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ORIGINAL ARTICLE

A Comparative Study of MTT and WST-1 Assays in Cytotoxicity Analysis

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Abstract

Introduction: Cytotoxicity assays are frequently used in cell culture and drug development studies. Some of these cytotoxicity assays may give incorrect results due to interactions of chemicals used in the assays. This study aimed to compare the results between the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (WST-1) assays, using Black Sea propolis extract (BSPE) and caffeic acid phenethyl ester (CAPE) on HCT-116 and DLD-1 colorectal cancer cell lines.

Methods: HCT-116 and DLD-1 cells were treated with different doses of CAPE and BSPE. The cell viability was analyzed by MTT and WST-1 cytotoxicity assays comparatively. Afterward, cell death was examined morphologically by acridine orange/ethidium bromide staining (AO/EB) and quantitatively by Annexin V/7AAD apoptosis detection method.

Results: MTT and WST-1 assays showed different viability results on two cell lines with the same doses of BSPE. However, there was no significant difference between the results of two assays on CAPE treatment with the same doses. AO/EB staining confirmed cell death following BSPE and CAPE treatment. All results from Annexin V/7AAD assay were consistent with the results of the WST-1 assay, particularly for BSPE treatment.

Discussion and Conclusion: In the determination of the cytotoxic effects of BSPE, WST-1 assay reflected more precise results than MTT assay. However, two assays showed similar results when the cytotoxic effects of CAPE were determined. Consequently, WST-1 assay is found to be more reliable than MTT assay in the cytotoxicity analysis of a natural product such as BSPE.

Keywords: Anticarcinogenic agents; apoptosis; propolis; toxicity tests.

Several assays have been developed for cell cytotoxicity studies over the years. These assays mostly rely on the measurement of either cell survival or cell proliferation. In a general aspect, metabolic signals might be altered by changes in growth conditions or addition of cytotoxic drugs to the medium. These alterations provide useful information about the efficacy of the drug used in the study[1].

In general, cell viability and proliferation assays are preferred to determine the percentage of viable cells in culture. Among these assays, tetrazolium salts such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) have been widely used in many studies[2-6]. Principally, yellow tetrazolium salts are reduced to form insoluble purple formazan crystals in viable cells by mitochondrial dehydrogenases. The absorbance of dissolved formazan crystals is measured by a spectrophotometer[7-9]. Several studies emphasize that MTT assay may give false-positive results[10-14]. According to studies, MTT is being reduced to form formazan crystals

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in the absence of living cells. This is due to the reduction of MTT by compounds that have antioxidant activities. In studies where flavonoid compounds were used, it has been observed that MTT reduction occurs while cell growth is inhibited[15-17]. Thus, there is a possibility of MTT to interact with some chemical compounds of natural products and gives false results in drug development studies in which cytotoxicity is extremely important.

WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay is a widely used colorimetric test for the determination of cellular viability. In principle, WST-1 reacts with mitochondrial succinate tetrazolium reductase to form water-soluble formazan dye and generally works similarly with MTT[18]. Differently, WST-1 contains two sulfonate groups which allow it to be kept out of the cell due to its net negative charge. Positively charged tetrazolium salts such as MTT have ability to enter the living cells. Therefore, they are reduced intracellularly by oxidoreductase enzymes. Conversely, negatively charged tetrazolium salts such as WST-1 are unable to enter the cell and are reduced extracellularly. The extracellular reduction takes place by transporting electrons along the plasma membrane with the help of an intermediate electron carrier[3].

Propolis is a bee product obtained from resin in the branches and trunk parts of plants. Approximately 300 different compounds have been defined in the propolis structure. Propolis exhibits a wide range of biological activity since it contains many chemical compounds. Propolis and its components have been used frequently in cancer research due to their ability to induce apoptotic pathways. The mechanism of action is thought to depend on the type and the concentration of propolis extract[19].

