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ORIGINAL ARTICLE

# Investigation of Antiproliferative, Apoptotic, and Migration Activity of Combination of Paclitaxel and Aloe Vera in Non-Small Cell Lung Cancer and Breast Cancer

**Tuğba Kul Köprülü[1,2], Jülide Balkan[3]**

[1]Experimental Medicine Application and Research Center, Validebağ Research Park, University of Health Sciences Türkiye, İstanbul, Türkiye
[2]Department of Medical Services and Techniques, University of Health Sciences, Hamidiye Vocational Faculty of Health Services, Medical Laboratory Techniques, İstanbul, Türkiye
[3]Department of Molecular Medicine, University of Health Sciences Türkiye, Hamidiye Institute of Health Sciences, Istanbul, Türkiye

## Abstract

**Introduction:** The aim of this study is to determine whether it is possible to increase the cytotoxic effects of known anti-cancer agents with natural compounds. For this purpose, the antiproliferative effect of Aloe vera (AVE) against human breast cancer (MCF-7) and lung cancer (A549) and the *in vitro* potential anticancer activity of its combination with Paclitaxel (PAX) were investigated.

**Methods:** The antiproliferative activity of the AVE and AVE-PAX combination was assessed against MCF-7 and A549 cancer cell lines and non-cancer cell (BEAS-2B) using xCELLigence real-time cell analysis. Cell apoptosis was determined by DNA laddering assay and Annexin V/FITC flow cytometer, and cell migration was evaluated by wound healing assay.

**Results:** Treatment with the AVE and AVE-PAX significantly increased the antiproliferative activity in A549, MCF-7, and BEAS-2B cells compared to the control group ($p<0.05$). AVE-PAX combination administration resulted in earlier antiproliferative activity than AVE in all three cell lines ($p<0.05$). It was determined that when 24 µg/mL AVE and 5 µM PAX were applied together, it triggered the apoptotic process in A549 and MCF-7 cells and showed an anti-migration effect.

**Discussion and Conclusion:** In our study, the combination of AVE-PAX reduced the growth and cell migration of MCF-7 and A549 cells with low PAX susceptibility *in vitro* and induced early apoptosis of A549 and MCF-7 cells. These results show that the use of AVE together with PAX is effective in NSCLC and breast cancer and has a synergistic effect.

**Keywords:** Aloe vera; Apoptosis; Breast Cancer; Migration; Non-Small Cell Lung Cancer; Paclitaxel; Real-Time Cell Analysis/ xCELLigence.

Cancer, defined as uncontrolled cell division, is a large group of diseases that can affect any part of the body and is one of the leading causes of death worldwide. Lung and breast cancer are among the most common cancers all over the world[1,2]. Worldwide, approximately 2.3 million breast cancers and 2.2 million lung cancers were observed in 2020 according to a World Health Organisation (WHO) report[3]. Non-small cell lung cancer (NSCLC), the major subtype of lung cancer and accounting for 80% of all lung cancer cases, is classified by WHO into three

**Correspondence:** Tuğba Kul Köprülü, PhD. Experimental Medicine Application and Research Center, Validebağ Research Park, University of Health Sciences Türkiye, İstanbul, Türkiye; Department of Medical Services and Techniques, University of Health Sciences Türkiye, Hamidiye Vocational Faculty of Health Services, Medical Laboratory Techniques, İstanbul, Türkiye
**Phone:** +90 505 857 40 34 **E-mail:** tugbakul.koprulu@sbu.edu.tr
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subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma[4,5]. Adenocarcinoma is notable because it accounts for about 40% of NSCLC[5]. Estrogen receptor-positive type luminal cancers constitute 70% of breast cancer, which is the most common cancer type and a heterogeneous disease with many subtypes[6].

For this reason, studies to develop new therapeutics and increase the effectiveness of existing therapeutics in lung and breast cancer, which have become a global health problem, constitute a dynamic field. Multi-drug resistance can develop in cancer cells during chemotherapy, and increased resistance drives the use of higher doses of drugs[7-9]. Higher doses of medication are also likely to produce serious side effects. The combination of molecules with different mechanisms can create potent therapeutics. Especially, drug combinations containing natural products exhibit similar effects to frequently used chemotherapeutic drugs but may show fewer side effects[10]. For this reason, natural and herbal compounds that have the potential to synergize with antitumor drugs constitute an area that needs to be investigated further. Paclitaxel (PAX), also known as Taxol, is the most widely used antineoplastic drug with a wide range of activity approved by the FDA in the treatment of many cancers, including breast cancer, NSCLC, bladder, cervix, and endometrial cancers[11,12]. Rosmarinic acid and ursolic acid were used in *ex vivo* breast cancer cells to increase the anticancer activity of PAX, which inhibits mitosis by interfering with microtubule organization during cell division and thus initiates the process resulting in the death of the cancer cell, and it has been found to increase the cytotoxicity of these cells by modulating the microenvironment of the tumor[13]. In another study, it was determined that Asparagus officinalis plant extract, when used together with PAX, reduced the viability and invasion of cancer cells[14]. Zhang et al.[15] (2020) concluded that when PAX is used together with Quercetin, it triggers apoptosis by inducing ER stress on prostate cancer cells and increasing ROS production, inhibits cell migration, and arrests the cell cycle in the G2/M phase. The results show that it is possible to increase the cytotoxic effects of known anticancer agents with natural compounds. Aloe barbadensis, commonly known as Aloe vera (AVE)[16], regulates cholesterol and blood sugar[17,18], promotes skin tissue regeneration[19], and also has antimicrobial[20], antiviral[21], chemopreventive[22], antitumor[23], antioxidant[20], and anti-inflammatory[24] activities. Although AVE is known to be a useful therapeutic herb, the exact mechanisms of how it induces cancer cell death have not yet been fully elucidated. Polysaccharides and anthraquinones, which are among the bioactive components of AVE, can inhibit the growth of cancer cells together. Acemannan, a mucopolysaccharide found in AVE, has been shown to have an immunomodulatory effect[25]. This activity includes anti-inflammation[26], immunological activation[27], and macrophages, which are pluripotent effector cells. Aloin and Emodin, two anthraquinones that have been extensively studied for their anticancer properties in AVE, have antiproliferative, apoptotic, chemopreventive, and radioprotective effects[28-30].

