) using the following equation: (absorbance treatment / absorbance control) × 100. The half maximal inhibitory concentration (IC_{50}) values for each cell line were calculated using the GraphPad Prism® software.

2.4 Hematoxylin-eosin staining (HE) qualitative analysis

The cells were seeded at a density of $5x10^3$ cells for MC3T3-E1 and $5x10^4$ cells for UMR-106 on 13 mm round glass coverslips inside a 24-well plate. After the adhesion and differentiation period for each cell line, the culture medium was removed and was replaced by the complete medium with the IC_{25} concentration of CAPE and Caffeic Acid, in addition to the control groups with a culture medium with 0.1% DMSO. After 72 h of treatment, the cells were fixed with formalin solution, neutral buffered,

10% for 30 min, and washed with PBS 1X. Then, the hematoxylin stain was added for 3 minutes and underwent color separation with 0.5% alcohol-acid. The cells were subsequently dyed with eosin 1% for 2 minutes, dehydrated with gradient ethanol, soaked with xylene and assembled with Entellan®. (SI et al., 2015). Representative images were captured using the digital camera Olympus U-TV0.5XC-3 (Olympus, Tokyo, Japan) microscope at 40X magnification [22].

2.5 Annexin V-FITC/DRAQ7 flow cytometry analysis for apoptosis quantification

The cells were plated at a density of $1.5x10^5$ cells per well into 6-well plates for the UMR-106 cell line. After the adhesion time, the culture medium was removed, and was replaced by the complete medium with the predetermined IC_{25} concentration of CAPE and Caffeic Acid, in addition to the control groups with culture medium with 0.1% DMSO. After 72 h, cells were harvested by trypsinization, then washed twice with cold PBS and centrifuged at 1200 rpm. About $5x10^5$ cells were then resuspended in 250 µL 1x Annexin V Binding Buffer and incubated with 5 µL of FITC Annexin V (Cat: 556419, BD Pharmingen™) and 2,5 µL of DRAQ7™ (Cat: 564904, BD Pharmingen™) in the dark for 10 minutes at room temperature. The cells fluorescence intensity was analyzed by BD FACSAria™ Fusion flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed in FlowJo v10 software (FlowJo LCC, OR, USA) and cells were quantified as live (AV-/DRAQ7-; Q4), necrosis (AV-/ DRAQ7+, Q1), early apoptosis (AV+/ DRAQ7-; Q3) and late apoptosis (AV+/ DRAQ7+; Q2), [23, 24].

2.6 CFSE flow cytometry for proliferation analysis

After the growth period of the UMR-106 cell line, the cell proliferation assay was performed by labelling cells with carboxyfluorescein succinimidyl ester (CFSE - Cat: 565082, BD Horizon™) followed by flow cytometry analysis. Cells were harvested by trypsinization and washed with PBS. $6x10^6$ cells were separated into a 15 mL tube, centrifuged at 1200 rpm for 5 min and resuspended in 1 ml PBS, and then stained with 10 µM of CFSE for 10 min at 37ºC, followed by its inactivation with 1 mL of FBS. Cells were washed with PBS 1x and $1x10^6$ cells were separated to have its fluorescence intensity measured by BD FACSAria™ Fusion flow cytometer (BD Biosciences, San Jose, CA) at day zero. The rest of the stained cells were plated $2.5x10^5$ cell per well

into 6-well plates. After 24 h, the culture medium was removed and replaced by the complete medium with the IC_{25} concentration of CAPE and Caffeic Acid, in addition to the control groups with culture medium with 0.1% DMSO. After 72 h, cells were harvested by trypsinization, then washed twice with cold PBS and centrifuged at 1200 rpm. The fluorescence intensity was analyzed by BD FACSAria™ Fusion flow cytometer (BD Biosciences, San Jose, CA), [25, 26].

2.7 Reactive Oxygen Species

After the growth period, the UMR-106 cell line was seeded at a density of $2x10^4$ cells per well into a 96-well black plate with a clear bottom in culture medium with 10% FBS and incubated for 24 h at 37°C and 5% CO_2 for cell adhesion. The culture medium was removed and replaced by the complete medium with the IC_{25} concentration of CAPE and CA, in addition to the control groups with culture medium with 0.1% DMSO. After 72 h, the cells were washed with PBS 1X and incubated with 25 µM in PBS of DCFH-DA (2′,7′-Dichlorofluorescein diacetate - Cat: D6883, Sigma-Aldrich) for 45 minutes (37°C, 5% CO_2) in the dark. Then the DCFH-DA solution was replaced with DMEM without phenol red and 10% FBS. The fluorescence intensity was measured with a spectrofluorometer at excitation at 495 nm and emission of 530 nm (monochromator based on Synergy MX) [27, 28].

2.8 Wound Healing Assay

The cell migration assay was based on the model described by Andrade Carvalho et al. [29] with some adaptations. UMR-106 cells were seeded at a density of $1.5x10^5$ cells/well in 12-well plates. The cells were kept in an oven for 72 h to acquire full confluence. Then the wells were treated with 5 µg/mL of mitomycin C (Sigma-Aldrich) for 2 h. The treatment with mitomycin C ensured that cells were migrating and not proliferating. The wells were washed with PBS 1X and a vertical slit was made in the monolayer with a 1000 µL tip. The wells were washed three times with PBS 1x, and the complete medium with the IC_{25} concentration of CAPE and Caffeic Acid, in addition to the control groups with culture medium with 0.1% DMSO

The plates were photographed at 0, 24, 48 and 72 h of exposure to the compounds using a phase contrast microscope coupled to the Olympus U-TV0.5XC-3

digital camera, with a 4X objective. The test was performed in biological triplicate. The percentage (%) of the slot area was calculated by the ImageJ Software and the percentage (%) of the closed slot area was calculated using the formula: % Slit closed area = (%A0 -%A72) x 100%A0. Where % A0 is the percentage of the gap area at 0 h and % A72 is the percentage of the gap area at 72 h.