Phenolic compounds are one of the most pharmacologically active compounds of propolis[20]. One of these phenolic compounds is caffeic acid phenethyl ester (CAPE), which possesses antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, and anticancer properties[21-27]. Whereas CAPE treatment kills cancer cells, healthy cells mainly remain unaffected[28,29]. This suggests that CAPE affects cancer cells selectively. These findings paved the way for CAPE to become a candidate therapeutic compound in cancer research.

In this study, we aimed to investigate the reliability of the MTT assay in treatment of Black Sea propolis extract (BSPE) and CAPE. In this context, we determined the cytotoxic effects of BSPE and CAPE on DLD-1 and HCT-116 colorectal cancer cell lines by MTT and WST-1 assays. We further analyzed the effects of BSPE and CAPE on cell morphology by AO/EB staining. We also determined the cellular apoptosis by Annexin V/7AAD assay.

Materials and Methods

Extraction of Black Sea Propolis Sample

Raw propolis sample was obtained from beekeepers in Pazar, Rize, Turkey. The sample was frozen in a deep freezer. Then, it was grinding with a coffee hand mill. Five grams of powder raw sample were dissolved in 100 mL 70% ethanol in a glass flask (500 mL), stirred on a shaker (Heidolph Promax 2020, Schwabach, Germany) at room temperature for 48 h. Afterward, the particles were removed by filtration and the extract was evaporated with a rotary evaporator at 40°C. Finally, the water was lyophilized, and the dry extract was stored at -20°C.

Cell Culture and Chemicals

Human colorectal carcinoma cell lines HCT-116 (from ATCC, no.CCL-247) and DLD-1 (from ATCC, no.CCL-221) were cultured in RPMI-1640 medium containing penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 µM/mL), and 10% fetal bovine serum (all from Thermo Scientific, Waltham, Massachusetts, USA). Cells were incubated at 37°C in a 5% CO_2 humidified incubator.

BSPE was prepared in 70% ethanol at a concentration of 0.2 g/mL. CAPE (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri, USA) at a concentration of 0.01 g/mL as the stock solution. Final concentrations of BSPE and CAPE were adjusted in culture medium. WST-1 was purchased in a ready-to-use format (Abcam, Cambridge, UK). MTT was purchased (AppliChem, Ottoweg, Darmstadt, Germany) and dissolved in phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, California, USA) at a concentration of 5 mg/mL as the stock solution.

Experimental Design

HCT-116 and DLD-1 colorectal cancer cells were seeded in cell culture flasks and checked properly. Cells were harvested when their confluency reached approximately 80%. This procedure was repeated until the end of the experiments. For cytotoxicity studies, HCT-116 and DLD-1 cells were seeded at a density of 5×10^3 cells per well in 96-well plates and incubated for 18 h at 37°C in 5% CO_2 humidified incubator. After incubation, culture medium was discarded from each well and cells were washed with PBS. Cells were then treated either with CAPE (e.g., 25, 50, 75, 100, 125, and 150 µM) or BSPE (e.g., 50, 100, 150, 200, and 250 µg/mL)

in a dose-dependent manner. Untreated cells contain cell medium only. After treatment, cells were incubated at 37°C in 5% CO_2 humidified incubator for 72 h.

MTT Cell Viability Assay

Following CAPE or BSPE treatment, the first group of HCT-116 and DLD-1 cells was incubated with MTT (0.5 mg/mL of final concentration) for 4 h at 37°C in 5% CO_2 humidified incubator. After incubation, DMSO was added in each well and cells were incubated for additional 30 min. Then, absorbance was measured by a microplate reader (Versa Max, Molecular Devices, San Jose, California, USA) at 570 nm wavelength.

WST-1 Cell Viability Assay

After CAPE or BSPE treatment, the viability of the second group of HCT-116 and DLD-1 cells was incubated with WST-1 reagent according to the manufacturer's protocol. Cells were incubated for 4 h at 37°C in 5% CO_2 humidified incubator. Absorbance was measured with a test wavelength at 440 nm and a reference wavelength at 600 nm.