Concomitant use of PAX and AVE may increase the sensitivity of cancer cells to the drug. In our study, the combined use of PAX, which is used in the treatment of lung and breast cancer, and AVE, which is used for various therapeutic purposes, was investigated in terms of anticancer activity.

## Materials and Methods

### Preparation of Cell Culture and Test Compounds

A549 (CCL-185™, human non-small cell lung cancer cell), MCF-7 (HTB-22™, human breast adenocarcinoma), and BEAS-2B (CRS-9609™, human bronchial epithelial cell) cells were incubated in cell culture flasks at 37°C and 5% $CO_2$ in DMEM High Glucose (4.5 g/L) medium supplemented with 10% Fetal Bovine Serum (Capricorn Scientific, Germany) and 1% Penicillin/Streptomycin (Capricorn Scientific, Germany). Cells that covered 80% of the cell culture flask were removed by Trypsin-EDTA (0.05%) (Gibco, New Zealand) and 10 µL of cell suspension stained with Trypan Blue at the same rate and transferred onto TC20 slide and counted with TC20 Cell Counter (BioRad, California, USA). Aloe barbadensis (Sarısabır in Turkish) was purchased from the Akdeniz Aloe Vera company in Antalya and is cultivated at the Experimental Medicine Application and Research Center of the University of Health Sciences. Fresh AVE leaves were cleaned with distilled water, peeled off by drying on filter paper, and homogenized by taking the gel inside. After the homogenized gel was centrifuged at 300g for 3 min, it was diluted by adding distilled water and mixed at 60°C for 2 h. The homogenized gel was filtered first through Whatman Grade 1 filter paper and then through a 0.22 µm filter (Sigma, Massachusetts, USA). At the end of the period, the entire supernatant was concentrated in the appropriate volume (3.01 g) under low pressure. The PAX (Sigma, Massachusetts, USA) to be used in the experiment was weighed in an appropriate amount and dissolved with DMSO and 10 mM stock solution was prepared and stored at -20°C.

### Determination of Cell Proliferation by Real-Time Cell Analysis

In order to evaluate the effect of AVE together with PAX on the proliferation of cells, cells were seeded in triplicate on a 96-well e-plate at 15,000 cells/well in a volume of 100 µL. AVE was applied alone at concentrations of 1.5, 3, 6, 12, and 24 µg/mL and together with PAX at concentrations of 1, 5, 10, and 20 µM and completed with DMEM for a total volume of 200 µL. The final concentration of DMSO in the medium was kept at less than 1% (v/v). Prepared cell culture special plates (e-plate) were incubated in an environment containing 37°C and 5% $CO_2$, and instant cell index values were recorded by the xCELLigence (RTCA MP) (Agilent, California, USA) device. Impedance measurements were recorded every 5 min for 72 h, and cell proliferation data were analyzed via RTCA soft (ACEA Biosciences, California, USA).

### DNA Laddering Assay

The method of Gong et al.[31] (1994) was applied with some minor modifications to determine whether the most effective dose application showing antiproliferative activity causes an apoptotic effect on cells. The working principle of the test is to show 180-200 bp DNA breaks that occur during apoptosis. For this purpose, a combination of AVE and PAX was applied to cells cultured in a 25 $cm^2$ cell culture flask and incubated for 24 h. At the end of the period, cells were removed from the flask using a scraper and then the content of the flask was transferred to a falcon tube. It was centrifuged at 300g for 5 min. After this process, 70% alcohol was added to the cell pellet at the bottom and precipitated at -20°C for 48 h. In the end, the alcohol was completely removed and the cell pellet was dissolved with phosphate-citrate buffer. The dissolved pellet was incubated at 37°C for 30 min with the addition of 5 µL of RNase A and 5 µL of Tween 20, then 5 µL of Proteinase K was added and incubated at 37°C for 30 min. Samples stained with 5 µL of loading dye were run in 2% agarose gel and imaged with ChemiDoc Imaging System (BioRad, California, USA). When the test substance induces the apoptotic mechanisms in cells, DNA will be cut by endonucleases and thus DNA will be fragmented into 180-200 bp pieces, which will provide a ladder-like image on the agarose gel and apoptotic evaluation of the relevant substances.

### Detection of Apoptosis in Flow Cytometry with Annexin V/FITC

The determination of apoptotic cells by flow cytometry was performed using Annexin V/FITC Apoptosis Detection Kit. Cells seeded in T25 cell culture flasks at $5\times10^6$ cells/mL were removed with Trypsin-EDTA and centrifuged at 300g for 5 min to form a cell pellet. Then, cold PBS was added to pass the cell pellet, and resuspended cells were centrifuged at 300g for 5 min to form a cell pellet. After the aspiration of the supernatant, the cell pellet was resuspended by adding 1 mL of 1× Binding Buffer. In the ongoing process on ice, 100 µL of the cell suspension was added to a new tube and 1 µL of Annexin V-FITC solution and 5 µL of PI were added to it. After adding 400 µL of 1× Binding Buffer to the tube, which was kept on ice for 15 min in a dark environment, measurements were recorded with CytoFlex Flow Cytometer (Beckman Coulter) with FITC (Absorption 492 nm-Emission 520 nm) and PI (Absorption 370/550 nm-Emission 560-680) stainings.

