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Evaluation Effects of Caffeic Acid and Methylseleninic Acid on Human Breast Cancer Cell Line MDA-MB-231

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ABSTRACT

Breast cancer is one of the most common cancers in women; whose mortality rate has remained high despite medical advances. The present study is aimed to evaluate the antitumor activity of caffeic acid (CA) and methylseleninic acid (MSA), separately and in combination, on the triple-negative human Caucasian breast adenocarcinoma cell line (MDA-MB -231). To evaluate the cytotoxicity of CA and MSA on the growth and proliferation of breast cancer cells, and the rate of cell survival, the MTT test was carried out. DAPI staining was used to determine the type of death induced in the MDA-MB-231 cells. The effect of caffeic acid and methyl seleninic acid on apoptosis was investigated in triple-negative Caucasian breast adenocarcinoma cell lines (MDA-MB- 231) by flow cytometry. Based on the results of the MTT assay, IC50 values of 40 and 30 µg ml^{-1} were calculated for both CA and MSA on MDA-MB -231 cell line after 24 and 48 h, respectively. Based on the results of this study, caffeic acid and methylseleninic acid inhibited MDA-MB-231cell proliferation and induced apoptosis in these cells in a dose-dependent manner by increased Bax/Bcl2 level. Morphological studies also showed apoptosis characteristic features in MDA-MB -231cell after treatment with CA and MSA. Our findings indicated that CA and MSA had significant anti-tumor activity separately and in lower dose in combination. CA is effective in treating breast cancer at lower concentrations if used in combination with MSA.

Keywords: Caffeic acid, Methylseleninic acid, Apoptosis, Breast cancer

INTRODUCTION

Cancer is a disease that disrupts intercellular relationships and order. Cancer is one of the most common threatening diseases worldwide and one of the leading causes of death [1]. Breast cancer is the second most common cancer in women; one in eight women suffers from breast cancer [2]. Apoptosis (literally meaning leaf fall) is a programmed cell death process. There are distinct biochemical and genetic pathways that play an important role in the development and homeostasis of normal tissues [3]. Polyphenols refer to a wide range of natural compounds and plant metabolites, with one or more benzene rings containing one or more hydroxyl groups [4-5]. The average daily intake of polyphenols is about one gram [6-7]. Comic hydroxy acids are polyphenolic compounds and aromatic acids. Caffeic acid (CA) with the chemical formula C9H8O4 is a member of this class. CA derivatives include chlorogenic acid, caffeic acid methyl ester, caffeic acid ethyl ester (CAPE), caffeic butyl ester, caffeine benzyl acid ester, caffeic acid phenyl ester, methyl caffeine, methyl dihydrophytic caffeine, octyl acid, and citric acid. Caffeic acid has shown anti-cancer effects on various cancer cells. However, different cytotoxic activities against normal cells have been shown in the presence of cancer cells [8]. Selenium is one of the rare non-metallic chemical elements. Consuming large amounts of Se is toxic, but in small amounts, it is necessary for cell activity [9]. Se metabolites and their compounds are sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), sodium selenide (Na_2Se), selenium dioxide (SeO_2), selenomitonin, selenocystein, selenodiglutithione (SDG), hydrogen selenide (H_2se) [10]. The chemical form of selenium is used in very important

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experiments and can have a significant impact on its biological activity, including its ability to inhibit cancer growth. Evidence suggests that selenium also protects against the development of cancer in humans [11-12]. Methylseleninic acid (MSA) is a monomethylated form of selenium. MSA is an organic species of selenium that is metabolized by animal cells [13]. The second generation is strong selenium compounds that have very different biological and medicinal activities (selenomitonin, the form of selenium used in vitamins) [14-15]. Previous publications reported selenium nutritional status in whole blood ranging from 0.71 to 3.24 µM [16]. Hence, the aim of this study was to evaluate the combination effects of CA and MSA as a new strategy with induction of apoptosis in breast cancer cell lines.