Acridine Orange/Ethidium Bromide (AO/EB) Staining

HCT-116 and DLD-1 cells were seeded at a density of 5×10^4 cells per well in 6-well plates and incubated for 18 h at 37°C in 5% CO_2 humidified incubator. Thereafter, cell medium was removed, and cells were treated with BSPE and CAPE at specific concentrations (150 µg/mL of BSPE, 100 µM of CAPE) which provide an effective decrease in cell number. After treatment, HCT-116 and DLD-1 cells were incubated once more at 37°C in 5% CO_2 humidified incubator for 72 h. Following this step, cells were harvested and suspended in PBS. The mixture of dyes containing 5 µg/µL of AO (Sigma-Aldrich, St. Louis, Missouri, USA) and 3 µg/µL of EB (Sigma-Aldrich, St. Louis, Missouri, USA) was prepared. Equal volumes of dye mixture (10 µL) and cell suspension (10 µL) were mixed and transferred to glass slides. Morphological changes of the cells were examined by fluorescence microscopy (Nikon, Minato, Tokyo, Japan).

Annexin V/7AAD Apoptosis Detection Assay

HCT-116 and DLD-1 cells were seeded in 6-well plates at a density of 2×10^5 and incubated with culture media for 18 h at 37°C in 5% CO_2 humidified incubator. Afterward, cell medium was discarded, and cells were treated with 150 µg/mL of BSPE and 100 µM of CAPE before 72 h of incubation. After incubation, cells were harvested and washed with PBS. Finally, cells were stained with Annexin V and 7AAD according to manufacturer's protocol (BD Pharmingen, Franklin Lakes, New Jersey, USA).

Statistical Analysis

Statistical data analysis was performed using GraphPad Prism (GraphPad Software, Version 6.0, La Jolla, USA). Unpaired t-test was applied to data from MTT and WST-1 assays. $P<0.05$ was considered as statistically significant.

Results

In our study, we analyzed the reliability of MTT and WST-1 assays on treatment of naturally obtained BSPE and commercially obtained CAPE.

In Figure 1, viability diagrams of BSPE on HCT-116 and DLD-1 cells are shown. Untreated cells exhibited nearly 100% viability. Higher viability values were obtained in the cells treated with the lowest concentrations of BSPE (50 µg/mL) compared to untreated cells. Cell viability decreased as the concentration of BSPE increased. However, MTT assay showed high viability values at doses that caused a high rate of death in cell culture. As an example; when BSPE was used at the highest concentration (300 µg/mL), MTT assay showed 67% viability in HCT-116 cells and 46% viability in DLD-1 cells. The viability values obtained by WST-1 assay for the same concentration of BSPE were 34% in HCT-116 cells and 12% in DLD-1 cells. Statistically significant differences were found between the MTT and WST-1 assays for all doses of BSPE (unpaired t-test, $p<0.05$).

As shown in Figure 2, MTT assay gave slightly higher results than WST-1 assay on CAPE-treated cells. However, there was no statistically significant difference between the results of two assays (unpaired t-test, $p<0.05$). Briefly, cell viability values measured by the MTT assay were significantly higher than the WST-1 assay for BSPE treatment, but the similar results were not observed for CAPE treatment.

The effective doses of BSPE (150 µg/mL) and CAPE (100 µM) were used in the following experiments. In case of 150 µg/mL of BSPE treatment, MTT assay detected 85% and 96% viability in DLD-1 and HCT-116 cells while WST-1 assay detected 56% and 75% viability in DLD-1 and HCT-116 cells, respectively. For 100 µM of CAPE treatment, the viability values of DLD-1 and HCT-116 cells were 52% and 59% according to the MTT assay. WST-1 assay detected 48% viability in DLD-1 cells and 53% in HCT-116 cells for the same concentration of CAPE.