2.9 Real-time qRT-PCR

After the growth period, UMR-106 cell line was seeded at density of $2x10^5$ cells in 6-well plates. After 24 h of adhesion time, the culture medium was removed, and was replaced by the complete medium with the IC_{25} concentration of CAPE and Caffeic Acid, in addition to the control groups with culture medium with 0.1% DMSO. After 72 h of treatment, the total RNA of the cells were isolated by the column extraction method, using the mini-kit RNeasy 74106 and DNase-79254 (QIagen), according to the manufacturer's instructions. The samples were resuspended in DEPC water and quantified by Nanodrop. The amount of 1.0 µg of RNA was reverse transcribed and the equivalent to 0.04 µg of cDNA was used for the PCR reactions by Sensi mix hi-Rox SYBR Green (Bioline). The results were expressed as times of increase in qRT-PCR and normalized by the reference gene (β-actin). PCR amplification was performed using the following primer sets: CYBB (NOX-2), 5'-CTCTTTGTGATCTTCATCG-3' (forward), 5'-TCCATTTCCAAGTCATAGGAG-3' (reverse); NOX-4: 5'-ACAACCTCTTCTTTGTCTTC-3' (forward), 5'-GTCTGCTATGG-ACATATTCTG-3' (reverse); β-actin: 5'-ATTGAACACGGCATTCTCACC-3' (forward), 5'-GGTCATCTTTTCACGGTTGGC-3' (reverse). The β-actin expression was used as a control [30].

2.10 Statistical analysis

Data are presented as a percentage of the mean and standard deviation (SD). The parameters were analyzed by one-way ANOVA coupled with Tukey's post-hoc test; for all analyzes, values of $P<0.05$ were considered statistically significant. Results are reported as means ± SD of 2-3 independent experiments with 5-8 wells of cells per treatment condition per experiment. All statistical tests were performed using GraphPadInStat and Prism (GraphPad, San Diego, CA).

3. Results

3.1 Viability Assays – MTT and Crystal Violet – CAPE was more efficient than CA and more selective to the osteosarcoma cells

The analysis of MTT reduction demonstrated that after 72 h treatment, there was a significant reduction in the cell viability compared to the control groups (Figure 1). The 72 h period was the one that showed more significant results in a time-concentration-depend manner. As shown, CAPE and CA's effects were concentration-dependent, and they were more cytotoxic to the osteosarcoma cell line. The results of the inhibitory concentration (Table 1) showed ($P<0.05$) that both compounds were more cytotoxic to the cancer cell line than the controls. Besides, CAPE was significantly more potent than CA (Figure 1).

The compounds' cytotoxicity profile remains for the crystal violet assay, with the DNA damage being slightly more pronounced than the mitochondrial one, observed in the MTT assay, for the highest concentrations (Figure 1). In this way, according to the results of the crystal violet assay, it was established that the next experiments would be carried out with the IC_{25} values since higher concentrations are more toxic to the tumor cell and resulted in a reduction of cell viability.

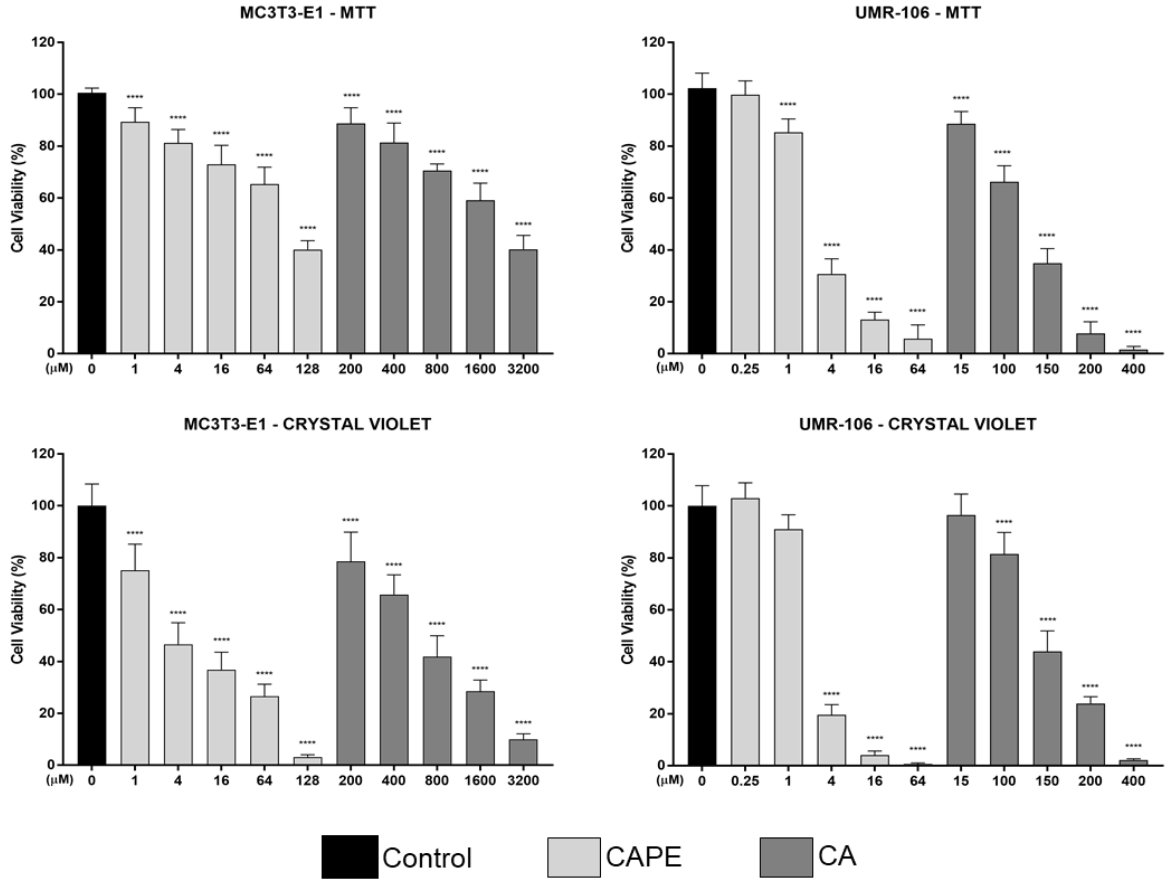

Figure 1. Effect of CA and CAPE on cell viability: MTT and crystal violet assays. Osteoblasts (MC3T3-E1) and osteosarcoma cells (UMR-106) were treated with CA and CAPE's indicated concentrations for 72 h. Values expressed as means ± standard deviation. ****P<0.0001 represents statistical differences when compared to the control.

