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Article

Comparison of Two Components of Propolis: Caffeic Acid (CA) and Caffeic Acid Phenethyl Ester (CAPE) to Induce Apoptosis and Cell Cycle Arrest of Breast Cancer Cells MDA-MB-231

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Abstract: 1) Background: Studies indicate that caffeic acid (CA), caffeic acid phenethyl ester (CAPE) are compounds with potent chemopreventive effects. Breast cancer is a common cancer among women worldwide. The study shows comparison of caffeic acid and its ester activity in the cells of breast cancer line MDA-MB-231; 2) Methods: The cells of MDA-MB-231 were treated by CA and CAPE with doses from 10 to 100 μM in time 24 h and 48 h. Cytotoxicity MTT test, apoptosis by Annexin V and cell cycle with Dead Cell Assay were performed; 3) Results: The cytotoxic activity was greater for CAPE comparing to CA, in both incubation time (same dosage). IC50 values for CAPE were 27.84 (24h) and 15.83 (48h) and >10000 (24h) and >1000 (48h) for CA. Polyphenols induced apoptosis, higher apoptotic effect observed for CAPE (dose dependent). CAPE induced cell cycle arrest in S phase (time and dose dependent). Dose dependent decline G0/G1 phase (48h) and elimination of phase G2/M (100 μM of CAPE). For CA, only after 48 hours, small effect of cell cycle at phase S (however dose dependent), and slight decline of phase G0/G1 and G2/M only for highest doses (50 and 100 μM); 4) Conclusions: Comparing CA and CAPE activity, on the MDA-MB-231, we clearly see better activity of CAPE, with the same dosage and experiment time.

Keywords: caffeic acid; caffeic acid phenethyl ester; CAPE; apotosis; cell cycle; proliferation; breast cancer; propolis.

1. Introduction

Breast cancer is a common cancer and is the leading cause of cancer-related deaths among women worldwide. Breast cancer studies have shown that this is a heterogeneous tumor with

different response to treatment. Radiation therapy is particularly effective in the treatment of breast cancer, but it carries the risk of normal cell damage and radioresistance of tumor cells. The development of radioresistance leads to a cancer recurrence with a more aggressive phenotype in patients [1-4].

Chemopreventive agents act as cell cycle inhibitors. Cellular stress can lead to the cell cycle inhibition as a result of activation of check points. G1/S phase control prevents replication of damaged DNA, while G2/M phase control does not allow segregation of damaged chromosomes into daughter cells during mitosis. Many chemopreventive factors inhibit the growth and proliferation of tumor cells by modulating the expression and/or activation of cell cycle regulatory proteins [5-7].

Apoptosis is a process of programmed cell death that plays a major role in the preservation of tissue homeostasis and the elimination of neoplastic cells. Chemopreventive agents can affect many effector and regulatory elements of the apoptosis process [8-10].

Many studies have shown that chemopreventive agents induce apoptosis in various cancer cell types by affecting multiple proteins involved in programmed cell death [11, 12].

The need for new compounds with effective antitumor action and high cancer cell selectivity and low normal cell toxicity is conducive to testing a wide variety of chemically and structurally related compounds. Recently, the interest in natural compounds has increased significantly and they show cytotoxic, antiproliferative and proapoptotic effects essential for cancer cell growth inhibitory effect.

Recent studies indicate that caffeic acid (CA), its phenethyl ester (CAPE) are compounds with potent chemopreventive effects, inter alia by cell cycle inhibition and proapoptotic action [13-16].

Caffeic acid phenethyl ester (CAPE) is the one of the polyphenol which is a component of honeybee propolis. Known CAPE properties are antiviral, anti-inflammatory, anti-cancer and antioxidant effects [17-19].

CAPE showed its anticancer effects on different cancer cell lines; however, it exhibited differential cytotoxic activity against cancer normal cells around [20-24].

Some researchers notified a significant role of CAPE in apoptosis and cell cycle arrest [25-27].

It has also been shown that CAPE can reduce expression of the mdr-1 gene and thereby increases the sensitivity of cancer cells to chemotherapy. A decrease in VEGF concentrations inhibits angiogenesis and cancer growth [28-30].

Inhibition of the nuclear factor κB (NF-κB) cell signalling pathway by CAPE is also known; it reflects in resistance to radiotherapy [31, 32].

Caffeic acid has been showed as a factor influencing in a variety of potential pharmacological effects in *in vitro* researches and in animal models, and also, it's been reported, that caffeic acid has an inhibitory effect on cancer cell proliferation [33-35].

However, the precise reports concerning cytotoxicity and apoptotic activity of propolis components: caffeic acid and its caffeic acid phenethyl ester in human cancer cells, still remain inconsistent and leave the field for further exploration.

Our *in vitro* study has been conducted in order to investigate and compare the cytotoxic effects of two phenolic substances of propolis: caffeic acid and caffeic acid phenethyl ester on the viability, apoptosis, and cell cycle arrest of breast cancer cell line MDA-MB-231.

2. Results

2.1. MTT

The effect of inhibiting growth of cell line MDA-MB-231 treated with caffeic acid (CA) and caffeic acid phenethyl ester (CAPE) was assayed by MTT cell viability. Accordingly, data were normalized and expressed as % of viability over controls.

For the MDA-MB-231, for CA, cell viability decreased with dose increasing from 97.9% for dose 10 μM, via 95.4% and 91.4% for 25 μM and 50 μM respectively, to reach the value 80.9% with the dose of CA 100 μM, all after 24-hours' time of incubation (Figures 1a and 1d). Comparing CAPE cytotoxic activity to CA for the MDA-MB-231 cell line in the same time (Figures 1a and 1c), cell viability values

for the dose of 10 μM was similar to CA (97,3%), what suggest yet low cytotoxic effect in this time of experiment. However, the value took even 50.3% at 25 μM, 11.9% for 50 μM and 11.6% for 100 μM.