Cytotoxic effects of BSPE and CAPE on cells are represented by AO/EB staining in Figure 3. BSPE and CAPE treatment caused cell death on both HCT-116 and DLD-1 cells with a decrease in cell number compared to untreated cells. Since

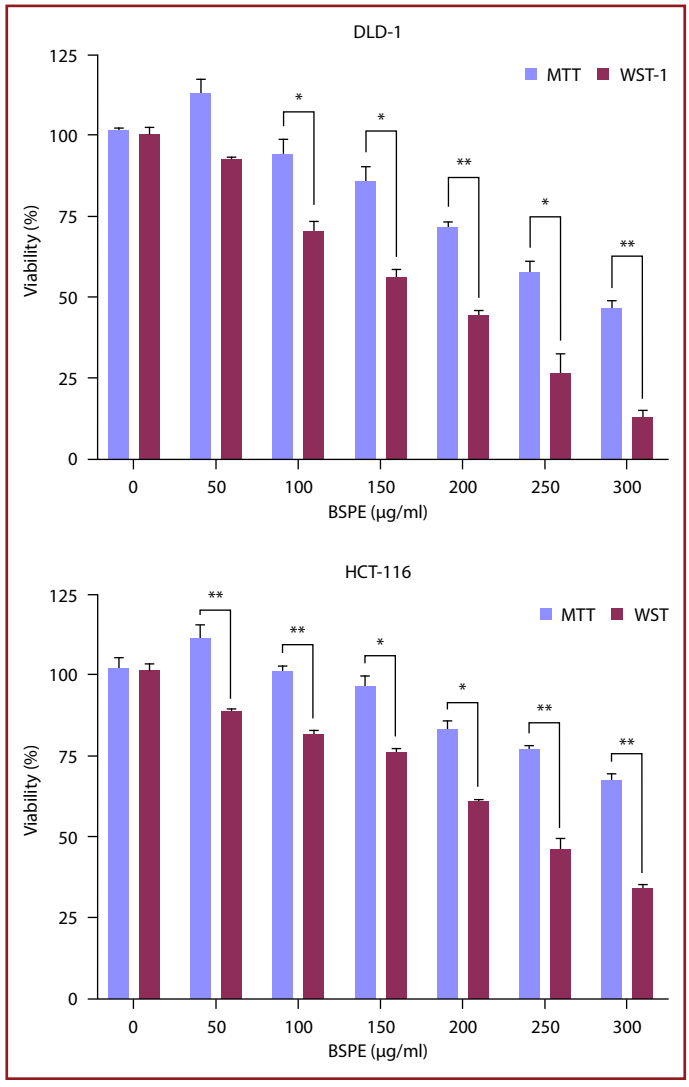

Figure 1. Concentration-dependent change of DLD-1 and HCT-116 cell viabilities after BSPE treatment using MTT and WST-1 assays. Three independent experiments performed in triplicate and all data are expressed as means±SD. *p<0.05, **p<0.01.

DLD-1, HCT-116: Human colorectal carcinoma cell lines; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WST: (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; monosodium salt); BSPE: Black Sea propolis extract.