Table 1. Inhibitory concentration of CA and CAPE on cell viability

		MC3T3-E1	UMR-106
CAPE (μM)	**IC_{25}**	18.7 ± 3.1	1.3 ± 0.5
	IC_{50}	66.3 ± 10.4	2.7 ± 0.1
CA (μM)	**IC_{25}**	620.8 ± 74.5	91.0 ± 1.3
	IC_{50}	2239.0 ± 219.7	119.5 ± 1.7

The cells were treated with CA and CAPE for 72 h. IC_{25} and IC_{50} expressed as means ± standard deviation of the means from three independent experiments.

Table 2. Effectiveness ratio between cell lines [a] and compounds [b,c].

$IC_{(MC3T3-E1)}$ / $IC_{(UMR-106)}$ [a]		
CAPE	IC_{25}	14
	IC_{50}	24
CA	IC_{25}	6
	IC_{50}	18
MC3T3-E1 - CA / CAPE [b]		
IC_{25}		33
IC_{50}		33
UMR-106 - CA / CAPE [c]		
IC_{25}		70
IC_{50}		44

3.2 Morphology Assay

The qualitative morphological analysis was performed by hematoxylin-eosin staining after 72 h of treatment with CA and CAPE (Figure 2) [20]. Compared with the controls, there was a reduction of the number of UMR-106 cells after treatment with the IC_{25} of both compounds and an increase of their size, which appear to be slightly bigger, more widely spaced, and with some cytoplasmatic damage. Regarding the MC3T3-E1 cell line, the decrease in the number of cells was not verified for both compounds. However, the cells appeared bigger, including the nuclei and the cytoplasm.

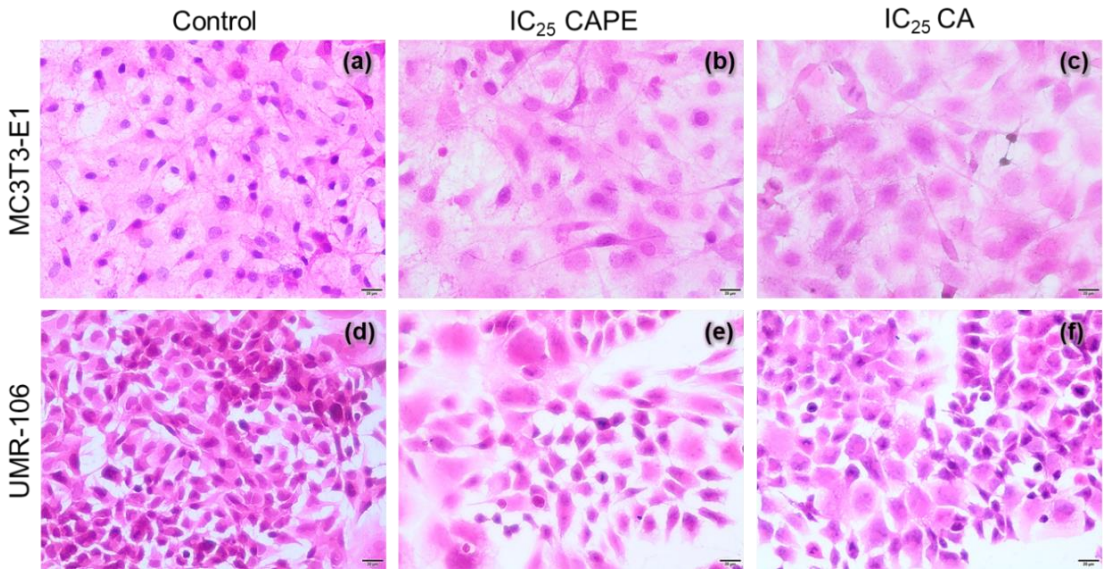

Figure 2. Cells morphological alteration promoted by CA and CAPE: Hematoxylin Eosin Staining of normal and osteosarcoma cells under *Olympus SC30 microscope* 40x magnification, after 72 h of treatment. (a) MC3T3-E1 control (DMSO); (b) MC3T3-E1 treated with IC_{25} CAPE; (c) MC3T3-E1 treated with IC_{25} CA; (d) UMR-106 control (DMSO); (e) UMR-106 treated with IC_{25} CAPE; (F) UMR-106 treated with IC_{25} CA.

3.3 Apoptosis Assay – CAPE promoted a 11.2% increase in apoptosis in osteosarcoma cells

The apoptotic profile was determined by flow cytometry after 72 h of the exposure to the IC_{25} of CA and CAPE (Figure 3). The cells were stained with Annexin V-FITC and DRAQ7 to determine live cells, early apoptosis, late apoptosis, and necrosis through differences in the cell's integrity and permeability plasmatic membrane. For the osteosarcoma cell line, UMR-106, there was a statistical decrease ($P<0.05$) in the percentage of live cells from 68.0% (control group) to 61.0%, and an increase ($P<0.001$) of early apoptosis from 28.5% to 39.7% for the CAPE treatment. However, neither CAPE nor CA showed statistical differences for late apoptosis and necrosis when compared to the control group.