MATERIAL & METHODS

Cell Lines and Reagents

This study considered one breast cancer line: MDA-MB-231 (human breast, adenocarcinoma, TNCB). MDA-MB -231 cells are adhesive. Cells (1×10^5 cells/ml) were cultured with RPMI 1640 medium containing 10% FBS and 1% antibiotics. The cell was transferred to an incubator containing 5% CO_2 and a temperature of 37 °C. The cell flask was examined daily for cell growth, count, morphology, and contamination. The cell culture medium was replaced with a fresh culture medium every two days. Caffeic acid (CA, Sigma: C0625) and methylseleninic acid (MSA, Sigma: 541281) were purchased from Sigma-Aldrich (USA) and used strictly according to the manufacturer's instruction.

Microculture Tetrazolium Test (MTT)

To evaluate the cytotoxity of CA and MSA on MDA-MB-231. MDA-MB-231 were cultured and then seeded in 96 well plate. The density was 5×10^3 cells per well. This methods is based on the mithochondria dehydrogenase **enzyme.** Only living cells with active mitochondria can participate in this reaction, and can conversion of the yellow solution to insoluble purple crystals of formazan. Whereas dead cells will remain inactive. In a typical process, MTT solution was prepared at a concentration of 5 mg ml^{-1} in PBS. This solution was then stored at 20 °C in the dark (screwed foil) for several months. The cells were treated with CA and MSA, cultured in the 96-well plate, and incubated for 24 and 48 h for MTT testing. After 24 and 48 h, the supernatants were removed, and 15 µl MTT solution was added to each well. Then, the plate was incubated for 3 to 4 h at 37 °C. Afterward, 100 µl DMSO was added followed by storing at room temperature for 5 to 10 min. The absorbance of the control (not treated) and samples were read at 570 nm using an ELISA device. According to the obtained data, the IC_{50} values (the concentration at which 50% of mortality occurred) and the survival percentage were calculated at different incubation times.

DAPI Staining

DAPI staining was performed to determine whether CA and MSA alone and in combination could induce apoptosis in MDA-MB 231 cells. DAPI is an easy and fast approach to diagnose the morphological assessment of apoptotic cells. DAPI contains fluoride that binds to DNA. Thanks to its fluorescence properties, the resulting dye can be seen under a fluorescence microscope. Due to the location of DNA inside the nucleus, staining with this dye can examine the morphology of the nucleus under a microscope. In control and healthy cells, the nucleus will usually be round. In apoptotic cells, however, because the nucleus is dense and fragmented, the nucleus is seen in bold fragments. In a typical procedure, the cells (100,000 cells) were treated with CA (30 µg ml^{-1}) and MSA (3.2 µg ml^{-1}) and their combination. They were then cultured in 96-well plates and placed in an incubator for 48 h. The well contents were removed and washed with PBS. The cells were then fixed with ethanol for 30 min followed by washing with PBS and ethanol. DAPI dye was then added to the cells and stored at laboratory temperature for 5 min in the dark. Afterward, DAPI was removed and washed with PBS, and finally, the cells were observed by a fluorescence microscope.

Flow Cytometry

The cells (100,000 cells) were treated with CA and MSA and then cultured in 6-well plates for 48 h. After that, the supernatants were removed and the adherent cells were collected followed by adding trypsin. The cells were centrifuged for 5 min (900 rpm) and the cell precipitates were suspended in cold PBS and transferred to the microtube. The microtube was centrifuged for 5 min. Then,

the cells were incubated in 500 µl of binding buffer for 3 min. After adding 5 µl of the annexine-v solution, they were incubated in the dark for 15 min followed by adding 5 µl of PI and incubation for another 15 min in the dark. The resulting suspension was transferred to a flow cytometry tube and analyzed for 1 h.