### Wound Healing Assay

A549 and MCF-7 cells were seeded in a culture-insert (ibidi GmbH, Germany) apparatus at $3.5\times10^4$ cells/well and incubated under cell culture conditions for 24 h. Then, the culture-insert apparatus was removed and 2 mL of DMEM was added to the cells, an effective dose of the test compound was added, and the cells were incubated under culture conditions again. Images of the cells were taken at 0 and 24 h under an inverted microscope (Zeiss Primovert, Germany) and evaluated.

### Statistical Analysis

Results were expressed as mean values±standard deviation (SD). All data collected from experiments were performed in three replicates and analyzed using the one-way analysis of variance (ANOVA) at a significance level of $p<0.05$.

## Results

### Evaluation of the Antiproliferative Activity of AVE and AVE-PAX on A549, MCF-7, and BEAS-2B cells by Real-Time Cell Analysis (xCELLigence)

The changes in cell proliferation occurring when only Aloe vera is used on BEAS-2B cells at concentrations of 1.5, 3.0, 6.0, 12, and 24 µg/mL and in combination with concentrations of 5, 10, and 20 µM PAX are shown in Figure 1 ($p<0.05$). When AVE was applied from a lower concentration of 1.5 µg/mL to a higher concentration of 24 µg/mL to BEAS-2B cells, it did not reduce proliferation for up to 16 h for all concentrations. It began to show antiproliferative activity from 16 h and it has been shown to seriously inhibit cell proliferation between 24-30 h depending on the concentration compared to control cells. Also, the 24 µg/

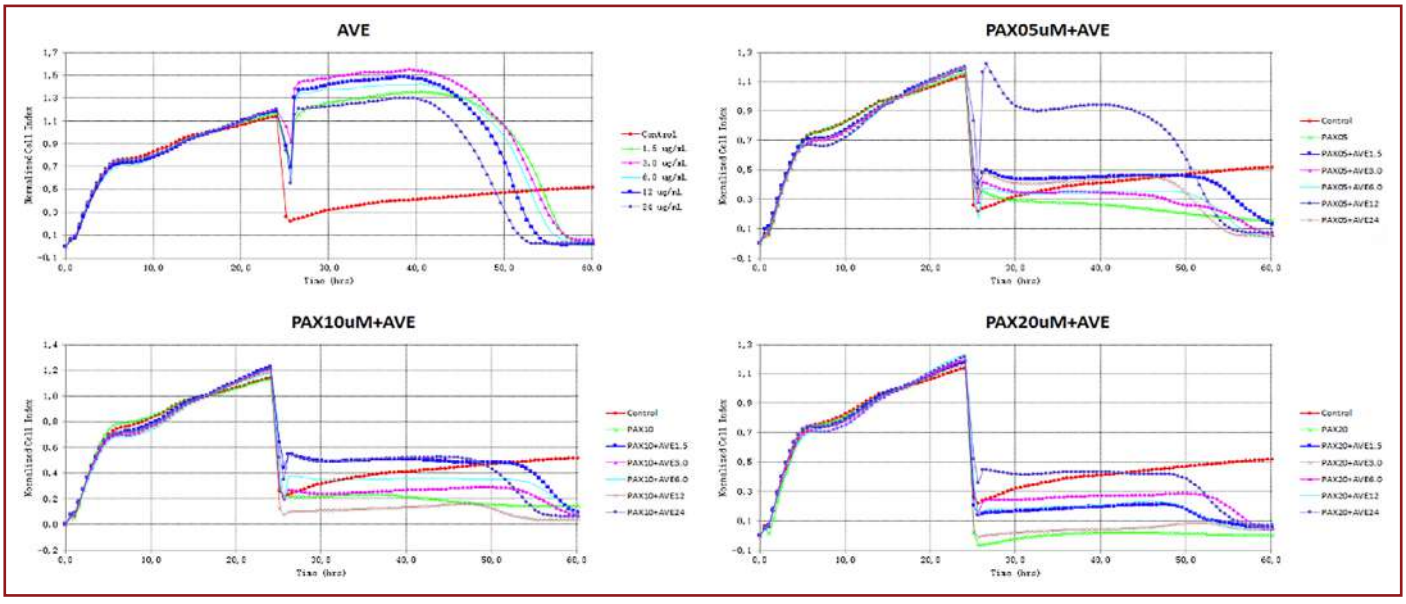


**Figure 1.** Determination of the antiproliferative properties of AVE on BEAS-2B bronchial epithelial cells when administered alone and with PAX with a real-time cell analyzer (xCELLigence). Proliferation results were obtained by applying AVE at 1.5, 3.0, 6.0, 12, and 24 µg/mL concentrations with 05, 10 and, 20 µM PAX concentrations ($p<0.05$).

AVE: Aloe vera; PAX: Paclitaxel.