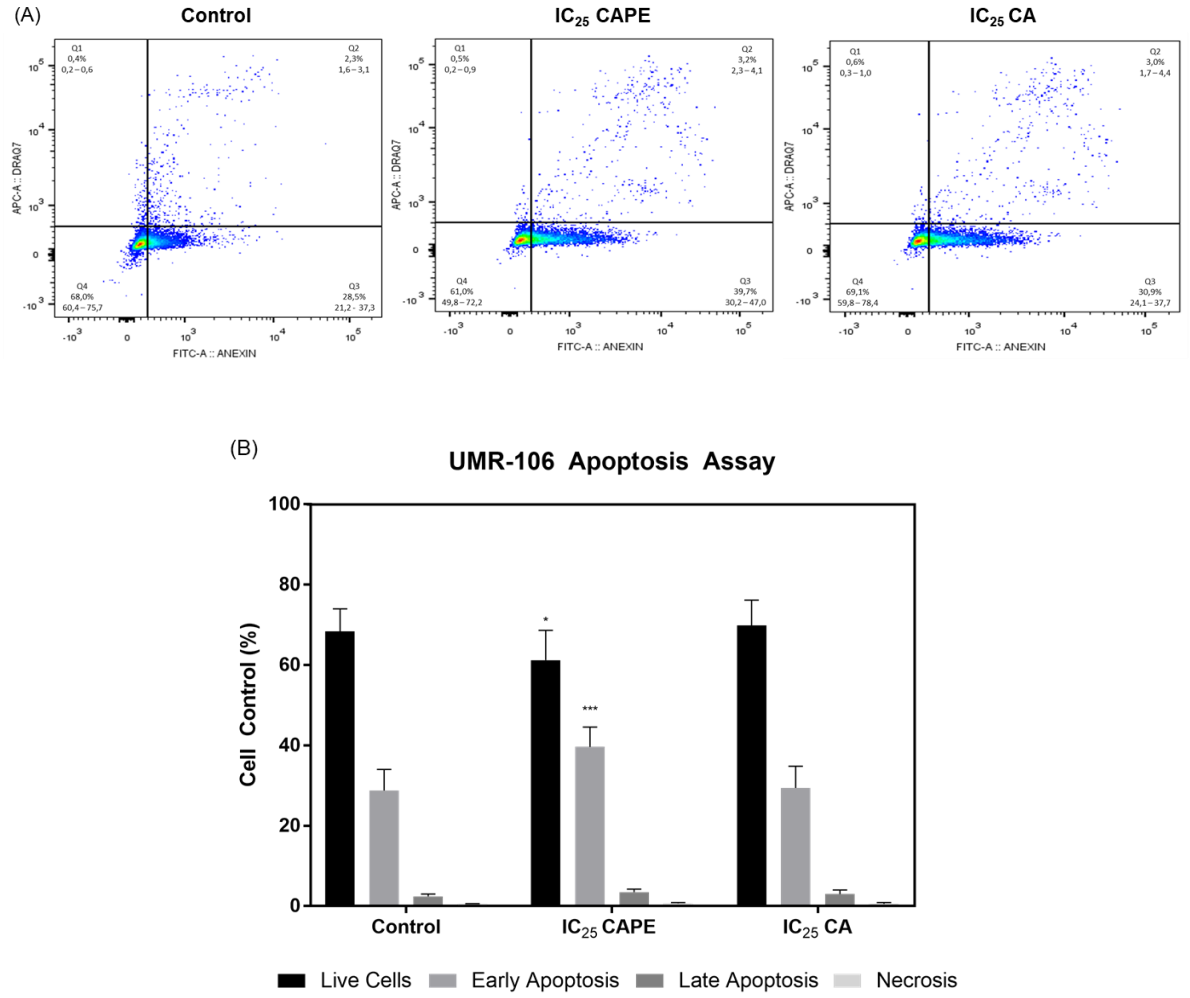

Figure 3. Apoptosis and necrosis promoted by CA and CAPE. Flow cytometry analysis (A) and statistical analysis (B) of osteosarcoma cells (UMR-106) treated with CA and CAPE within 72 h. Values expressed as means ± standard deviation. *P<0.05 and ***P<0.001 represents statistical differences when compared to the control.

3.4 Proliferation Assay

CFSE-staining cell proliferation assay was performed by flow cytometry with UMR-106 in two periods, 0 and 72 h of treatment (Figure 4). The peak control 0 h represents the number of cells without treatment. The peak control 72 h represents the number of cells incubated with 0.1% DMSO for 72 h. The treatment peaks represent the number of cells incubated with CAPE CA for 72 h. According to the results, it was not possible to distinguish the number of cell divisions after staining. Also, within 72 h, there were so many cells divisions that the signal's intensity could no longer be read in all three groups. It is possible to observe that the number of cells increased equally to

the DMSO, CAPE, and CA compared to the control group of 0 h. In short, the treatments were not capable of decreasing cell proliferation.

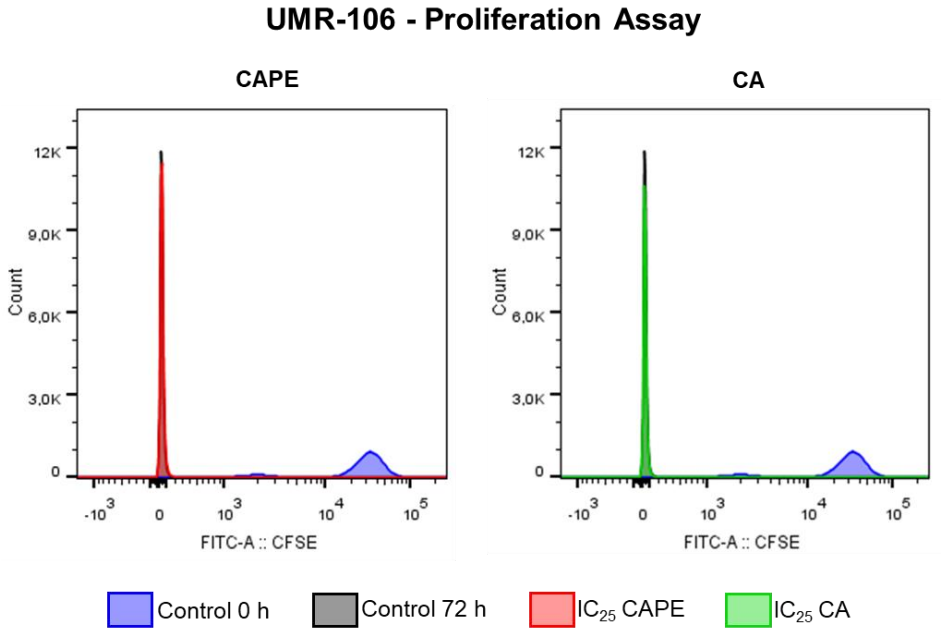

Figure 4. Effect of CA and CAPE on cell proliferation - Flow cytometry analysis of osteosarcoma cells (UMR-106) stained with CFSE fluoresce die and treated with CA and CAPE's indicated concentrations for 72 h.

3.5 Reactive oxygen species - treatment with CAPE's IC_{50} promoted a reduction in the generation of ROS in osteosarcoma cells

After UMR-106 treatment with the IC_{25} and IC_{50} of CA and CAPE, the intracellular ROS level was measured by DCFH-DA assay (Figure 5). At IC_{25}, there were no statistical differences between the treatments and the control group for both cell lines. However, for the cell treated with CAPE, at IC_{50} level, a statistically significant decrease (***P<0.001) was obtained.