Statistical Analysis

All data on cell growth and apoptosis were analyzed using Gen.52.01 software, analysis starter, FLW.J, IMGE, and Prism. ANOVA test, One-way analysis of variance, and Tukey's Multiple Comparison Test were used to analyze the data. E-cell software was employed to draw the graphs.

RESULTS

Cell Proliferation

After the MDA-MB-231 cell line was treated with CA and MSA, survival and cell proliferation were examined by MTT assay. Cell viability decreased by incrementing the treatment dose, decrease from 96.11% at a dose of 10 µg ml^{-1} to 92.93%, 61.77%, 58.11%, 47.92%, 32.5%, and 31.32% at CA doses of 20, 25, 30, 40, 50, and 100 µg ml^{-1} at 24 h, respectively (Fig. 1a). Also the cell viability decreased when MDA-MB- 231 were treated with MSA in a dose dependent manner. Decrease from 89.31% at MSA dose of 1.6 µg ml^{-1}, to 81.07%, 76.51%, 74.45%, 52.25%, 47.41%, and 37.5% at MSA contents of 2, 2.5, 3.2, 3.5, 5, and 100 µg ml^{-1}, at 24 h, respectively (Fig. 1c). The cell viability of MDA-MB-231 declined when treated with CA at 48 h in a dose-dependent manner; decrease from 72/29% at CA dose of 10 µg ml^{-1} to 62.85%, 52/97%, 46/31%, 28.68%. 27.5%, and 15/42% at CA doses of 20, 25, 30, 40, 50, and100 µg ml^{-1}, respectively (Fig. 1b). The cell viability of MDA-MB-231 were decreased when treated with MSA at 48 h in a dose-dependent pattern, dropping from 64/9% at a dose of 1.6 µM to 49.69%, 43.67%, 38.73%, 25.30%, 20.12%, and 12.08% at 2, 2.5, 3.2, 3.5, 5, and 100 µg ml^{-1}, respectively (Fig. 1d). These results showed that CA and MSA decreased cell viability and induced cell death in a time- and dose-dependent manner. CA led to 50% cell death at 40, 30 µg ml^{-1} at 24 and 48 h.

MSA led to 50% cell death at 5, 3.2 µg ml^{-1} at 24 and 48 h. The dose of CA and MSA significantly decreased at

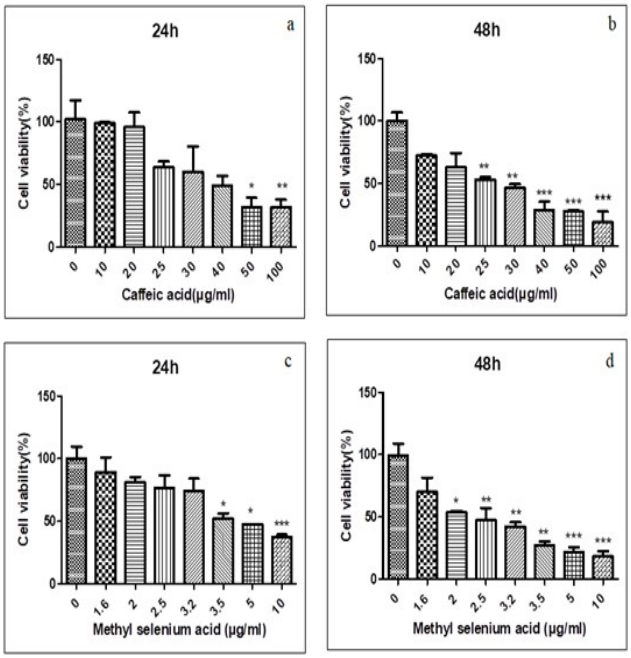

Fig. 1. Survival percentage of MDA-MB 231cells treated with different concentrations of CA (0-100 µg ml^{-1}) after (a) 24 h and (b) 48 h. Survival percentage of MDA-MB 231 cells treated with different concentrations of MSA (0-10 µg ml^{-1}) after (c) 24 h and (d) 48 h. Cell viability was analyzed by MTT assay. Data are reported as means ± SD. (*P < 0.1, **P < 0.01, ***P < 0.001; ANOVA test, TUKEY test).