After 48 hours of incubation time (Figures 1b and 1d), for the CA cell viability had dose-dependent effect, and the values were, as follow: 99.0% for the dose 10 μM, 93.6% for 25 μM, 89,2 for 50 μM, and finally 78.0% for 100 μM. However, if we compare CAPE vs. CA viability effect after 48-hours of incubation time (Figures 1b and 1c) the values were statistically different, starting from 71.2% for 10 μM of CAPE dose, via 27.2 per cent for 25 μM, 9.6% for 50 μM and reaching 5.6% for 100 μM, the strongest cytotoxic effect. For CAPE we see high dose-dependent effect.

Comparing CA vs CAPE, the cell viability values were statistically lower for CAPE (it means CAPE has higher cytotoxic effect than CA). Our results showed dependent trend in dose domain, for both substances, and time dependent for CAPE with one remark, that CAPE reached lower viability for higher doses earlier, it means CAPE cytotoxic activity occurs earlier, respectively.

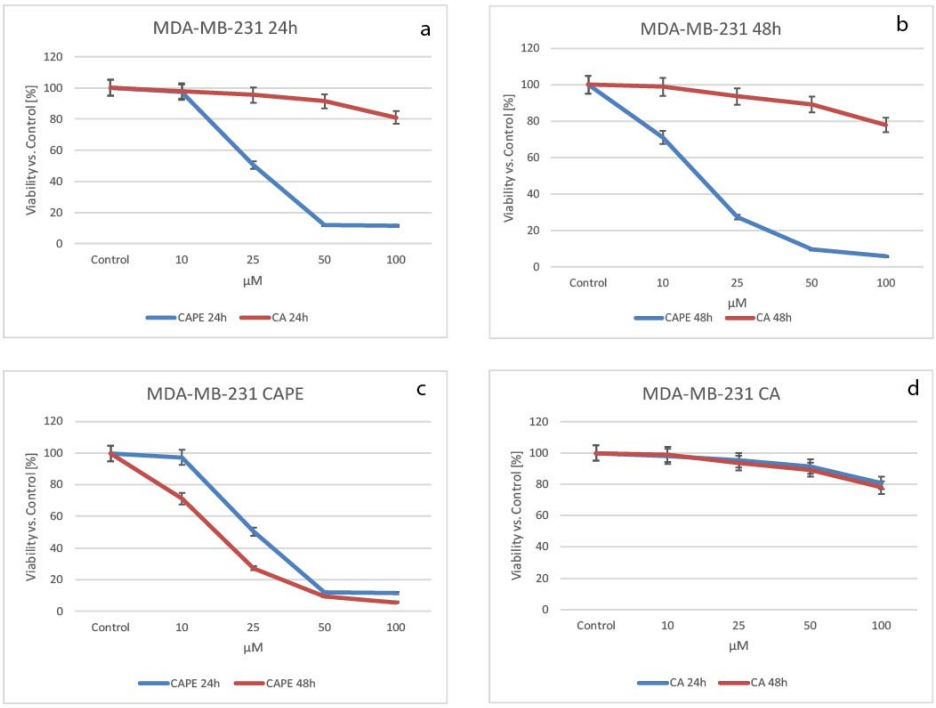

Figure 1. Cytotoxic effects of caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) at concentrations of from 10 to 100 μM by 24- and 48-hours' incubation time of the breast cancer cell line MDA-MB- 231 with the tested compounds. Visible dose-dependent effect for both substances. Stronger activity of CAPE vs. CA starting from dose of 25 μM of tested compound (Figure 1a) for 24-hour incubation time. For 48-hours' time of experiment (Figure 1b), all doses of CAPE resulted much stronger cytotoxic effect than CA with corresponding doses. In Figure 1c, for dose 10 μM of CAPE, 48h-experiment gave visible cytotoxic effect comparing to 24 hours, and conspicuous stronger effect for 25 μM; however, succeeding dose increase of CAPE (50 and 100 μM) didn't give significant difference in viability factor but both reached very low level of viability. CA cytotoxic activity has no significant difference in the time manner and generally, has low cytotoxic activity (Figure 1d) for the MDA-MB-231 cell line. The cell viability was analyzed by MTT assay. The results were presented as mean and standard deviation of three independent experiments, 12 wells each ($p < 0.05$; Friedman ANOVA test).

During the experiment, the half maximal inhibitory concentration (IC50) was calculated, for both substances on the MDA-MB-231 breast cancer line. The results are showed in Table 1.

50%-mortality of breast cancer cells of MDA-MB-231 were obtained at a dose of CAPE 27.84 μM for 24-hours' time of incubation, and for 48-hours - 15.84 μM. For CA, the values reached more than 10000 μM for 24 hours and more than 1000 μM for 48-hours' experiment time. These results showed that CA has lower cytotoxic activity than CAPE on MDA-MB-231 cells during 24-hours' and 48-hours' experiment.

Table 1. IC50 values of caffeic acid (CA) and caffeic acid phenethyl ester (CAPE) in relation to breast cancer MDA-MB-231 for 24 h and 48 h. The obtained data allow to conclude that CAPE has far bigger activity than CA on the line MDA-MB-231, during 24h and 48h.

IC50 (MDA-MB-231) [μM]		
	Time of Incubation	**Time of Incubation**
Compounds	**24h**	**48h**
Caffeic acid	>10000	>1000
Caffeic acid phenethyl ester	27.84	15.83

2.2. MUSE® ANNEXIN V AND DEAD CELL ASSAY

The cells of the breast cancer line: MDA-MB-231 after exposure to CA and CAPE was stained with Annexin V bound to FITC and analyzed by flow cytometry to assess the apoptotic cells' percentage.