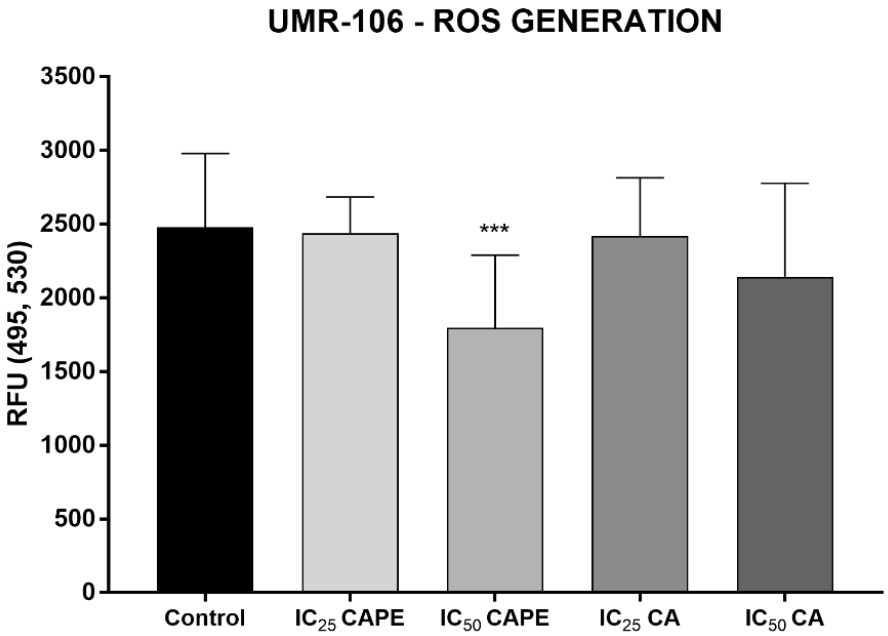

Figure 5. Effect of CA and CAPE on the production of reactive oxygen species – Osteosarcoma cells were treated with CA and CAPE's indicated concentrations for 72 h. Values expressed as means ± standard deviation. ***P<0.001 represents statistical differences when compared to the control.

3.6 Quantitative reverse transcription PCR (RT-qPCR)

The effect of CAPE and CA on NADPH oxidase was evaluated by measuring the relative mRNA expression of CYBB (NOX-2) and NOX-4 in relation to β-actin (reference gene) in UMR-106. CAPE did not provoke any effect in the tested concentration. On the other hand, CA was able to negatively regulate the CYBB ($P<0.001$) and NOX-4 ($P<0.05$) levels (Figure 6).

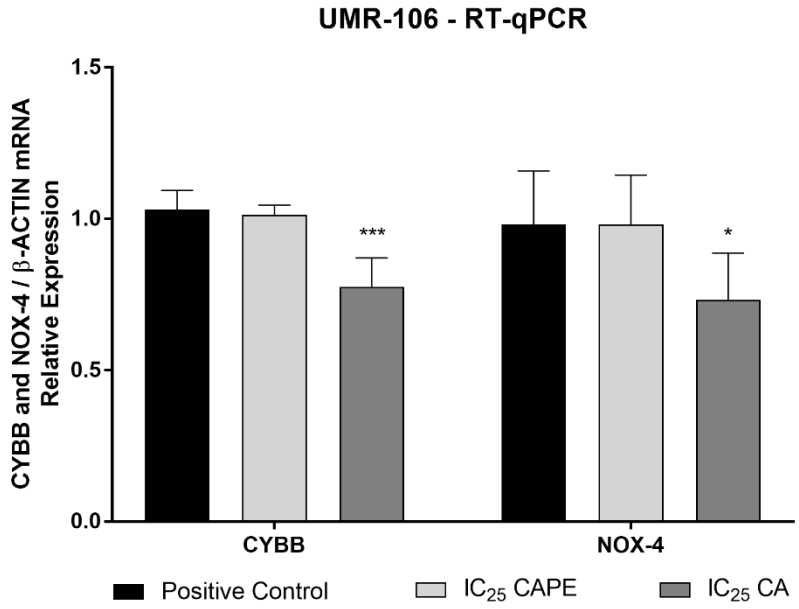

Figure 6. Effect of CA and CAPE on NOX gene expression - Levels of CYBB (NOX-2) and NOX-4 gene expression in osteosarcoma cells (UMR-106) after treatment with CA and CAPE's indicated concentrations within 72 h. Values expressed as mean ± standard deviation. *P<0.05 and ***P<0.001 represent statistical differences when compared to the control.

3.7 Wound healing assay - CAPE and CA promoted a reduction of cell migration in osteosarcoma cells

Once the control cell line does not show migratory properties, the wound-healing assay was only performed with the cancer cell line (Figure 7 A, B). The cells were treated with the IC_{50} of the compounds, and the images were obtained in the periods of 0, 24, 48, and 72 h. For the 24 h incubation period, the wound closure was 24, 15, and 20%, for control, CAPE, and CA, respectively. At 48 h, they reached 42, 22, and 29%, and at 72 h, 56, 27, and 31%. Significant differences (P<0.001) were seen in all periods for both treatments compared to each control group period. The assay was only performed with the IC_{50} of the compounds because the treatment with the IC_{25} did not show a difference compared to the control group.