48 h (Table 1). Results of MTT indicated that CA inhibited cell proliferation by the lowest IC_{50} value 30 µg ml^{-1}. The IC_{50} value of MSA was 3.2 µg ml^{-1}. Our result demonstrated that the combination of these compounds significantly reduced the proliferation of MDA-MB-231 cells (Fig. 2).

DAPI Staining

DAPI staining was used to determine the type of death induced in the MDA-MB-231 cells. Comparison of the treated sample with the control sample showed that the controls had a spherical core with smooth edges and uniform staining but the apoptotic cell exhibited irregular edges around the core, more potent coloring, chromatin density in the core, and collapse on the nuclear membrane or the morphology of the core fragmentation. The results showed the apoptotic death in the MDA-MB-231 Fig. 3.

Table 1. IC_{50} Values (μg ml^{-1}) of CA and MSA in MDA-MB-231 for Different Treatment Times

Compounds	Time of incubation (24)	Time of incubation (48)
Methylseleninic acid	5 μg ml^{-1} (37.5%)	3.2 μg ml^{-1} (25/30%)
Caffeic acid	40 μg ml^{-1} (32.5%)	30 μg ml^{-1} (28/68%)

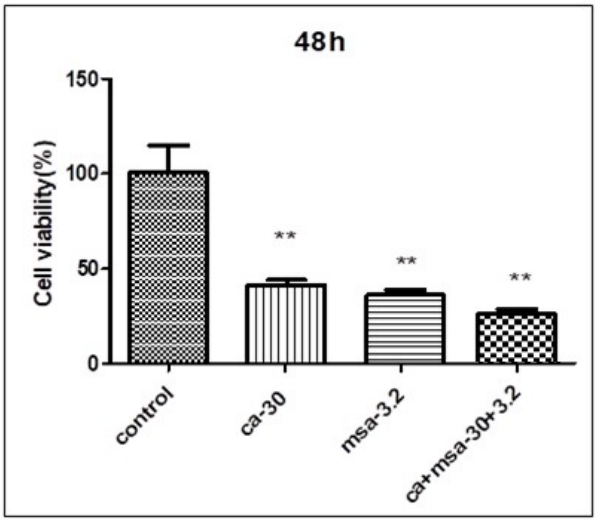

Fig. 2. Survival percentage of MDA-MB 231 cells treated with suitable dose of CA (30 μg ml^{-1}) and MSA (3.2 μg ml^{-1}) after 48 h. Combination effect of CA and MSA compared to the control or even single compound could significantly decrease cell proliferation in MDA-MB-231 cell lines. Data are mean ± S.E of three independent experiments (**P < 0.01; ANOVA test, TUKEY test).

Apoptosis Assay

Flow cytometry was performed to assess the effect of cited compound on apoptosis induction. Annexin-V/PI staining indicated that CA (30 μg ml^{-1}) and MSA (3.2 μg ml^{-1}) induced the apoptosis. According to the obtained data, we observed significant increase in percentage of early apoptotic cells and minimal percentage of necrotic as compared with the Fig. 4. In addition, significant increase of apoptotic cells (53%) were seen in combination of CA and MSA (Table 2).