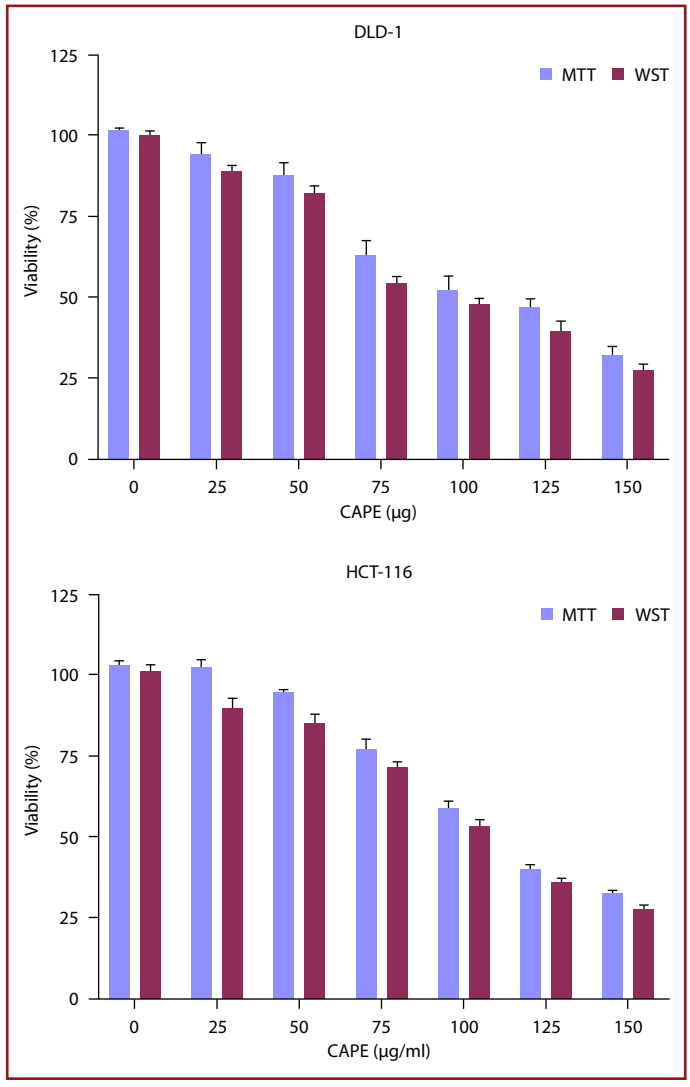

Figure 2. Concentration-dependent change of DLD-1 and HCT-116 cell viabilities after CAPE treatment using MTT and WST-1 assays. Three independent experiments performed in triplicate and all data are expressed as means±SD.

DLD-1, HCT-116: Human colorectal carcinoma cell lines; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WST: (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; monosodium salt); CAPE: Caffeic acid phenethyl ester.

live cells are the only cells that are permeable to AO, green-stained nuclei containing cells were live cells. Apoptotic and necrotic cells, which are shown by arrows, were red or orange stained under the influence of both fluorescent dyes.

The results of the flow cytometric Annexin V/7AAD assay are shown in Figure 4. In the density plot of Annexin V/7AAD analysis, viable cells were obtained as Annexin V negative and 7AAD negative while early apoptotic cells were Annexin V positive and 7AAD negative. In addition, late apoptotic/necrotic cells were both Annexin V positive and 7AAD positive. According to Annexin V/7AAD assay, viable cell percentage of DLD-1 and HCT-116 cells after 150 µg/mL of BSPE treatment was 52.1% and 75%, respectively. Besides, the viability of DLD-1 cells was 45.9% after 100 µM of CAPE treatment while the viability of HCT-116 cells was 53.2%. Annexin V/7AAD assay showed highly different results compared to MTT assay. However, all results were compatible with WST-1 assay, especially for BSPE treatment.

Discussion

Cytotoxicity assays are widely used in drug screening studies to measure the cytotoxic potential of drugs. Although