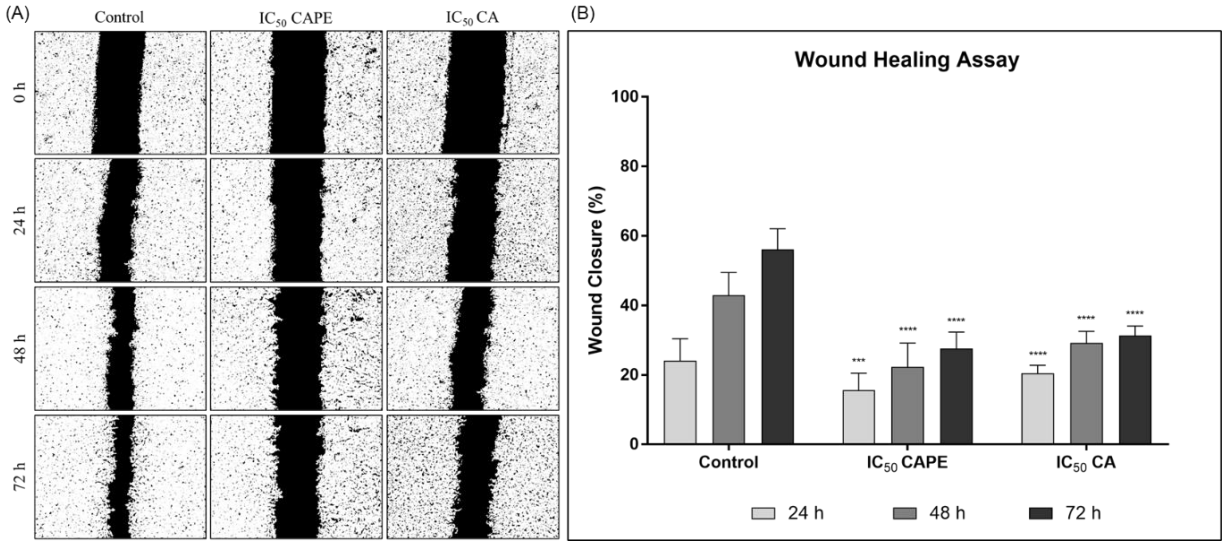

Figure 7. Cell migration provoked by CA and CAPE – Wound healing assay of osteosarcoma cells (UMR-106) after the periods of 0, 24, 48 and 72 h of treatment with CA and CAPE. (A). Percentage graph of the wound closure analysis. Values expressed as means ± standard deviation (B). ***P<0.001 and **** P<0.0001 represent statistical differences when compared to the control.

4. Discussion

This study shows the potential anticancer properties of CAPE and highlights as a simple chemical modification can improve the pharmacological potency of a phytochemical. As shown, compared to its precursor CA, CAPE was more efficient in the reduction of viability of the osteosarcoma cells (UMR-106), induction of apoptosis, and decrease of cell migration. For the half-maximal inhibitory concentration, calculated after 72 h of treatment, CAPE displayed 44-fold higher toxicity than CA. The improvement was still higher if it is taken into account the quarter-maximal inhibitory concentration, where CAPE was 70-fold more cytotoxic than CA. Our proposal for explaining this property of CAPE relies on its increased hydrophobicity compared to CA. This physicochemical feature has been demonstrated to be essential in different biological models and also for other phytochemicals [15, 31]. For instance, for head and neck squamous carcinoma, CAPE's efficacy was 2-fold higher than CA [32]. The same tendency was reported using triple-negative breast adenocarcinoma cells (MDA-MB-231) and breast cancer cells (MCF-7) [33]. According to Kudungunti et al. [34], this efficacy could be explained by the compound's lipophilicity since CAPE is 40 thousand-fold more lipid-soluble than CA. This property enables an efficient partition in the lipid membrane and access to the cell interior and organelles. In the same investigation

line, gallic acid and synthesized analogues were tested against wild-type human ovarian cancer (A2780). It was found that almost all synthesized analogues presented better anticancer activity. This effect was due to introducing an ester oramide linkage that reduced the hydrophilicity and enhanced the lipophilicity [35].

CAPE was not only more cytotoxic to the UMR-106 cells but also more selective. Indeed, CAPE was able to interfere with tumor cells' viability while preserving the viability of normal ones (MC3T3-E1). Similar results were observed for natural phenolic compounds such as quercetin, hesperidin, and epigallocatechin-gallate, which showed more pronounced and evident effects on tumor cells [36].

Regarding the mechanism by which CAPE [37, 38], CA [36], and other natural phenolic compounds display selectivity to tumor cells, the involvement of NF-kappa β transcription factor [38, 39] and signaling pathways that control the proliferation process [36, 40] and cell cycle [41, 42] have been proposed. Corroborating, our results of cell viability and migration behavior suggest that similar pathways could be involved in CAPE's action on UMR-106 cells.

Several authors have demonstrated the effect of NOX inhibitors on the expression of the p53 gene, increasing its expression and consequently inducing tumor cell apoptosis [40–42]. The surviving is due to a protein with anti-apoptotic action highly overexpressed in tumor cells and involved in invasion and metastasis. Interestingly, its expression was reduced due to the effect of CAPE [42]. These mechanisms of action of phenolic compounds and NOX inhibitors on the activation of the apoptosis process [36, 42] are also present in osteosarcoma cells [41], demonstrating remarkable similarity to our findings.

The apoptosis increase after treatment with CAPE (IC_{25}) might be related to reducing ROS (IC_{50}) due to the CAPE capacity to counterbalance the oxidative stress produced in cancer cells and regulate the apoptosis process. The consequence would be preventing cell proliferation and metastasis. It is worthy of note that metastasis is quite common in osteosarcoma [43]. In agreement, CAPE was able to decrease the cell migration behavior in UMR-106 cells. This result agrees with those reported to breast cancer cells [44] and pancreatic ductal adenocarcinoma [45]. In the last case, lower doses reduced cell migration, and higher doses decreased cell growth. In short, the interference in the ROS generation pathway caused by the inhibition of the NOX enzymes could lead to an increase of apoptosis and cell migration reduction due to the overcome of the oxidative stress.

In summary, our findings highlight the promising effectiveness of CAPE as a potential anticancer drug. This beneficial health property seems to be related to its lipophilicity and antioxidant properties. The reduction of UMR-106 cell viability appears to be related to acute mitochondrial and DNA damage, an increase of apoptosis, a decrease of ROS generation, and cell migration behavior. Further studies must be carried out to clarify the relationship between cytotoxicity and the ROS vias that CAPE has altered.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

This study was based on a thesis submitted by the first author to the Bauru School of Dentistry, University of São Paulo, to obtain the title of Master in Applied Dental Sciences. We would like to thank to FAPESP for its financial support (#2018/10321-2). “This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) Finance Code – 001”.

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3 DISCUSSION

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Polyphenols act as anti-tumor agents regulating numerous molecular targets related to survival, proliferation, angiogenesis, invasion and metastasis (MILEO; NISTICÒ; MICCADEI, 2019). Caffeic acid (3,4-dihydroxycinnamic acid, CA) has been reported for presenting antioxidant, antibacterial, antifungal and anticancer properties. A derivative of CA, Caffeic acid phenethyl ester (phenethyl 3- [3,4- dihydroxyphenyl] acrylate, CAPE), which is a component of propolis, is a phenolic compound that possess a catechol group. CAPE has also been reported for presenting several biological activities, such as antioxidant, anticancer, anti-inflammatory and antimicrobial (QIN et al., 2021). Therefore, investigations for new sources of biologically active compounds are very important for the discovery of new drugs that can be used as treatments and/or adjuvants against cancer (AKYOL et al., 2013).