In order to investigate the apoptotic effect of CA and CAPE, MDA-MB-231 cells were treated with both substances in the time of 24 h and 48 h, and apoptotic cells were assessed by staining with Annexin V bound to FITC and analyzed by flow cytometry to assess the apoptotic cells' percentage. To determine whether CA/CAPE treatment results in apoptosis in MDA-MB-231 cells, we used a Muse Annexin V and Dead Cell kit to measure the changes in cell apoptosis after experimental times: 24 and 48 hours.

We observed that both investigated substances induced cell death through apoptosis in MDA-MB-231 cells (Figure 2).

For CAPE, after 24 hours of experiment (Figure 2a), the significant decrease in the number of live cells (with 93.96% for control), starting from 84.33% for 10 μM; via 73.79% for 25 μM and reaching for 50 and 100 μM respectively 52.90 and 48.52 per cent. Early apoptosis (at a control's value 0.65%) reached the value of 7.46 per cent already for 10 μM, and not changed significantly with the dose of CAPE up to 50 μM, but reached 14.00 for 100 μM. Late apoptosis (control's value 3.38%) fluctuated up to 30.11% for highest dose. Taking into the consideration all apoptotic cell phenotype, we observed that apoptotic number of cells started at 13.33% for 10 μM, having increased value of 24.91 for 25 μM, and reached significant growth of 32.43% for 50 μM and 44.11 per cent for 100 μM. Similar situation with live cells number decreasing we observed in longer, 48 hours' time of incubation (Figure 2b). However, with control value of 92.24 per cent, after 10 μM-dose treatment of CAPE we reached decreasing number of live cells number up to 62.23%. Then, respectively, obtained results were as follows: 49.04 for the dose 25 μM, 43.18 for 50 μM, and for the highest concentration – 100 μM – 24.85 per cent. It resulted also in faster gradient of apoptotic cell number increasing. Early apoptotic cell number was quite stable with the dose increasing (control: 2.72, but after dosage the values fluctuated between 9.26 and 12.51), but the late apoptosis was significantly changed. With the control 3.32 per cent, after dosage of 10 μM we obtained the value 24.15, for 25 μM - 32.85, having similar value of 37.29 for 50 μM, and reached finally 53,35 per cent for 100 μM of CAPE, after 48 hours. Taking into the consideration all apoptotic cells phenotypes we observed significant apoptotic cells number growth (control total: 6.04): already after CAPE treatment with the dose of 10 μM, we obtained the value 33.41 per cent, and this value reached up to 63.76 with the dose of 100 μM of CAPE, for 48h.

For caffeic acid, after 24 hours of experiment (Figure 2c), also significant decrease in the number of live cells (control value: 93.03 per cent) obtained in the dose depended manner. Starting from 86.15% for 10 μM of CA, via 71.65% and 64.35% for 25 and 50 μM respectively, ending with 57.17 per cent for the dose 100 μM. Apoptotic effect of CA was not so significant as for CAPE, however we observed increase of early apoptotic cells number with the treatment of this agent, starting with the dose of 10 μM and reaching the value of 3.87, then 9.84 per cent for 25 μM. Only for 50 μM we observed decreasing of early apoptosis (back 3.98), but we clearly saw in the same time unnormal growth of dead cells number (14.31%, when for the rest of doses fluctuated from 3.56 up to 7.27). For 100 μM, early apoptosis had the highest value of 12.47. Late apoptosis phenotype existed during this experiment and was dependent in the dose domain, starting from 6.39 per cent for 10 μM and

reaching max. value for 100 μM – 23.01 (control: 2.77). Total apoptotic cells number increased also with dose dependent manner, starting from 10.26 per cent for 10 μM and reaching its extremum on 100 μM with 35.47%, for 24-hours' time of experiment. During the 48 hours of incubation (Figure 2d), the apoptotic activity of CA was slightly similar in total, the apoptotic cells number reached 36.35 per cent for 100 μM, however starting from 20.25% for 10 μM. The distribution between early and late apoptosis was different then in 24-hours' time, fluctuated for early phenotype only from 5.91 up to 7.35% (with the control: 2.38). The majority of apoptotic cells was given by late apoptosis phenotype, starting from 14.34% for 10 μM and reaching 29.84% for CA dosage of 100 μM having dose dependent effect.

Representative plots of apoptosis are shown on the Figure 3.