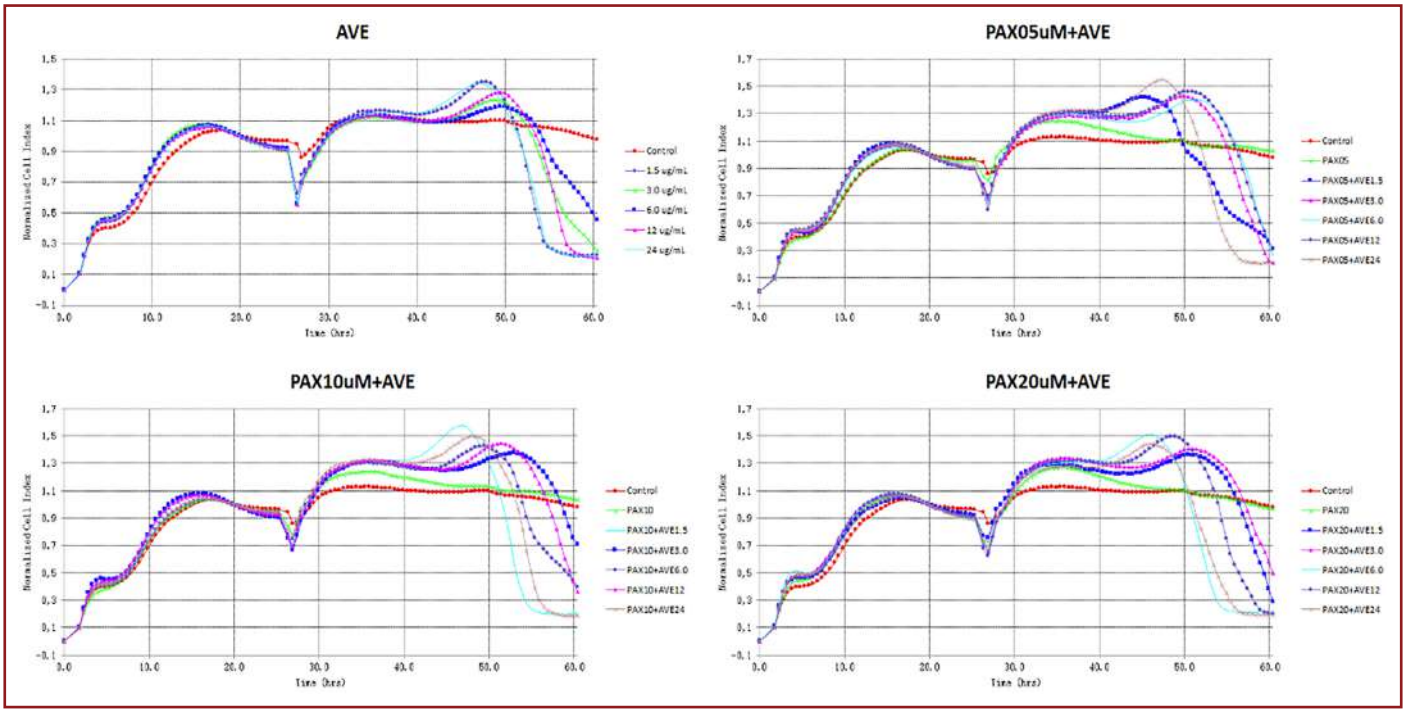


**Figure 2.** Determination of the antiproliferative properties of AVE on A549 lung cancer cells when administered alone and with PAX with a real-time cell analyzer (xCELLigence). Proliferation results were obtained by applying AVE at 1.5, 3.0, 6.0, 12, and 24 µg/mL concentrations with 05, 10, and 20 µM PAX concentrations ($p<0.05$).

AVE: Aloe vera; PAX: Paclitaxel.

mL concentration of AVE inhibits cell proliferation earlier than other concentrations. It was determined that the antiproliferative activity started significantly much earlier when AVE was combined with PAX at all concentrations.

As shown in Figure 2, depending on the concentration, AVE began to reduce proliferation in A549 cells between 22-25 h for all concentrations, while antiproliferative activity began to be observed between 26-30 h when