This study shows the potential anticancer properties of CAPE and highlights as a simple chemical modification can improve the pharmacological potency of a phytochemical. As shown, compared to its precursor CA, CAPE was more efficient in the reduction of viability of the tumor cells (UMR-106), induction of apoptosis, and decrease of cell migration. For the half-maximal inhibitory concentration, calculated after 72 h of treatment, CAPE displayed a 44-fold higher toxicity compared to CA. The improvement was still higher if it is taken into account the quarter-maximal inhibitory concentration, where CAPE was 70-fold more cytotoxic than CA. Our proposal for explaining this property of CAPE relies on its increased hydrophobicity compared to CA. This physicochemical feature has been demonstrated to be essential in different biological models and also for other phytochemicals (M.Q.G. DE FARIA et al., 2012; PARACATU et al., 2014). For instance, for head and neck squamous carcinoma, CAPE's efficacy was 2-fold higher than CA (DZIEDZIC et al., 2017). The same tendency was reported using triple-negative breast adenocarcinoma cells (MDA-MB-231) and breast cancer cells (MCF-7) (KABAŁA-DZIK et al., 2018). According to Kudungunti et al. (KUDUGUNTI et al., 2010), this efficacy could be explained by the compound's lipophilicity since CAPE is 40 thousand-fold more lipid-soluble than CA. This property enables an efficient partition in the lipid membrane and access to the cell interior and organelles. In the same investigation line, gallic acid and synthesized

analogs were tested against wild-type human ovarian cancer (A2780). It was found that almost all synthesized analogs presented better anticancer activity. This effect was due to introducing an ester oramide linkage that reduced the hydrophilicity and enhanced the lipophilicity (SHERIN; SOHAIL; SHUJAAT, 2019).

Other reports showed a more selective effect of CAPE (FRENKEL et al., 1993; MURTAZA et al., 2014) and CA (MULLER et al., 2019) against tumor cells. In the viability assay through MTT reduction it was possible to observe that the treatment with the compounds promoted a decrease in cell viability in a dose-dependent manner for both cells. The MC3T3-E1 presented more resistant to the compounds than UMR-106. According to the viability results, the half maximal inhibitory concentration (IC_{50}) was 24-fold for CAPE and18-fold for Caffeic acid higher for the normal cell compared to the osteosarcoma. In this way, it was possible to work with concentrations specially selected for UMR-106 without affecting MC3T3-E1 control cells. A similar result was found when CAPE inhibited the growth of multiple myeloma cells in a dose-depend manner without altering the viability of the control group, although the concentrations used were higher than the ones in this study (MARIN et al., 2019).

This selectivity, that also occurs for other types of natural phenolic compounds, seems to be associated with the mechanisms involving the NF-kappa β transcriptional factor (MURTAZA et al., 2014; NATARAJAN et al., 1996) and other signaling pathways that control the proliferation process (LIN et al., 2015; MULLER et al., 2019) and cell cycle (KITAMOTO et al., 2018; SARI; SÜMER; CELEP EYÜPOĞLU, 2020). In this way, our results involving cell viability and migration behavior are compatible with previous studies with different cells lines and phenolic compounds, which indicates similar cell mechanisms.

It is acknowledged that apoptosis and cell cycle dysregulation are closely associated events and a disturbance in cell progression can result in cell death. Alterations in cell cycles checkpoints and uncontrolled cell proliferation are frequent characteristics of many cancers (PELINSON et al., 2019). Studies report that CAPE and CA cytotoxicity is due to their capacity to induce apoptosis. Studies show these properties in human cervical cancer (CHANG et al., 2010), breast cancer cells (KABALA-DZIK et al., 2017), nasopharyngeal carcinoma (LIANG et al., 2019), ovarian (LIU; HAN; LIU, 2018), oral (KUO et al., 2015), prostate (TSENG et al., 2016) and lung

cancer (CHEN et al., 2004). The results obtained in this study revealed that CAPE (IC_{25}) affected the apoptotic process that occur in the osteosarcoma cells increasing the early apoptosis in 11.2%, although CA did not affect the apoptosis profile in these cells. A distinct result was found when CA was tested in different concentrations through mitochondrial apoptotic pathway in human cervical cancer (CHANG et al., 2010).

The control mechanisms of apoptosis specifically appear to be regulated and/ or modulated in tumor cells that are under the effect of phenolic compounds (MULLER et al., 2019). Several authors demonstrated the effect of NOX inhibitors on the expression of the p53 gene, increasing its expression and consequently inducing tumor cell apoptosis (KITAMOTO et al., 2018; LIN et al., 2015; SARI; SÜMER; CELEP EYÜPOĞLU, 2020). The survivin is a protein with anti-apoptotic action highly overexpressed in tumor cells and involved invasion and metastasis. Interestingly, its expression was reduced due to the effect of CAPE (SARI; SÜMER; CELEP EYÜPOĞLU, 2020). These mechanisms of action of phenolic compounds and NOX inhibitors on the activation of the apoptosis process (MULLER et al., 2019; SARI; SÜMER; CELEP EYÜPOĞLU, 2020) are also present in osteosarcoma cells (KITAMOTO et al., 2018) and demonstrate remarkable similarity to our findings.

The loss of reproductive ability and proliferation are common characteristics in the mechanisms of cell death for cancer cells. A cell that loses the capacity to divide itself into others and consequently proliferate could be considered dead and this is identified as loss of reproductive integrity (PELINSON et al., 2019). In the proliferation assay, as the intensity peaks overlapped between the control and the treatment with CAPE and CA after 72 h, it could be affirmed that neither of the compounds were able to decrease the division cycle of the cells in these parameters. These results differ from other published studies using polyphenols that showed their capacity to alter cell proliferation through mechanisms such as inhibition of DNA synthesis, modulation of reactive oxygen species, regulation of tumor expression genes, among others (CHIANG et al., 2018; D'ARCHIVIO et al., 2008; KUO et al., 2015; LIANG et al., 2019; REN et al., 2019).