Table 2. Survival Percentage of MDA-MB-231 Cells Treated with Different Concentrations of CA Acid and MSA after 48 h Based on Flow Cytometry Test

	Control	MSA	CA	MAS + CA
Viable cell	97.4%	66.4%	70.9%	36.6%
Early apoptosis	1.45%	30%	27/1%	31.4%
Late apoptosis	0.828%	2.72%	1.78%	21.6%
Necrotic cell	0.299%	0.860%	0.146%	10.4%

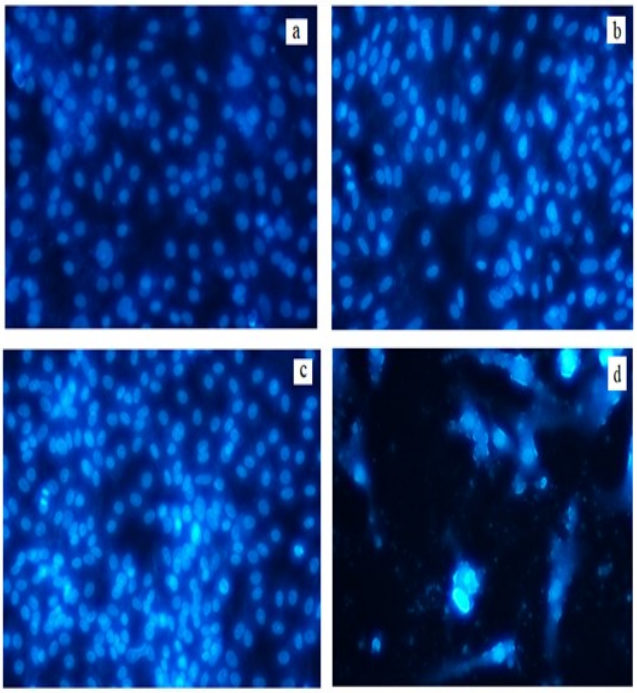

Fig. 3. The cells were visualized by fluorescence microscopy. DAPI stained cells treated with control showed the result as in (a), which shows the rounded nuclei and uniform color, (b) MDA-MB-231 cells treated with CA (30 μg ml^{-1}) after 48 h which shows the apoptotic nucleus cells morphology. The nucleus of living cells is round; (c) MDA-MB-231 cells treated with MSA (3.2 μg ml^{-1}) 48 h exhibiting the apoptotic cell nucleus morphology. The nucleus of living cells is round; (d) MDA-MB-231 cells treated with CA (30 μg ml^{-1}) and MSA (3.2 μg ml^{-1}) after 48 h showing the apoptotic cell nucleus morphology. The nucleus of living cells is round (Observation with a fluorescence microscope at x 400 magnification).

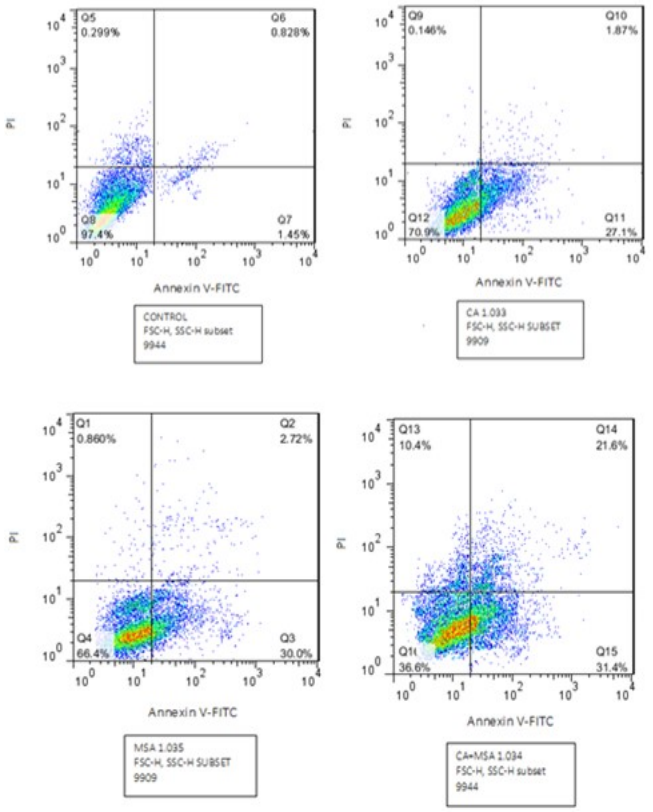

Fig. 4. Flow Cytometry of MDA-MB-231 cells treated with CA (30 µg ml^{-1}) and MSA (3.2 µg ml^{-1}) and their combination after 48 h. The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells.