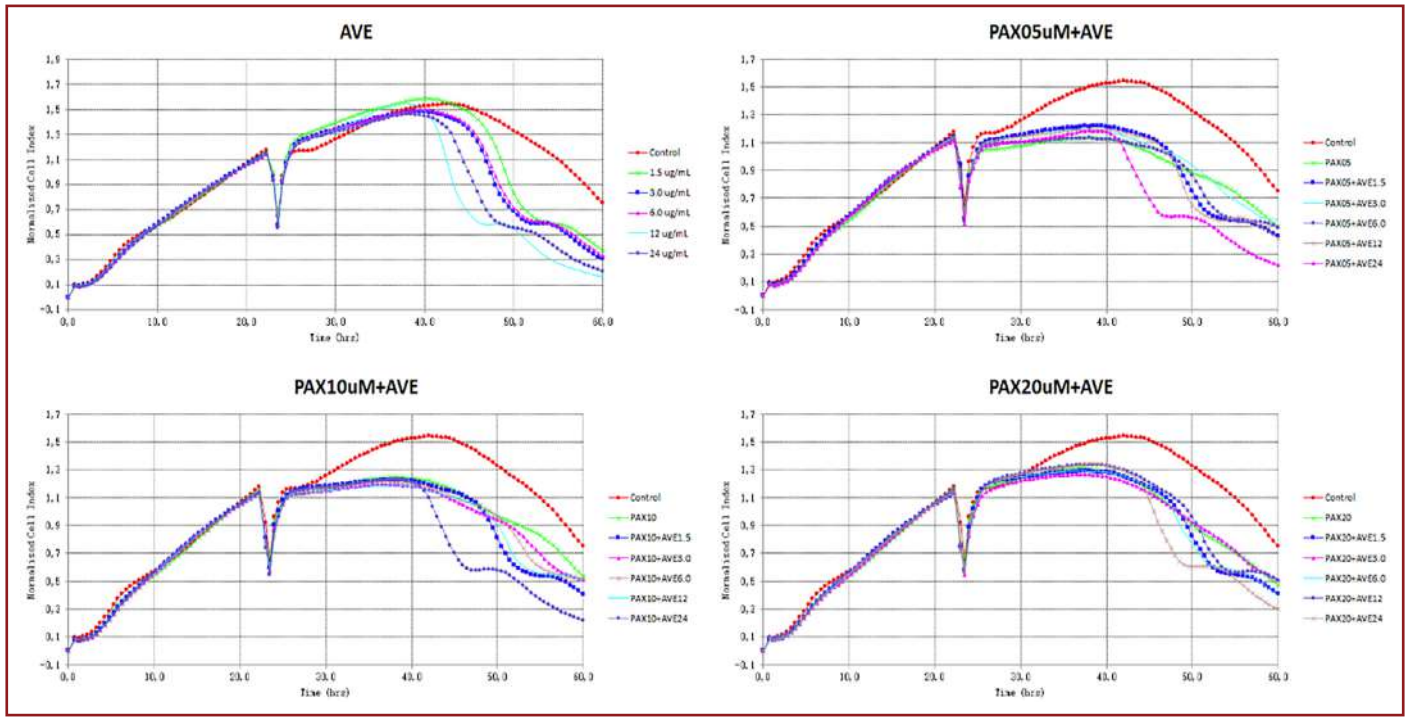


**Figure 3.** Determination of the antiproliferative properties of aloe vera (AVE) on MCF-7 breast cancer cells when administered alone and with PAX with a real-time cell analyzer (xCELLigence). Proliferation results were obtained by applying AVE at 1.5, 3.0, 6.0, 12, and 24 µg/mL concentrations with 05, 10, and 20 µM PAX concentrations ($p<0.05$).

PAX: Paclitaxel.

used in combination with PAX ($p<0.05$). The fact that AVE shows antiproliferative activity in A549 cells at a later time (26-30 h) than BEAS-2B cells explains that cancer cells have resistance mechanisms compared to healthy cells. At the same time, when looking at the graphs, the 24 µg/mL concentration of AVE is more effective in A549 cells. When AVE and PAX are administered at the same time, it is observed that the effect of PAX increases with increasing concentration. Also, as the concentration of PAX increases, it is observed that the antiproliferative effect of AVE on cells increases, even at low concentrations. The response of MCF-7 cells to AVE application with a decrease in the number of cells began within 15 h depending on the concentration and AVE began to affect cell proliferation much earlier when used in combination with PAX, especially at a higher concentration of AVE ($p<0.05$). It is observed that the sensitivity of MCF-7 cells to both AVE and PAX is higher than other cell lines, which are A549 and BEAS-2B. The graphs show that AVE shows an antiproliferative effect even at low concentration. The graphs in which PAX and AVE are administered together indicate that the antiproliferative effect also increases as the amount of concentration increases. In addition, MCF-7 cells are more sensitive to AVE treatment and in combination with PAX than A549 cells, and this is defined by the appearance of antiproliferative activity very soon after administration (Fig. 3). When all the graphs are examined, it is seen that AVE has an antiproliferative effect on A549 and MCF-7 cells, it shows an effect much earlier when used in combination with PAX, and MCF-7 cells are more sensitive to AVE than A549 cells. AVE showed antiproliferative activity on BEAS-2B cells at a concentration of 24 µg/mL at the earliest. PAX showed an earlier effect on BEAS-2B at all concentrations where it was used in combination with AVE, so the lowest concentration used in the experiment (5 µM) was combined with 24 µg/mL AVE and applied in other experiments.

## Determination of DNA Fragmentation by DNA Laddering

DNA fragmentation is one of the main features of apoptosis, so it is used as a marker in the detection of the apoptotic process. DNA laddering is a technique that makes the presence of apoptosis visible by creating a DNA ladder model in agarose gel electrophoresis of DNA fragments that occur during apoptosis. Since the genomic DNA of apoptotic cells is divided into fragments of about 180 bp, a characteristic ladder image is obtained in agarose gel electrophoresis. DNA fragmentation assay was applied to both A549 and MCF-7 cancer cells to examine the molecular mechanisms underlying the antiproliferative