The induction of ROS overproduction and the onset of oxidative stress by mitochondrial pathway of apoptosis is activated by different agents, which can induce cells to proliferate uncontrollably and obtain metastatic potential (D'ARCHIVIO et al., 2008). The intracellular sources of ROS include the mitochondria, the electron transport chain, the cytochrome p450, the NADPH oxidase (NOX), lipoxygenase and peroxisomal oxidases. The NOX family is a group of enzymatic complexes that generate ROS as a product of its activity. Studies show that NOX isoforms are overexpressed in several types of cancer, suggesting that NOX can promote cancer advancement by promoting oxidative stress and regulating cell signaling, resulting an increase of cell proliferation, invasiveness, metastasis and angiogenesis. In the same way, the increase of NOX expression and activity are associated to several neurodegenerative and inflammatory diseases (GÀO; SCHÖTTKER, 2017; SKONIECZNA et al., 2017). The regulation of these complexes can contribute to counterbalance the oxidative stress in the cancer cells regulating consequent processes. A study with human osteosarcoma cells showed that NOX-2 mediated ROS generation promoted the cancer cell survival and the ROS depletion through NOX-2 knockdown along a treatment with flavoenzyme inhibitor led to cell apoptosis (KITAMOTO et al., 2018).

In this manner, antioxidants as CAPE and CA can contribute to counteract oxidative stress and control cell proliferation, apoptosis resistance and metastasis. In the reactive oxygen species assay, it was observed a reduction in the ROS production after the CAPE treatment (IC_{50}). This result could be related to the increase of the early apoptosis. Therefore, the decrease of reactive oxygen species enabled the cells to counterbalance the oxidative stress produced in cancer cells and regulate the apoptosis process, in order to prevent further proliferation and metastasis.

In the present study, Caffeic acid slightly downregulated the expression of NOX-2 and NOX-4 which could be related to the capacity of the compound in stall the migration process. A study with lung cancer cells has reported that the inhibition of the NOX activity decreased their invasive potential in vitro. Yet, in cancer stem cells the NOX downregulation provided capacity to prevent metastasis through cell growth and proliferation modulation (SKONIECZNA et al., 2017).

Metastasis is a complex mechanism that involves the detachment of osteosarcoma cells from the primary tumor. In osteosarcoma, metastasis occurs in 10-25% of the cases. In order for cancer cells to spread and disseminate throughout the body, they must migrate and invade the extracellular matrix to reach the blood stream and get to distant sites (JUSTUS et al., 2014). In this type of cancer, metastasis is considered the main problem for patients, as it affects their prognosis and survival rate. The mechanisms of this process are still considerably unclear, for which reason it is crucial to identify new targets for new therapies that not only can kill the primary tumor but can also prevent and suppress the metastasis occurrence in osteosarcoma (CHIAPPETTA et al., 2019).

In this way, through the would-healing assay it was observed the migratory behavior of the osteosarcoma cells under treatment with the compounds. The findings confirmed that both compounds were able to delay cell migration. Within the 72 h of treatment, the stilt closure was 56% for the control, 27% for CAPE and 31% for Caffeic Acid, resulting in a setback of average 29% between the control and the treatments. The wound closure percentage was very much alike for both compounds. As follows, similar effects were seen in breast cancer cells in a dose-time-depend manner, with the same compounds, where they were able prevent and also halt the wound closure with high doses (KABALA-DZIK et al., 2017; KABAŁA-DZIK et al., 2018).

In summary, our findings highlight the promising effectiveness of CAPE as a potential anticancer drug than CA. This beneficial health property seems to be related to its lipophilicity and antioxidant properties. CAPE showed to be more efficient than CA in the cell viability through more acute mitochondrial and DNA damage, an increase of apoptosis, a decrease of ROS generation and of migration behavior. As for the regulation of NOX-2 and NOX-4, only Caffeic acid seemed to affect these genes regulation pathway. For this reason, CAPE was more effective presenting better anticancer properties through induction of apoptosis and possible involvement of oxidative stress. Regarding future studies to be carried out will be able to demonstrate which generation pathways of cytotoxicity and reactive oxygen species have been activated for the presentation of antioxidant properties, while improving our findings on anticancer properties.

There may be some possible limitations in this study as each assay required a different number of cells the concentrations of the compounds had to be adjusted to each one of them. It is possible that the elevated number of cells that some assays required affected the performance of the stablished concentrations.

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ANNEXES

Participation in scientific event

Event name: Reunión Anual de SOCIEDADES DE BIOCIENCIA SAIC. SAIFE. SAB. SAP. AACYTAL. NANOMED-ar. HCS. Mar del Plata, Argentina, November 13th to 19th, 2019.

Presentation of work in poster form:

“Increased resistance of MC3T3-E1 cells differentiated or not compared to UMR-106 in response to Caffeic Acid and Caffeic Acid Phenethyl Ester (CAPE)”. **PAGNAN, A. L.**; PESSOA, A. S.; TOKUHARA, C. K.; FAKHOURY, V. S.; LIESSA, M. R. S.; OLIVEIRA, G. S. N.; XIMENES, V. F.; OLIVEIRA, R. C.

“Evaluation of *Myrcia bella* in murine osteosarcoma cells: effect of the crude extract and fractions of ellagitannins and flavonoids”. FAKHOURY, V. S.; PESSOA, A. S.; **PAGNAN, A. L.**; TOKUHARA, C. K.; OLIVEIRA, G. S. N.; LIESSA, M. R. S. ; SALDANHA, L. L. ; DOKKEDAL, A. L. ; OLIVEIRA, R. C.

“Cytotoxicity of methyl vanillate and methyl divanillate in breast cancer cells”. PESSOA, A. S.; TOKUHARA, C. K.; **PAGNAN, A. L.**; FAKHOURY, V. S. ; LIESSA,M. R. S.; OLIVEIRA, G. S. N.; XIMENES, V. F.; OLIVEIRA, R. C.

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Se deja constancia que el trabajo

- INCREASED RESISTANCE OF MC3T3-E1 CELLS DIFFERENTIATED OR NOT COMPARED TO UMR-106 IN RESPONSE TO CAFFEIC ACID AND CAFFEIC ACID PHENETHYL ESTER (CAPE)

de los autores

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ha sido presentado en la **Reunión Anual de Biociencia 2019**, llevada a cabo en el Hotel 13 de Julio de la ciudad de Mar del Plata, del 13 al 16 de noviembre de 2019

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