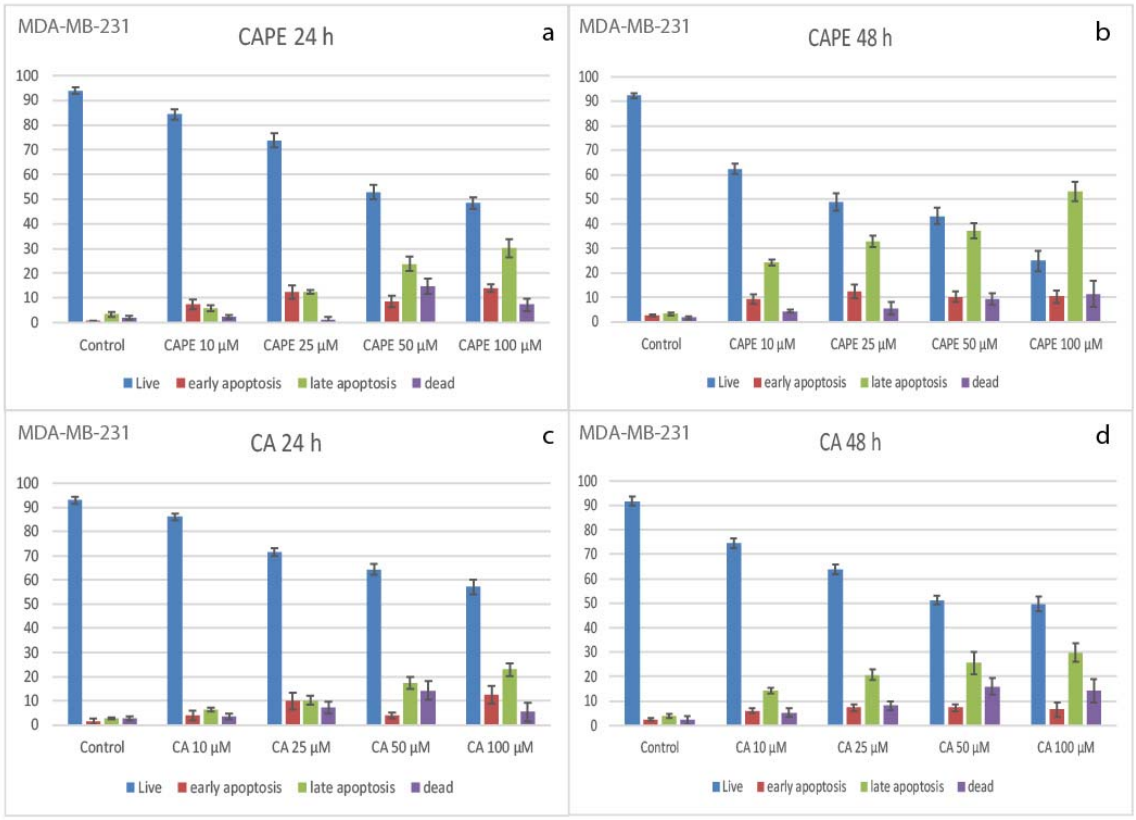

Figure 2. Apoptotic effect of using CA and CAPE with concentrations 10, 25, 50 and 100 μM after 24h and 48h incubation on MDA-MB-231 cell lines. For CAPE, after 24 hours of experiment (Figure 2a), the significant decrease in the number of live cells observed. Early apoptosis stable after the treatment of CAPE (24h) and not changed significantly with the dose. Late apoptosis reached up to 30.11% for highest dose. Visible dose dependent effect, also taking into consideration total cells number with apoptotic phenotypes. Stronger apoptotic effect in the case of 48 hours of incubation (Figure 2b). Similar situation with live cells number decreasing we observed. Early apoptosis number quite stable, but late apoptosis increasing with the dosage, reaching 53.35%. In total, for CAPE, 48 h, highly dose depended effect with apoptosis. For CA, 24h of experiment (Figure 2c), negative gradient of live cells number obtained, decreasing with the dosage of CA. Apoptotic effect of CA was not so significant as for CAPE, however increase of early apoptotic cells number observed. Dose dependent effect for late apoptosis. In total, for CA, 24h, with dose dependent manner, apoptotic phenotype reached the number of 35.47% for the dose 100 μM. 48-hour time of experiment didn't change significantly the results with live apoptosis, increased dead cells number and increase of occurring late apoptotic phenotype cells number reaching with dose dependent effect up to 29.84% for 100 μM. Vertical bars represent the standard deviation of means (SD) (n=3 experiments), $p < 0.05$ value.

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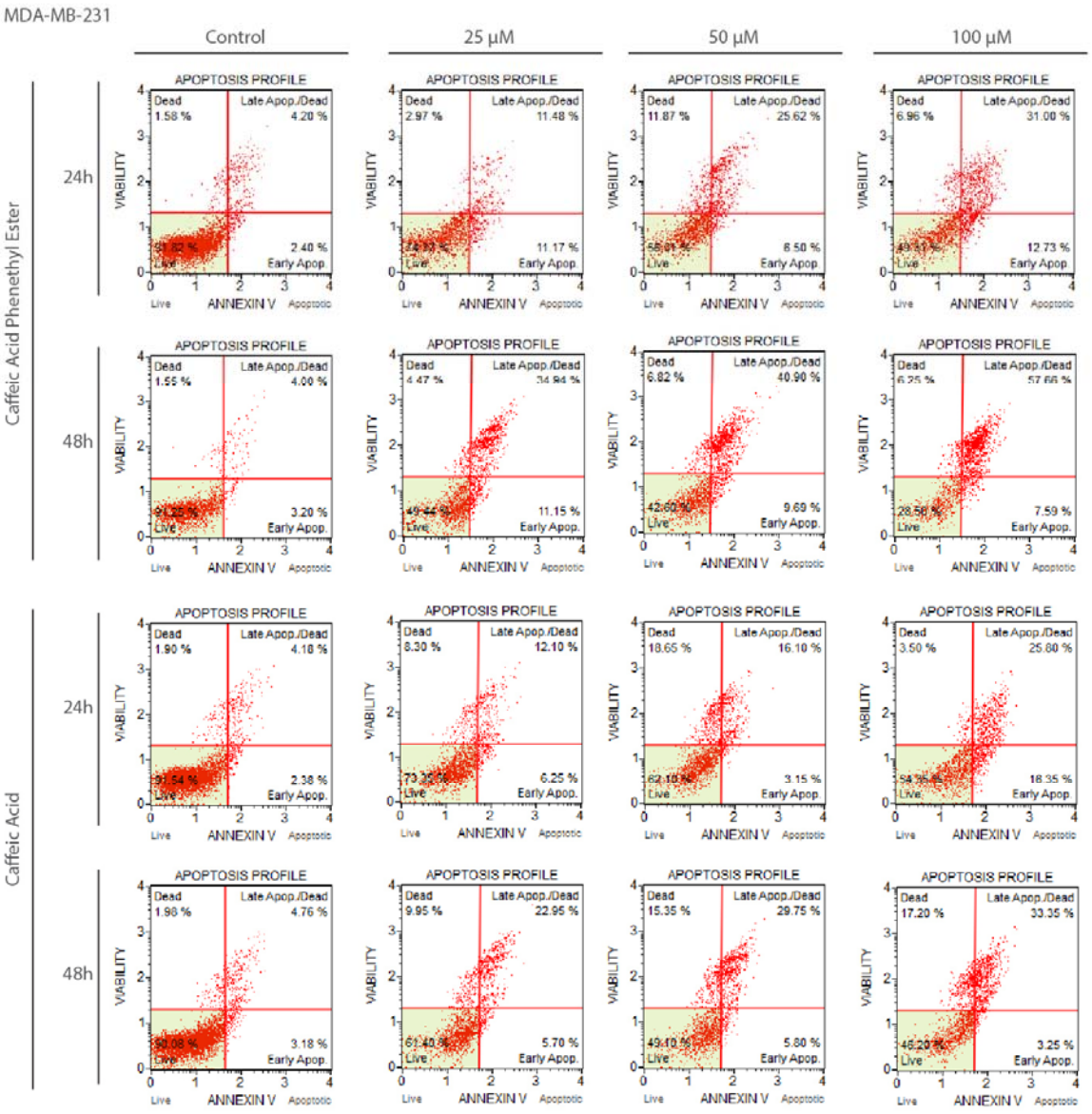