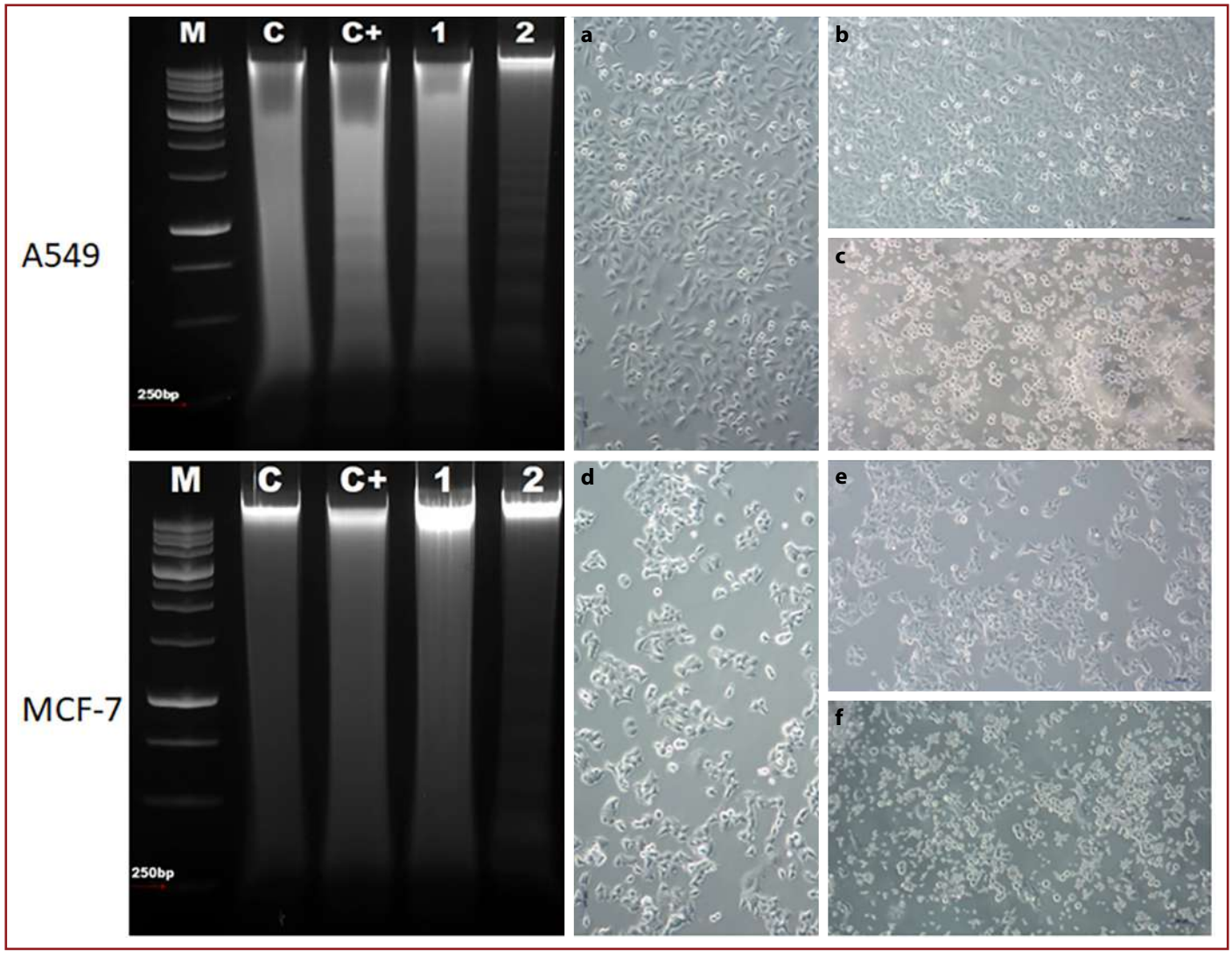


**Figure 4.** Demonstration of DNA Fragmentation in A549 and MCF-7 cells by Agarose Gel Electrophoresis and Representative Cell Images. M: 1 kb DNA Marker; C: Control, untreated cell line; C+: Positive Control, Cell Line+PAX; 1: Cell Line +5 µM PAX; 2: Cell Line+5µM PAX + 24 µg/mL. (a) Control (untreated A549) 0 hour; (b) control (untreated A549) after 24 hours; (c) A549 + 5 µM PAX + 24 µg/mL AVE after 24 hours, (d) control (untreated MCF-7) 0 hour; (e) control (untreated MCF-7) after 24 hours; (f) MCF-7 + 5 µM PAX + 24 µg/mL AVE after 24 hours. Scale Bar= 500µm.

PAX: Paclitaxel; AVE: Aloe vera.

activity and to check whether the effect is due to cell death via apoptosis. In the experiment, when A549 and MCF-7 cells were treated separately with 5 µM PAX+24 µg/mL AVE for 24 h, it was observed that apoptotic cell death occurred in both cell lines (Fig. 4).

## Flow Cytometric Measurement of the Percentage of Apoptotic Cells

A common feature of apoptotic cells is the exposure of phosphatidylserine on the outer part of the plasma membrane. To detect phosphatidylserine, Annexin V staining labeled using fluorescein isothiocyanate (FITC) and Annexin V/FITC binding is assessed by using flow cytometry. Translocation of phosphatidylserine to the outer cell membrane occurs not only in apoptotic cells but also in necrotic cells. In the assay, double staining with Annexin V and propidium iodide (PI) was evaluated as apoptosis. Annexin V-positive cells were considered to be in the early stage of apoptosis, whereas Annexin V and PI-positive cells were considered to be in the late stage of apoptosis ($p<0.05$). From cytometric data, it was shown that the AVE-PAX combination induced early-stage apoptosis in both A549 and MCF-7 (Fig. 5) ($p<0.05$). AVE+PAX has been observed to increase the rate of early apoptosis by at least 20% in A549 and MCF-7 cells. In addition, as a result of AVE+PAX treatment, necrosis of A549 cells is also observed.

