



# Enhanced anticancer potency of Aloe vera in combination with Royal jelly in non-small cell lung cancer and colorectal adenocarcinoma

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## Abstract

**Aim:** The aim of this study was to determine the anticancer activity of royal jelly (RJ) and Aloe vera (AVE) separately and in combination on human non-small cell lung cancer (A549) and colorectal adenocarcinoma cells (HT29) and to investigate whether the side effects of AVE can be ameliorated by RJ.

**Materials and Methods:** The antiproliferative activity of RJ, AVE, and the combination of AVE and RJ was performed in vitro on A549 and HT29 cells using a Real-Time Cell Analyzer (xCELLigence). To determine the mechanisms underlying the antiproliferative activity, cell apoptosis was performed by flow cytometry using the annexin V-FITC/PI apoptosis detection kit, while cell migration was determined by wound healing assay.

**Results:** Treatment with varying concentrations of AVE, RJ, and the combination of AVE and RJ was concluded to have dose-dependent antiproliferative activity and that the AVE-RJ combination induced 41% of HT29 and 11.79% of A549 early apoptosis ( $p < 0.05$ ). However, the inhibition effect of the combination of AVE and RJ on cancer cell migration was not detected.

**Conclusion:** Taken together, our results showed that the AVE-RJ combination could be a promising therapeutic combination in A549 and HT29 by inducing apoptosis. The obtained results revealed that the AVE (40  $\mu\text{g/mL}$ ) and RJ (300  $\text{mg/mL}$ ) combination in the A549 cell line exhibited earlier antiproliferative activity than the 40  $\mu\text{g/mL}$  AVE. Also, it has been found that the antiproliferative activity of AVE is enhanced when used in combination with RJ.



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## Introduction

Cancer, defined as uncontrolled cell division and growth which are caused by a wide range of factors, is one of the most important health problems of in the past decades. Among the different types of cancer, lung and colon cancer are the most common cancers. According to the World Health Organization (WHO), lung cancer ranks first in cancer-related deaths in both men and women worldwide. There are two main subtypes of lung cancer, non-small-cell lung carcinoma (NSCLC) accounting for 85%, and small-cell lung carcinoma accounting for 15% of all lung cancer [1]. Colorectal cancer (CRC) is the third most common cancer worldwide after breast and lung cancer, resulting

in approximately 2 million new cases and 1 million deaths each year. In addition, the lung is the second most common site of CRC metastases due to its abundant circulation [2]. Although one of the most critical approaches in the fight against cancer is traditional treatments such as radiotherapy, chemotherapy, and surgery, their accessibility is limited due to developing drug resistance, multiple recurrences, severe side effect, and so on [3]. For this reason, approaches that completely eliminate life-threatening lung and colon cancer still constitute a dynamic field of research [1, 2]. In recent years, natural products and their combinations have been evaluated as potential anticancer drugs. While natural products produced naturally by living organisms such as plants, animals, insects and microbes show special properties compared to traditional synthetic molecules, they can also increase their anticancer activities and chemosensitizing properties when

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used with synthetic molecules [4, 5]. Aloe barbadensis, known as Aloe vera (AVE), has many biological activities such as antiviral [6], antimicrobial [7], antitumor [8], anti-inflammatory [9], antioxidant [10, 11, 12], and has been used to accelerate the healing of wounds [12, 13, 14]. However, recent *in vitro* and *in vivo* studies have raised concerns about whether AVE prevents tumorigenesis due to its side effects [15]. Using AVE with other natural products can increase its effectiveness and reduce its side effects. One of the most important factors in cancer treatment is to create a treatment option with minimal side effects. In a study where royal jelly (RJ) and Cisplatin (CP) were used together, it was shown that RJ can protect liver tissue by reducing the toxic effects of CP [16]. RJ is a yellowish, creamy, highly acidic, water-soluble substance secreted by the jaw and pharynx glands of worker bees. Although all larvae are genetically identical, queen bees are formed from larvae fed only RJ, and worker bees are formed from other larvae [17]. RJ which shows antimicrobial [18], antioxidant [19], anticancer [20], and anti-inflammatory [21] properties thanks to the bioactive peptides in its content, is a health-improving nutritional supplement due to its low toxicity compared to other bee products [17, 22, 23]. It has been shown that RJ reduces cell viability when used with 5-fluorouracil (5-FU) [24], reduces DNA damage when used with cyclophosphamide [25], and induces apoptotic mechanisms of cells when used with thymoquinone [26]. The use of AVE and RJ may reduce the side effects of AVE, and the combination of AVE-RJ may exhibit higher antiproliferative activity by triggering apoptotic mechanisms in cancer cells. In our study, the effectiveness of the combined use of two natural products, AVE and RJ, on human lung cancer and colon cancer was investigated *in vitro*.

## Materials and Methods

### *Establishment of cell culture and preparation of test materials*

A549 (CCL-185TM) and HT29 (HTB-38TM) cells were cultured in DMEM high glucose (4.5 g/L) medium prepared by adding 10% FBS (Capricorn Scientific, Germany) and 1% Penicillin/Streptomycin (Capricorn Scientific, Germany) in cell culture flasks at 37°C and 5% CO<sub>2</sub>. Cells that had 80% coverage of cell culture flasks were removed with Trypsin/EDTA (Gibco, New Zealand). Cells stained with trypan blue at a ratio of 1:1 were transferred to a TC20 slide and counted with the TC20 Cell Counter (BioRad, USA). After the AVE leaves were dried on filter paper, the gel obtained was homogenized and centrifuged. After the reconstituted gel was stirred and filtered, the appropriate dose was obtained under low pressure. RJ was obtained from Macahel (Artvin, Turkey) and protected from light, and kept at 4°C. 10 gr RJ was dissolved in 10 mL PBS for 1 h. at 4°C to make 1 g/mL stock concentration.