Figure 3. Apoptotic effect of CAPE and CA substances on MDA-MB-231 cell in the 24- and 48-hours' time of experiment (representative plots). CAPE and CA induce apoptosis in the breast cancer cells of examined line. Early apoptotic cells are shown in the lower right quadrant and late apoptotic phenotype cells in the right upper quadrant of the plot. Dose-dependent effect visible. Measured by Muse Annexin V and Dead Cell assay.

2.3. MUSE® CELL CYCLE ASSAY.

In different series, MDA-MB-231 cells were treated with doses: 10, 25, 50 and 100 µM of CAPE and CA for 24 and 48 hours and cell cycle progression was determined using flow cytometry. All results are shown in Figure 4.

For 24 hours' incubation time, CAPE induced cell cycle arrest in MDA-MB-231 cells at the S phase, changing from 15.6% at control (0 µM of CAPE) to 60.2 % at 100 µM dosage. CAPE with a concurrent declined with dosage increasing in the G0/G1 phase from 60.7% at control (0 µM of CAPE) to 31.1% at 100 µM of CAPE. Parallelly, the G2/M phase decreased from 23.7 at control ((0 µM of CAPE) to 8.6% at 100 µM of CAPE (Figure 4a). At the same time, CA activity in cell arrest was not so spectacular. G0/G1 phase started (at control: 0 µM of CA) from 61.0% and didn't change its value with increasing the CA dose, ending with 59.4% at 100 µM of CA. The S phase changed from 16.8%

(at control: 0 μM of CA) up to 22.6% for 100 μM of CA dosage. Parallelly, G2/M phase decreased from 22.0% at control (at control: 0 μM of CA) down to 17.7% for 100 μM of CA (Figure 4c).

For the 48-hours' incubation time, CAPE induced cell arrest in MDA-MB-231 cells at the S phase, strongly changing the values from 19.5% at control (0 μM of CAPE) to 89.7 % at 100 μM dosage. The G2/M phase started from 9.9% (at control: 0 μM of CAPE) to be practically completely eliminated (0.2%) for the 100 μM of CAPE (Figure 4b). Respectively, for CA, the decline of phase G0/G1 was observed: starting from 69.6% (at control: 0 μM of CA) to 59.0% for 100 μM of CA. CA induced cell arrest in MDA-MB-231 cells at the S phase, however the cell arrest activity of CA with results, starting from 17.2% (at control: 0 μM of CAPE) up to 32.6% for 100 μM of CA dosage weren't so spectacular as using CAPE.

Simultaneously, the G2/M phase decreased from 13.1% (at control: 0 μM of CAPE) down to 8.4% for 100 μM of CA in the dose-dependent manner (Figure 4d).