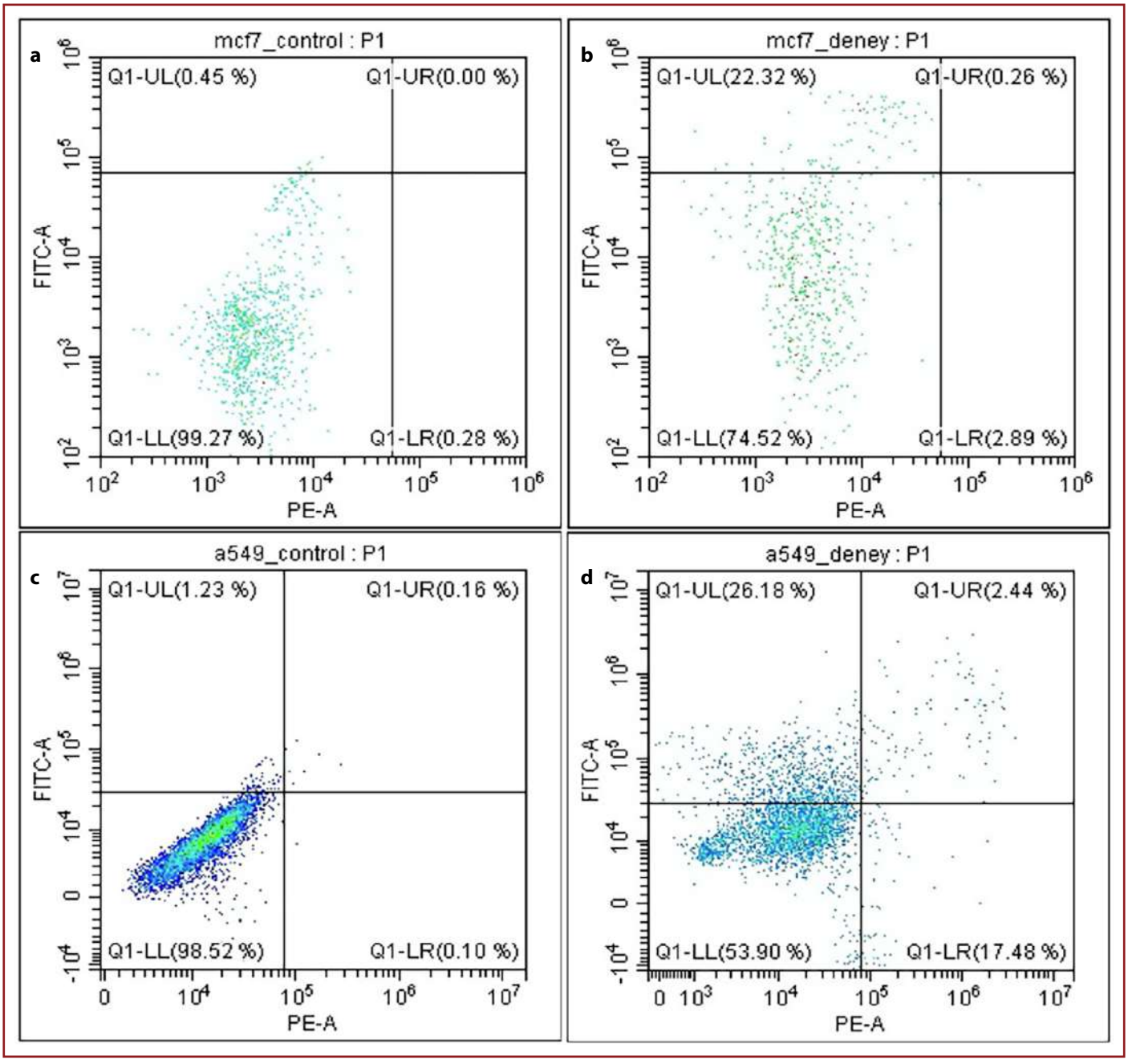


**Figure 5.** Quantification of apoptosis by using Flow Cytometer in A549 and MCF7 cells treated with 24 µg/mL AVE and 5µM PAX for 16 hours. The percentage of apoptosis in MCF-7 control was 0.45% (a). It was increased to 22.32% with AVE+PAX supplement (b). The percentage of apoptosis in A549 control was 1.23% (c). It was increased to 26.18% with AVE+PAX supplement (d) ($p<0.05$).

AVE: Aloe vera; PAX: Paclitaxel.

## Wound Healing Assay

The effects of 24 µg/mL AVE and 5 µM PAX combination on cell migration in A549 and MCF-7 cancer cells were examined using a wound-healing assay. It was observed that less migration and wound closure occurred when cells were treated with AVE-PAX (Fig. 6). Compared to control A549 cells, the combination of AVE-PAX appears to have more anti-migration effects on MCF-7 cells.

## Discussion

Natural compounds are used in cancer chemotherapies to expand the therapeutic window of chemotherapeutic drugs and to reduce the formation of chemotherapy resistance[32]. The synergistic effect of chemotherapeutic drugs with natural compounds can enhance the tumoricidal effect of chemotherapeutic drugs. The synergistic effect, which is defined as the increase in the effectiveness of a combination