DISCUSSION

The main goal of cancer biologists is to develop antitumor drugs that prevent breast cancer and reduce oxygen and nutrients required for tumor growth [17-18]. The major challenge in breast cancer is the lack of proper treatment options for a particular type of breast cancer, triple-negative breast cancer. Despite significant progress in cancer treatment and the introduction of new chemotherapy methods, the development and synthesis of new efficient and cancer-selective chemicals are highly demanded.

Accordingly, the interests have significantly increased in studying the therapeutic potential of natural compounds [19-20]. Over the years, research allows us to identify important factors in the diagnosis of breast cancer, understanding its progress, and most importantly, the wise choice of the most effective treatment. Numerous recent studies have introduced natural compounds as a treatment for cancer due to their ability to inhibit the growth of cancer cells and their metastases and induce apoptosis. Many complementary (natural) treatment methods offer new substances that seem to support the treatment of certain types of cancer, even proving their effectiveness through clinical trials. Recently, MSA (one of the selenium-like-toxic derivatives) and CA (part of polyphenol compounds and components of propolis) have been considered in the treatment of various types of cancers and tumors. MSA and CA are investigated in different categories, and the results showed that MSA and CA can induce apoptosis in many cell lines. MSA is a potent antioxidant and anti-cancer agent which can be used to treat various types of cancers. The results of MSA effects on human breast cancer cells of MDA-MB231 showed that this compound is more toxic toward these cells compared to the CA, which inhibited this cell growth in a dose-dependent pattern in these cells. Their combination could also induce cell death in these cells. The results of the MTT assay confirmed that MSA inhibited the proliferation of MDA-MB-231 cells in a dose- and time-dependent pattern. The results of cell viability showed that the MSA (5 µg ml^{-1}) caused 50% cell death in these cells at 24 h, While 3.2 µg ml^{-1} of MSA caused the same cell death in these cells at 48 h. Juilana *et al.* reported that the fatal dose of MSA on MCF-7 cells was 2 µg ml^{-1} at 96 h [21], which is less than the dose reported that HUVECs by Zhihui Cai. *et al.* Based on Zhihui Cai *et al*. (2017), 5 µg ml^{-1} of MSA had a fatal effect on these cells at 24 h [22]. However, the treatment time required to induce cell death in MDA-MB231 cells by MSA is lower than that of HUVECs and MCF-7 cancer cells, indicating that the faster cytotoxic effect of MSA. Different responsive may be due to biological and genetic differences of various cell types. Here, DAPI staining was used to determine the type of cell death induced by MSA. The results of MDA-MB-231 morphological examination after treatment with 2.3 µg ml^{-1} of MSA showed that MSA altered the cell morphology which is consistent with the psychological measures of apoptosis as also confirmed by DAPI staining. The appearance of the nucleus with irregular edges and its