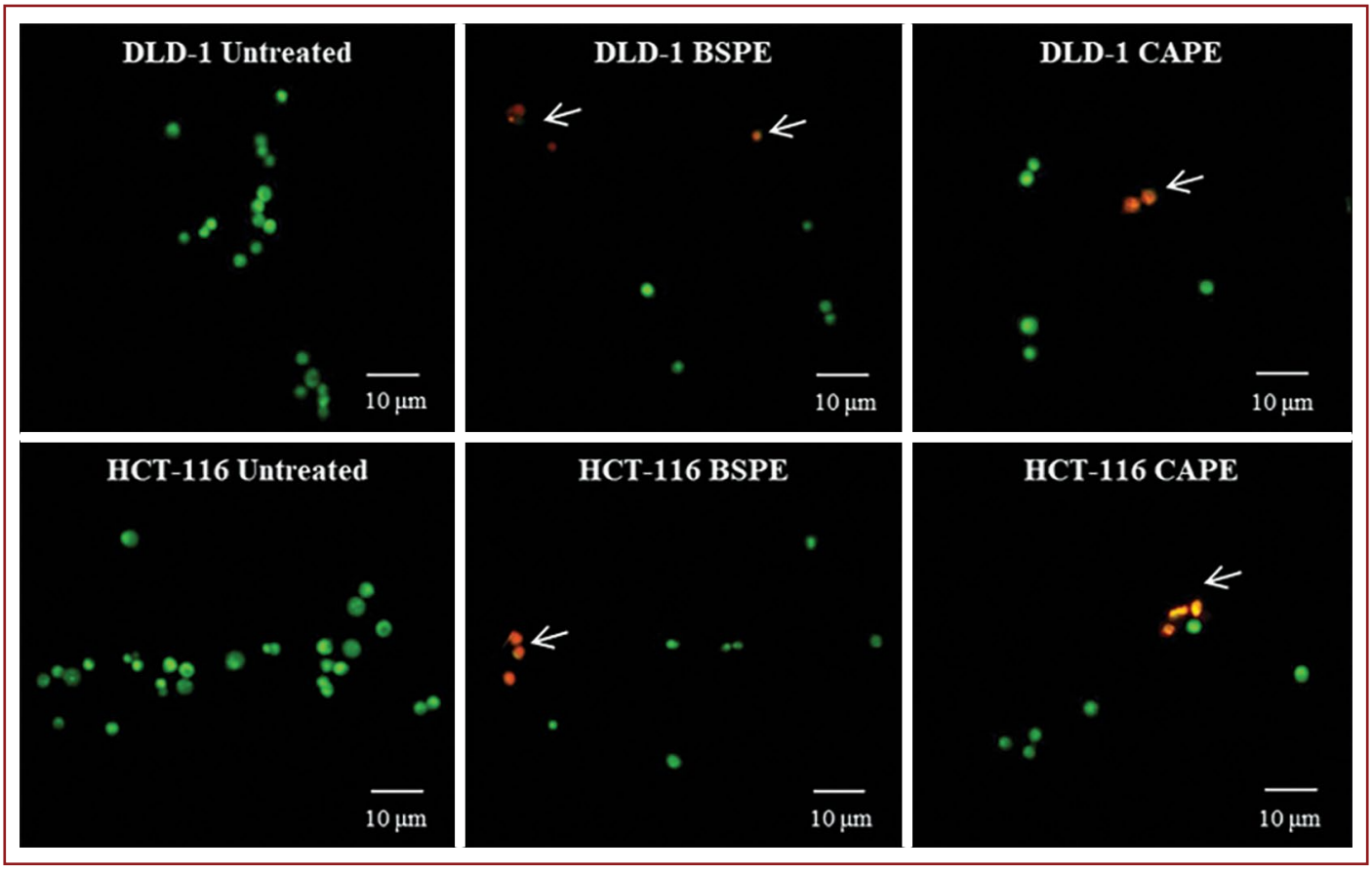

Figure 3. Fluorescence imaging for demonstration of cell death by treatment of BSPE and CAPE. Arrows show apoptotic cells. Magnification: 200×.
BSPE: Black Sea propolis extract; CAPE: Caffeic acid phenethyl ester.

MTT assay is a well-known cytotoxicity assay, its validity should be investigated in certain conditions. Some experimental systems in the previous studies have shown that MTT assay is incapable to measure the cytotoxic effects of several drug candidates and might give false-positive results[11,14,16,30]. It is known that the limitations of the MTT assay are usually due to direct interaction between tested compounds and MTT or the interference of tested compounds with mitochondrial dehydrogenase activity[31-33].