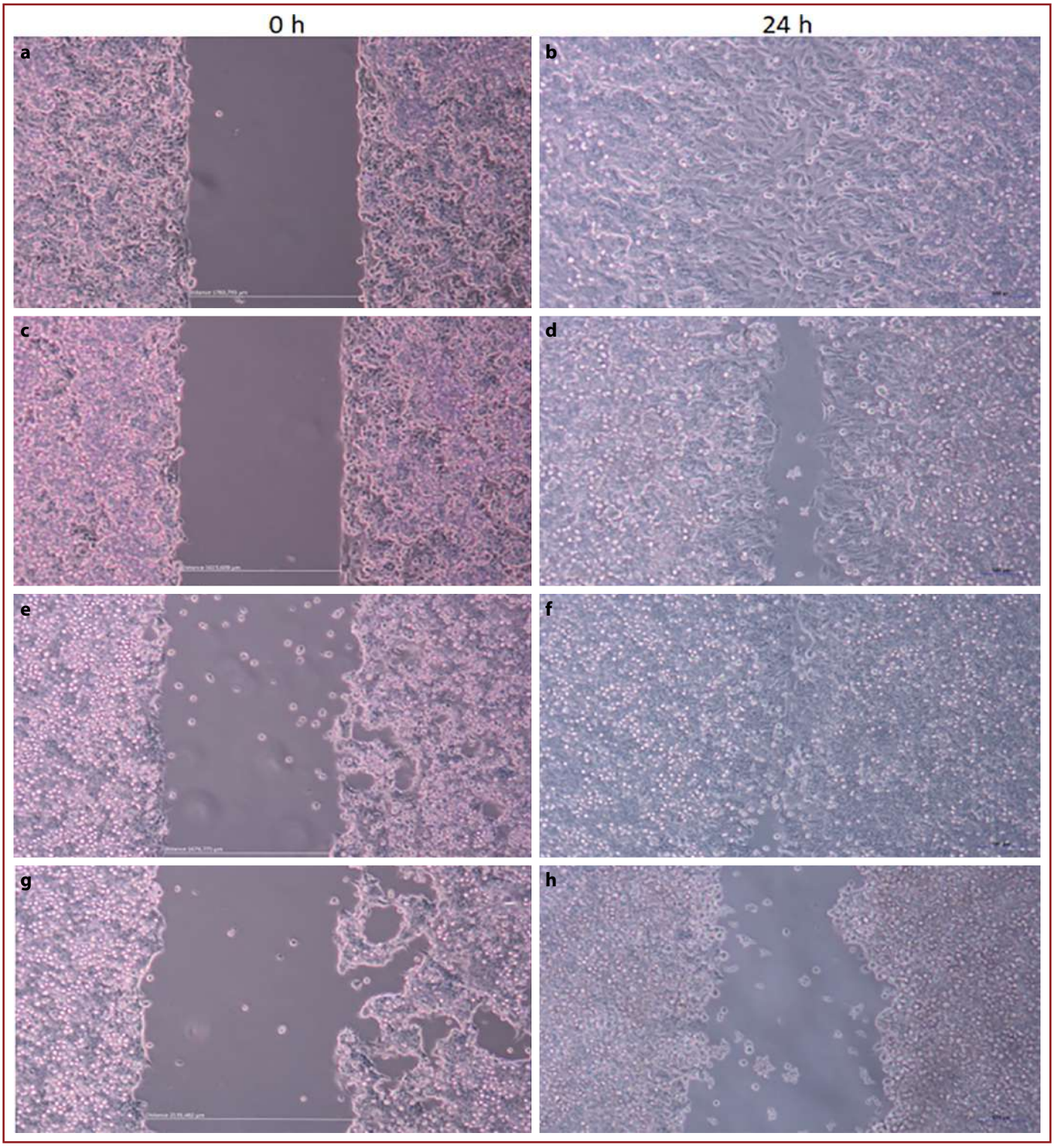


**Figure 6.** ffect of AVE -PAX combination on migration in A549 and MCF-7 cells. (a-c), A549 cells, control (0 h); (b), A549 cells, control (after 24 h.); (d), A549 cells treated with PAX-AVE (after 24 h) (e-g), MCF-7 cells, control (0 h); (f) MCF-7 cells, control (after 24 h); (h) MCF-7 cells treated with PAX-AVE (after 24 h) Scale Bar: 500µm.

PAX: Paclitaxel; AVE: Aloe vera.

of components compared to a single component, creates a dynamic research area[10]. The synergistic effects of natural compounds and traditional chemotherapeutic agents have been demonstrated in various studies[14,33,34]. Although AVE alone has been shown to have anticancer activity[34], AVE in combination with PAX has not been evaluated in previous

studies. This study aims to determine the synergistic effect of the combination of PAX and AVE, a chemotherapeutic agent, on A549 and MCF-7 cells. Both lung and breast cancer are common malignancies. The luminal subtype of ER-positive breast cancer, which has many subtypes, is the most common among breast cancer. Due to its high ER expression and hormone sensitivity, MCF-7 is often used in research. In NSCLC, which is a major subtype of lung cancer, accounting for 80% of all lung cancer and having metastatic characteristics, studies are being conducted on A549. PAX is an anticarcinogenic agent that stops cell division by stabilizing microtubules and is often used in combination with cisplatin both in metastatic breast cancer and during the initial period of NSCLC. However, the rapid development of resistance to PAX treatments in almost all cases significantly limits the use of PAX and generates a significant challenge, especially in the treatment of NSCLC. High doses of PAX are required to prevent the occurrence of drug resistance, in which case the side effects that develop limit the use of PAX. For this reason, it is necessary to evaluate the use of PAX in combination with molecules that can increase its effectiveness. In our study, it was shown that AVE has anticancer activity on A549 and MCF-7 cells, and it has been found that PAX increases its effectiveness when used with AVE. Considering the anticancer activity of the combination of 24 µg/mL AVE and 5 µM PAX, it seems that this effect is due to apoptotic processes affecting cells. It was determined that MCF-7 cells were more sensitive to the applied combination than A549 and showed anti-migratory activity in both cell lines. The results show that AVE has antiproliferative properties in A549 and MCF-7 cells and can be considered a more effective agent when used in combination with PAX.

## Conclusion

In our study, the combination of AVE-PAX reduced the growth and cell migration of MCF-7 and A549 cells with low PAX susceptibility *in vitro* and induced early apoptosis of A549 and MCF-7 cells. These results show that the use of AVE together with PAX is effective in NSCLC and breast cancer and has a synergistic effect.

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**Conflict of Interest:** None declared.

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