shrinkage and chromatin condensation were changed by MSA. Therefore, it shows that MSA induces apoptosis in the MDA-MB-231 cell line. These results were also similar to the findings reported by Zhihui Cai *et al.* Caspases, a family of cysteine proteases, are internal components of the apoptotic pathway whose activation cause the morphological features of apoptosis. In apoptosis, caspase-3 is essential for cell death components in response to various stimuli. Other researchers have reported that the antitumor effects of MSA and the induction of apoptosis on different cell lines. Previous studies have shown that methylmalonic acid induces apoptosis in the HUVECs cell line [22] and MDA-MB-231, BT-549 [23] by increasing caspase-3 activity in prostate cancer cells. Other studies also revealed that MSA induces apoptosis in human skin cancer and prostate cancer cells by increasing caspase-3 activity [24, 25]. Yan Feng and colleagues (2012) investigated that the effect of MSA on MDA-MB-231, MDA-MB-157, and BT-549. They reported that methyl seleninic acid can induce apoptosis in these cells by arresting the cell cycle at G2/M stage and activating and increasing caspase-3 and 7 activity (the main pathway involved in apoptosis) [23]. Investigation of the effect of methylmalonic acid on HUVECs cell line (Human umbilical vein endothelial cells), showed that methyl seleninic acid reduced the number of viable cells and induces morphological changes associated with apoptosis in a dose-dependent and time-dependent manner. Therefore, it can be said that methyl seleninic acid is capable of inducing apoptosis in the MDA-MB-231 cell line. These results are in line with the findings reported by Zhihui Cai *et al.* about the effect of MSA (2 μg ml^{-1}) on the HUVECs cell line. Xia Ojing *et al.* showed that MSA can inhibit the growth of MDA-MB-231 cells by inducing apoptotic cell death as well as the *in-vivo* xenograft model. These effects reduce the amount of VEGF protein and Ang-2 protein expression and secretion which can ultimately inhibit Ang-2 mediated protein. VEGF protein helps tumor growth and its nutrition and oxygenation; thus a decline in VEGF protein can reduce tumor size [26]. However, MSA may induce apoptosis in cancer cells through a caspase-3-independent pathway. In 2014, after investigating the effect of MSA on MCF-7 human breast cancer cells, it was reported that MSA induced apoptosis in this category through epigenetic mechanisms [21]. Caffeic acid is a powerful antioxidant that has been used to treat a variety of cancers. It inhibited cell growth in a dose- and time-dependent pattern. It also induced cell death in caner cells. According to MTT results, 40 μg ml^{-1} dose of this compound can induce 50% cell death at 24 h. While 30 μg ml^{-1} of CA caused this rate of cell death on this cell line at 48 h. Agata *et al*. (2017) A 50 μg ml^{-1} doses of caffeic acid inhibited cell growth in the MDA-MB-231 cell line at 24 h [27]. Morphological examinations of MDA-MB-231 cells treated with caffeic acid showed characteristic morphological changes such as chromatin condensation and nucleus fragmentation. These findings were similar to the results of a study of caffeic acid on MCF-7, T47D cell line [28]. Studies have shown that caffeic acid can induce apoptosis in many cancers. The ability of CA to induce apoptosis on MDA-MB-231 human breast cancer cells was investigated in 2017 by Agata *et al.* The results showed that caffeic acid is a known agent for the treatment of breast cancer and induces apoptosis in breast cancer cells [27], they examined the effect of this compound on MCF-7 breast cancer cell line. The study revealed that at 50 μg ml^{-1} of caffeic acid induced apoptosis [28]. In MCF-7 and MDA-MB-231 cells, caffeic acid-induced apoptosis in associated with decreased cyclin D1 expression and AKT phosphorylation [27]. According to the results of this study at 48 h, 30 μg ml^{-1} of CA along with 3.2 μg ml^{-1} of MSA induced 50% apoptotic cell death in MDA-MB-231 cells. In other words, the lethality of caffeic acid was enhanced in the presence of methylselenic acid. Anne *et al.* study also showed that the effect of caffeine in MCF-7 cells, as a DNA-damaging agent, could be amplified in the presence of CA. This study indicated that caffeine inhibits growth and proliferation and possibly through inhibiting AKT and inducing apoptosis. The effect of CA in cells is inhibit cell growth and proliferation. According to the results, a combination of caffeic acid with methyl seleninic acid has high anti-cancer effects also inducing apoptosis in MDA-MB-231 cells. In conclusion, CA in combination with MSA has a synergistic impact on the inhibition of cell proliferation and promotion of apoptosis in MDA-MB-231 cell lines.

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