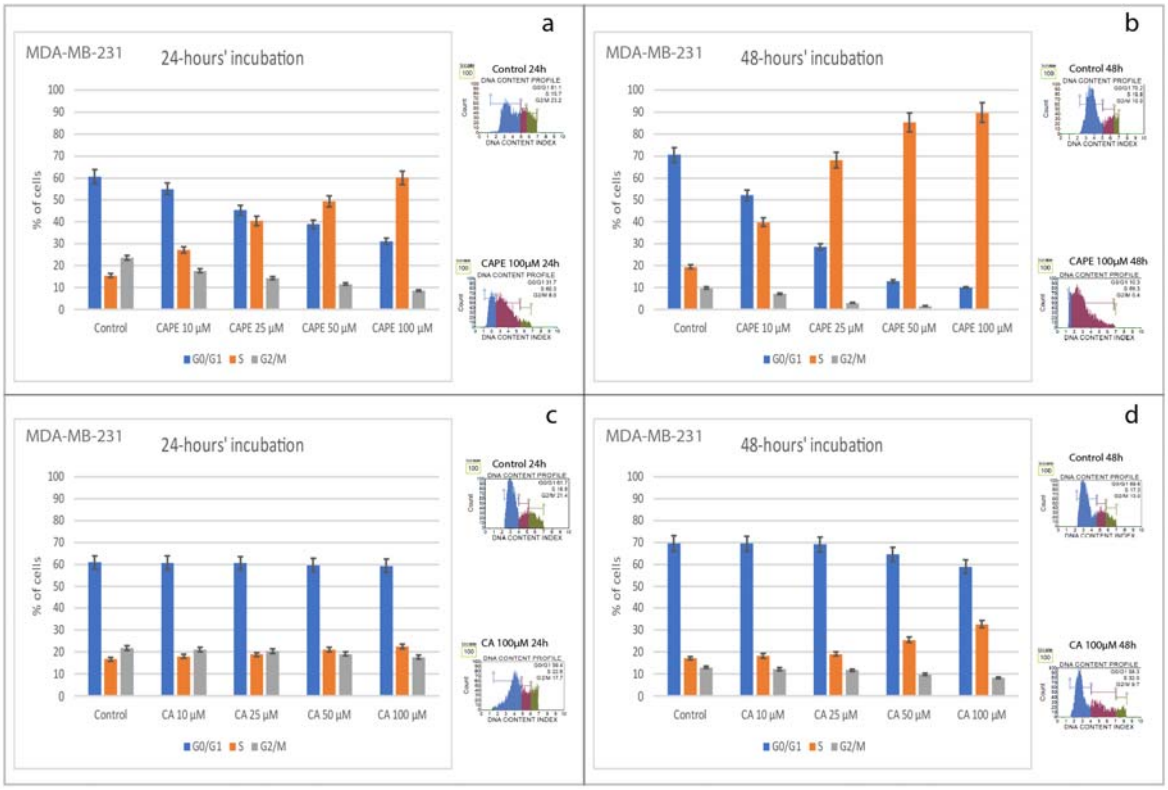

Figure 4. Cell cycle arrest of using CA and CAPE with concentrations 10, 25, 50 and 100 μM after 24h and 48h incubation on MDA-MB-231 cell line. The representative cytometric plots shown repectively. CAPE induces cell cycle arrest in S phase, in dose and time dependent manner (Figure 4a and 4b). Decline of G0/G1 phase and G2/M (Figure 4a), strong dose-dependent decline G0/G1 phase for 48h and complete elimination of phase G2/M for highest dose of CAPE (both Figure 4b). For CA, in the incubation time of 24 hours no significant effect in cell cycle arrest within MDA-MB-231 cells observed (Figure 4c). After 48 hours of experiment slight effect of cell cycle at phase S in dose-dependent manner, and slight but significant decline of phase G0/G1 and G2/M only for highest dosage (50 and 100 μM) (Figure 4d). Comparison of these two poliphenols evidently shows bigger influence on cell cycle arrest induction in MDA-MB-231 of CAPE vs. CA. Lower doses, also time testify in favor of CAPE anticancer activity on breast cancer cells. Cells were stained with Muse Annexin V and Dead Cell kit and were subjected to flow cytometric analysis collecting 10,000 events. Vertical bars represent the standard deviation of means (SD) (n=3 experiments), $p < 0.05$ value.

3. Discussion

Despite the noticeable progress in the treatment of cancer and the introduction of new chemotherapeutics into the clinic, modern medicine is still struggling with the problem of fully effective chemotherapy. There is therefore a strong need to make better use of existing knowledge in

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the development and synthesis of new potential chemotherapeutic agents that are characterized by their efficacy, selectivity and specificity for cancer cells, and therefore interest in natural compounds has increased significantly [36, 37].

Many years of research have allowed us to identify the important factors in the breast cancer diagnosis, its development, and in the choice of dedicated and most effective treatment. There are biological factors, include the presence or absence of hormonal receptors in cancer cells and overexpression of the HER2 receptor or even its absence. There are cancers that do not show hormonal receptors (ER, PGR) and overexpression of the HER2 receptor; these belong to the specific type of breast cancer, termed triple negative. TNBC can develop much faster than other cancers. It is therefore important to diagnose this disease and to introduce dedicated and specific patient-specific treatment because estrogen, progesterone and HER2 therapy are not so effective in that case. [38-41].

Exceptionally, in the breast cancer, estrogen receptor signalling plays important role in cell proliferation and vitality. It has been observed, that MDA-MB-231 is the ideal cell line for triple-negative breast cancer since minimal expression of estrogen receptor β and lack of estrogen receptors α [42].

In our research, we compared cellular response of breast cancer line MDA-MB-231 to two constituents which normally occur in propolis: caffeic acid (CA) and its derivative – caffeic acid phenethyl ester (CAPE), in line with our best knowledge, for the first time.

Natural agents are quite popular in complementary medicine at the moment, and appear to be well suited as potential novel substances for the treatment support of certain forms of cancer, even performed with clinical trials [39].

Our results obtained from flow cytometric assay clearly showed that CA and notably CAPE induced apoptosis and growth inhibition in time- and dose-dependent manner in the breast cancer MDA-MB-231 line. Clear changes of cell cycle namely, if compare these two phenolic compounds of propolis, in CAPE treatment were observed. Cell cycle arrest in the S phase, with reducing (and even removing) of G2/M phase for CAPE, and with at the same time, weak CA influence on MDA-MB-231 cell cycle (for 24h insignificant, and for 48h slight effect) leads us to conclusion, that CAPE comparing to CA, induces stronger and faster cell cycle arrest. For our best knowledge, this study presented comparison of CA and CAPE is one of the first, which compares inducing of cell cycle arrest and apoptotic effect, in the cells of MDA-MB-231 line.

The study also identified dead cells tests, and CA/CAPE treatment resulted in diminishment of life of MDA-MB-231 cells. Novel action of our research was to compare the cytotoxic effects of CA and CAPE in MDA-MB-231 cell line, with the conclusion that CA and CAPE inhibited the proliferation and reduced the viability of NDA-MB-231 cells. However, CAPE cytotoxic activity was stronger, in line of doses, and faster, in line of time than CA, respectively.

These results confirm, the phenolic compounds could be found as supportive chemotherapeutic agent for certain conditions of breast cancer [40].

Natural compounds, especially phenolic once, also have been demonstrated to soften the chemotherapeutic effect in tumour cells and subsequent treatment of caffeic acid and paclitaxel induce strong synergistic effects, antiproliferative and apoptosis of lung cancer cells, including the NF-κB pathway [41].

The results obtained showed that treatment of breast cancer cells with these two phenolic acids effectively induced apoptosis with condition indicated above, with stronger apoptotic effect. Chemotherapeutic agents, including propolis constituents, are expected and already confirmed to inhibit the growth of some cancer types.

Chen *et al.* showed in the research that CAPE acts as a radiation sensitizer in some types of cancer. Because CAPE's activity destination is radioresistance signalling pathway, it improves the efficiency of the radiation response [43].