In the present study, we compared the cytotoxic effects of BSPE and CAPE on colorectal cancer cell lines using MTT and WST-1 assays. Statistically significant differences were observed between the MTT and WST-1 assays for all concentrations of BSPE. However, there were slight differences in CAPE treatment that was not statistically significant. Bruggisser et al.[10] have shown that antioxidant compounds can interfere with MTT assay. They have noted that the use of MTT assay to detect the effects of natural products requires precaution. Natarajan et al.[34] have reported that different antioxidants might reduce MTT and the results of MTT assay should be reviewed carefully. In this case, different results obtained by MTT and WST-1 assays in our study may indicate a reduction of MTT with BSPE.

Flavonoids and phenolic compounds in propolis content contribute to the variety of biological activity of propolis[35,36]. Wisman et al.[37] have stated that MTT assay may produce false-positive results on treatment of some phenolic compounds. In a study by Peng et al.[16], MTT assay has been used to investigate the antiproliferative effects of luteolin and quercetin on cancer cells. These two flavonoid substances were shown to inhibit cell growth according to microscopic examination. However, contrary to this finding, high cell viability data have obtained in MTT assay. In addition, concentration and incubation time of flavonoids have been shown to alter the absorbance values. They concluded that flavonoids might be directly reduced MTT. Besides, Talorete et al.[15] have suggested that the chemical structure of flavonoids might be an effective factor in determining MTT reduction potential. Hence, further confirmation of the antiproliferative activity of BSPE and CAPE was required in our study. Fluorescence microscopy imaging after AO/EB staining showed that cells started to