Study of Omene *et al.* showed, that CAPE, in a dose-dependent manner inhibits MCF-7 (hormone receptor positive, HR+) and MDA-MB-231 cells growth, both breast cancer lines, *in vitro* and even *in vivo* without much effect on normal mammary cells. Additionally, CAPE strongly influenced gene and protein expression. It induced cell cycle arrest, apoptosis and reduced growth

expression and transcription factors like e.g. NF-κB. What was significant, CAPE downregulated mdr-1 gene, which were shown as responsible for the resistance of cancer cells to chemotherapeutic agents. Furthermore, CAPE in dose dependent manner suppressed VEGF formation in MDA-231 cells and formation of capillary-like tubes by endothelial cells, what implicated inhibitory effect on angiogenesis. Their results strongly suggested that CAPE inhibits MDA-231 and MCF-7 human breast cancer growth via its apoptotic effects, and modulation of NF-κB, the cell cycle, and angiogenesis [44].

Khoram *et al*., in their research noted, that CAPE decreased the viability of cell lines: MDA-MB-231 and T47D in a dose- and time-dependent manner. In the clonogenic assay, pretreatment of cells with CAPE before irradiation significantly reduced the surviving fraction of MDA-MB-231 cells at doses of 6 and 8 Gy. A reduction in the surviving fraction of T47D cells they observed in at lower doses of radiation, comparing to MDA-MB-231. Additionally, CAPE maintained radiation-induced DNA damage in T47D cells for a longer period than in MDA-MB-231 cells. They suggested, that induction of radiosensitivity by CAPE in radioresistant breast cancer cells might be caused by prolonged DNA damage [45].

Interesting results were presented by Onori *et al*. They found, caffeic acid phenethyl ester (CAPE) inhibited the growth of cancer cells and acts as known inhibitor of NF-κB, which is constitutively active in cholangiocarcinoma(CCH) cells. They evaluated the effects of CAPE on CCH growth both *in vitro* and *in vivo*. Inhibition of NF-κB DNA-binding activity was confirmed in nuclear extracts treated with CAPE (at doses 50, 40 and 20 μM). CAPE decreased the expression of NF-κB1 (p50) and RelA (p65) and decreased the growth of a number of CCH cells but without any activity on normal cholangiocytes. Cell cycle decrease was seen by a decrease in PCNA protein expression and the number of BrdU-positive cells treated with CAPE at 20 μM. Inhibition of growth and increased cell cycle arrest of Mz-ChA-1 cells by CAPE were conjugated with apoptotic effect. Bax expression was increased, Bcl-2 was decreased in the same time, in cells treated with CAPE. In the *in vivo* cancer growth was decreased and tumor latency was increased 2-fold in CAPE compared to vehicle-treated nude mice. In tumor samples taken, decreased CCH growth was coupled with apoptotic effect. CAPE, both *in vivo* and *in vitro* decreased the growth of CCH cells by increasing apoptosis [46].

Sanderson *et al*., showed in their interesting research, the activity of caffeic acid (CA) and its different derivates, including CAPE, on human androgen-dependent prostate cancer cells LNCaP. They compared them with results, that certain caffeic acid derivatives and, in particular, CAPE had potent cytotoxic effects in LNCaP cells. They compared 19 synthetic derivatives of caffeic acid and CA itself, but only three of them decreased the cell viability of LNCaP cells concentration-dependently after a 24 h exposure, including CAPE. CA-related results weren't definitely so optimistic than obtained with CAPE, what is in line with our research. Comparing CA and CAPE IC50 value, IC50 for CAPE was extremely lower than for CA [47].

The research of Rosendahl *et al.* showed that in breast cancer cell lines MCF-7, T47D (both ERα+) and MDA-MB-231 (ERα-), caffeine or caffeic acid reduces human breast cancer cell growth *in vitro*. Cell cycle arrest in similar doses of CA (10 and 50 μM) on MDA-MB-231 was in line with our experiment, not showing big differences. They compared CA with caffeine which this last one induced cell cycle arrest and decreased G2/M phase and increased G0/G1, but having in mind completely different dosage than CA: 1 and 5 mmol/L, respectively. With the doses 10 and 50 μM of CA, they didn't observe any changes with cell cycle on MDA-MB-231 cell line. Our research also confirmed only slight effect of caffeic acid on MDA-MB-231 cells [48].

The caffeic acid was also discovered in HCT 15 Colon Cancer Cells by Jaganathan research. Antiproliferative effect of caffeic acid against colon cancer HCT 15 cells growth *in vitro* was showed in the dose-dependent manner. However, he used in the experiment different doses: 100, 200, 300, 500, 600, 800, 1000, 2500 μM. In the matter of fact, that those big doses could lead him to conclusion, that caffeic acid could be promoted as a likely candidate in the chemoprevention of colon cancer. Also, the IC50 calculations showed that fact. Further mitochondrial membrane potential fall was also observed in the treated cells in his research. Dose- and time-dependent staining by Yo-pro-1 demonstrated increasing accumulation of apoptotic cells after caffeic acid treatment. Caffeic acid can

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be considered as a potential candidate for inducing apoptosis in colon cancer cells through ROS and mitochondrial mediated mechanism [49].

Caffeic acid has been also under our consideration in the previous research. In the study of Dziedzic *et al.*, caffeic acid reduced the viability and migration rate of oral carcinoma cells (SCC-25) together exposed to low concentration of ethanol. A significant variation of MTT absorbance values compared to ethanol alone and dose-dependent effect of combination of EtOH with caffeic acid (CA) on SCC-25 cells proliferation absorbance measurement were showed. The results demonstrated in this study, that caffeic acid had a cytotoxic effect on the tested oral carcinoma cell line [50].