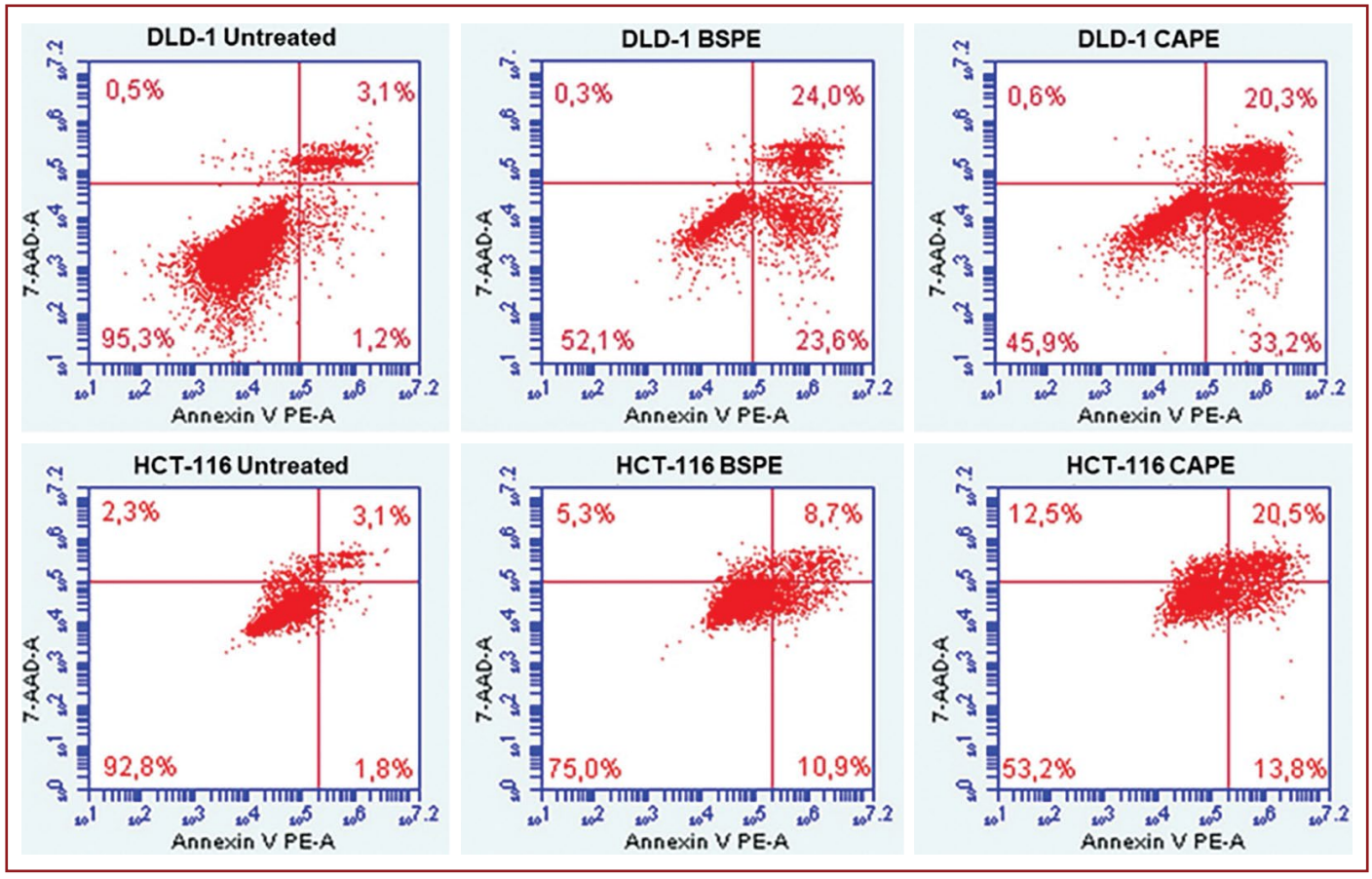

Figure 4. Flow cytometric analysis of HCT-116 and DLD-1 cells after BSPE treatment and CAPE treatment.

DLD-1, HCT-116: Human colorectal carcinoma cell lines; BSPE: Black Sea propolis extract; CAPE: Caffeic acid phenethyl ester.

exhibit apoptotic morphology and cell number decreased when BSPE and CAPE were used on DLD-1 and HCT-116 cells. These microscopic observations prove the efficacy of BSPE and CAPE. Considering the observation of apoptotic cells and the decrease in the total number of cells, it can be said that fluorescence microscope images were not consistent with MTT assay which gave high cell viability results.

In a study by Shoemaker et al.[17], several herbal extracts that inhibit cancer cell growth have found to be no longer inhibitory according to MTT assay. Karakas et al.[14] have stated that MTT assay exhibited false-positive results when some plant extracts were used on cancer cells. Wang et al.[38] have shown that the results of MTT assay have not precisely reflected the antiproliferative effects of green tea polyphenols. Since MTT-based analysis should be quantitatively investigated using different methods, we needed to confirm our data with cell death markers. In flow cytometric Annexin V/7AAD assay, DLD-1 and HCT-116 cells showed a viable cell population which is overlapped with WST-1 assay at certain concentrations of CAPE and BSPE. Particularly in treatment of BSPE, Annexin V/7AAD results were highly consistent with WST-1 results but inconsistent with MTT results. Thus, it was confirmed that MTT assay is an error-prone analysis method and might give false-positive results in case of BSPE treatment.

Conclusion

Our study indicates that MTT assay causes contradictory results in BSPE treatment. Several studies suggest that the chemical compounds included in the assay should be reviewed for their interactions with MTT reagent[10,39]. In addition, the structure of the compounds might influence their interaction with MTT[15]. It is known that various natural extracts containing polyphenols and flavonoids are strongly reduced MTT in cell culture[10,16,17,37,38]. Likewise, it should be noted that MTT assay may cause false-positive results if a naturally derived compound such as BSPE is used. Since propolis is a complex compound, our results suggest that different components of BSPE rather than CAPE might perform a reduction with MTT assay. Further studies with other active components of propolis will contribute to the literature. In conclusion, we believe that MTT

assay is not a reliable method to detect the cytotoxic effects of BSPE. We suggest that WST-1 assay is more precise than MTT assay in this respect.

Ethics Committee Approval: There is no requirement of Ethics Committee Approval for this study.

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Conflict of Interest: None declared.

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