Additionally, in our previous research (Dziedzic *et al.*) we tested induction of cell cycle arrest and apoptotic response of head and neck squamous carcinoma cells (Detroit 562). In particular, the results indicated that CAPE had a greater apoptotic effect in Detroit 562 cells than did caffeic acid. Also, that findings suggest that lower doses of CA and CAPE (up to 25 μM) acting for 24 hours may not affect Detroit 562 cancer cells' viability and cell cycle. The results show that CAPE at concentration of 100 μM has a mild effect on cell cycle arrest by Detroit 562. However, cell number in the S phases and G2/M phase was decreased to 31% and 18%, respectively, when exposed to 100 μM of CAPE for 48h of experiment time. We see still better potential in CAPE than CA, in the respect of tested cell lines and the same dosage [13].

4. Materials and Methods

4.1. Cell lines and Reagents

4.1.1. Breast Cancer Cell Line MDA-MB-231

In our research, we used one breast cancer line: MDA-MB-231 (human breast adenocarcinoma, TNBC, no. 92020424 SIGMA from Sigma-Aldrich), which is a model of human triple-negative breast cancer. We fully followed manufacturer's recommendations and cultured them according to these recommendations. The breast cancer line type MDA-MB-231 was cultured with Leibovitz's L-15 medium with 10% of inactivated fetal bovine serum (FBS, Sigma-Aldrich) at 37 °C without CO2.

The cultured cells were supplemented with antibiotics with the following final concentrations: penicillin 100 U·mL−1, streptomycin 100 µg·mL−1 and a fungistatic amphotericin B with a concentration of 0.25 µg·mL−1. We changed the medium every 48-72 hours, and the passage was carried out with a confluence of 80%–90%.

4.1.2. CA and CAPE

Caffeic acid (CA, Sigma: C0625) and caffeic acid phenethyl ester (CAPE, Sigma: C8221) were purchased from Sigma-Aldrich and they were stored and collected, and used according to the manufacture 's instruction.

4.2 MTT TEST

Cytotoxicity of tested compounds (CA and CAPE) for the MDA-MB-231 line was measured by MTT (3- (4,5-dimethylthiazol-2-yl) -2-5 diphenyl tetrazolium bromide). Test of viability shows the cells' ability to reduce of MTT. After 24-hour and 48-hour incubation of the cells with the test compounds, medium was decanted and each well of MTT reagent was added at a final concentration of 1 mg / mL, and then incubated for 4 hours at 37 °C in a 5% CO2. After this time the supernatant was removed, and the water-insoluble formazan crystals were dissolved in 150 mL of DMSO. We used an ELISA plate BioTek's reader at a wavelength of 570 nm in order to absorbance reading. The same procedure we used when tested 48 hours' time of incubation. As a control group we used nutrient medium.

4.3. Muse® Annexin V and Dead Cell Assay

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When taking into consideration the cell population, the degree of apoptosis is one of most important parameter which can show the state of cells health and the morphological changes.

Changes in the cell membrane structure are one of the significant factors of apoptosis. That leads to abnormal asymmetry in the membrane phospholipids distribution. In the normal cell, there prevail inert phospholipids on the surface of membrane. We can mention them: sphingomyelin and phosphatidylcholine. The inner layer contains the predominant anionic phospholipids, such as it is phosphatidylserine. In the apoptotic cells, phosphatidylserine is occurred in the outer layer of the cell membrane. This phenomenon is used in order to mark apoptotic cells using Annexin V, which has the binding ability itself to the negatively charged phospholipids, such as a phosphatidylserine. In our study, we used the Muse cell analyzer in order to research apoptosis using Annexin V & Dead Cell assay. The assay simply detects a phosphatidylserine on the surface of apoptotic cells.

For the analysis of flow cytometric, MDA-MB-231 cells in the amount of 5 × 105 cells / well were plated in 6 well plates and allowed to stand for 24 and 48 hours to obtain a logarithmic growth. The cells were incubated in a complete culture medium containing 10, 25, 50 and 100 μM of tested compounds for 24 and 48 hours. For the apoptotic assay, 1 × 106 of cells in suspension were transferred to the new tube and incubated with 100 μL of Annexin V & Dead Cell reagent (Millipore) for 20 minutes at room temperature. The apoptosis was determined by Muse Cell Analyzer (Emission max.: Yellow- Red- 576 nm and 680 nm, Excitation max.: 532 nm).

4.4. Statistical analysis

All results are expressed as means ±SD obtained from three separate experiments performed in quadruplicates (n=12). The results were performed with independent sample t-tests. The experimental means were compared to the means of untreated cells harvested in a parallel manner. Differences between 24 h-incubated samples and also for 48 h-incubated samples were tested for significance using the one- and multiple-way Friedman ANOVA test. A p-value less than 0.05 were considered statistically significant.

5. Conclusions

With all limitations of in vitro study, we are ready to set the thesis, that summarize that the current evidence of human breast cancer adjuvant therapy and/or chemoprevention with the use of caffeic acid or CAPE is positive but still inconclusive. However, lot of promising results have been obtained for selected biologically active substances isolated from bee propolis, especially polyphenols and propolis itself, but to define the hard conclusions is too early, mainly because of incoherent results. Further advanced studies are required, in particular clinical trials, to confirm the clinical effectiveness of polyphenols on breast cancer treatment and prevention. Comparing CA and CAPE activity, on the MDA-MB-231 we clearly see better activity of CAPE, with the same dosage and experiment time.

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Author Contributions: Agata Kabała-Dzik conceived the study idea, designed and performed the experiments, analyzed the data, and wrote the manuscript. Anna Rzepecka-Stojko carried out the experiments protocol, Robert Kubina performed the experiments and organized the data. Żaneta Jastrzębska-Stojko analyzed the data and made discussion research. Rafał Stojko consulted current treatment of breast cancer and analyzed the results, Robert D. Wojtyczka organized the data and revised the manuscript. Jerzy Stojko contributed reagents and tools and critically revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsor had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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