### *Real time cell analysis*

Cells were seeded in triplicate, with 5000 cells per well, and 100 µL each in 96-well e-plates. In order to examine the effect of AVE and RJ on cells, AVE alone at 5, 10, 20 and 40 µg/mL doses, RJ 50, 100, 200 and 300 mg/mL alone

and each dose of the AVE and RJ combined, were applied to the cells. The special e-plates, which were seeded with cells, were incubated for 96 h. in an environment containing 37°C and 5% CO<sub>2</sub>, connected to Real-Time Cell Analyzer (xCELLigence) (Agilent, USA). Thanks to the gold electrodes located under the e-plates of the RTCA, the electrical impedance of the cells is measured and the cell index value is obtained by calculating this impedance with the software of the device. The viability, number and biological status of cells can be analyzed by the cell index value. In this experiment, the device recorded the proliferation status by measuring the cell indices every 30 min. Obtained data were analyzed with RTCA soft (ACEA Biosciences).

### *Apoptosis detection in flow cytometry*

Apoptosis after the interaction of the cells with the substance was detected using the Annexin V-FITC Apoptosis Detection kit. Cells that were seeded in T25 cell culture flasks at 5×10<sup>6</sup> cells/mL and treated with the substances were removed with Trypsin/EDTA and centrifuged at 1500 rpm for 5 min., and the obtained cell pellet was washed with PBS. After centrifugation again, the formed pellet was dissolved with 1 mL of 1X Binding Buffer and 100 µL of the suspension was taken into a microcentrifuge tube. 5 µL of PI and 1 µL of Annexin V-FITC were added to the tubes. After staining, the cells were kept on ice and in the dark for 15 min. and the data were recorded on the basis of FITC (Absorption 492 nm-Emission 520 nm) and PI (Absorption 370/ 550 nm-Emission 560-680) measurements in the Cytoflex Flow Cytometer (Beckman Coulter).

### *Wound healing assay*

A migration experiment was designed to observe the migration movements of A549 and HT29 cells after their interaction with the substances. 3.5×10<sup>4</sup> cells were seeded in each culture-insert (ibidi, GmbH, Germany) well and the culture-insert was removed after the cells had adhered. After adding 5 mL of DMEM, 100 mg/mL RJ was added separately, 20 µg/mL AVE separately, and RJ and AVE in combination. The effect of the substances on the cells after 24 h. was observed with an invert microscope (Zeiss Primovert, Germany).

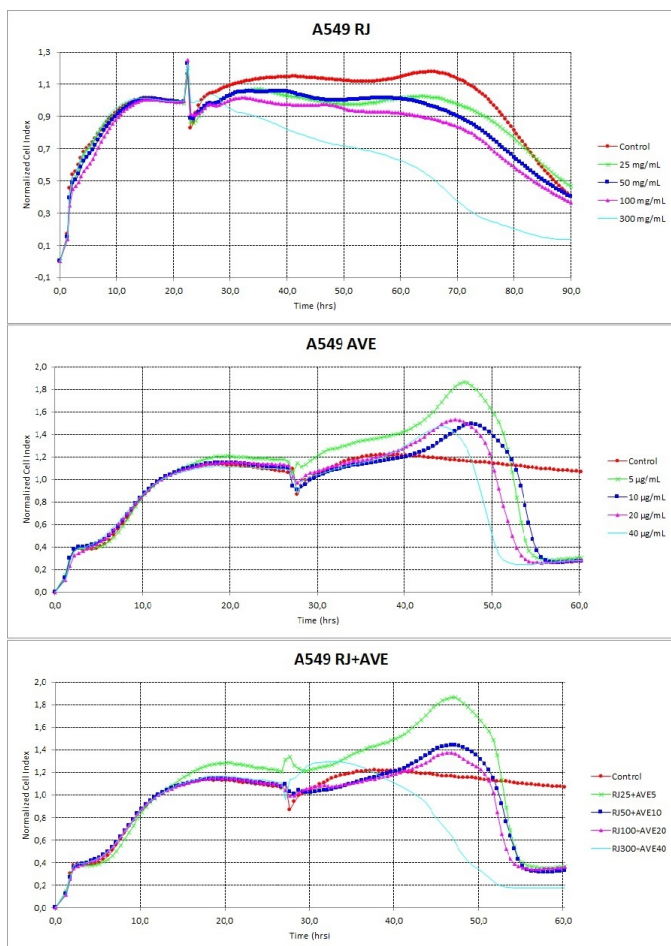
### *Statistical analysis*

All data collected from experiments were performed in three replicates and analyzed using the one-way analysis of variance (ANOVA). Post hoc analyses of group differences were performed using the Tukey test. Curves represent the mean Cell Index value from >3 wells ± SD. P value < 0.05 was considered statistically significant.

## Results

### *Antiproliferative effect of AVE and RJ alone and in combination on A549 and HT29 cells by Real-Time Cell Analyzer (xCELLigence)*

Figure 1 shows the concentrations of 25, 50, 100 and 300 mg/mL of RJ alone, 5, 10, 20 and 40 µg/mL of AVE alone, and the effects of combined doses of both substances on A549 cells. RJ started to show an antiproliferative effect



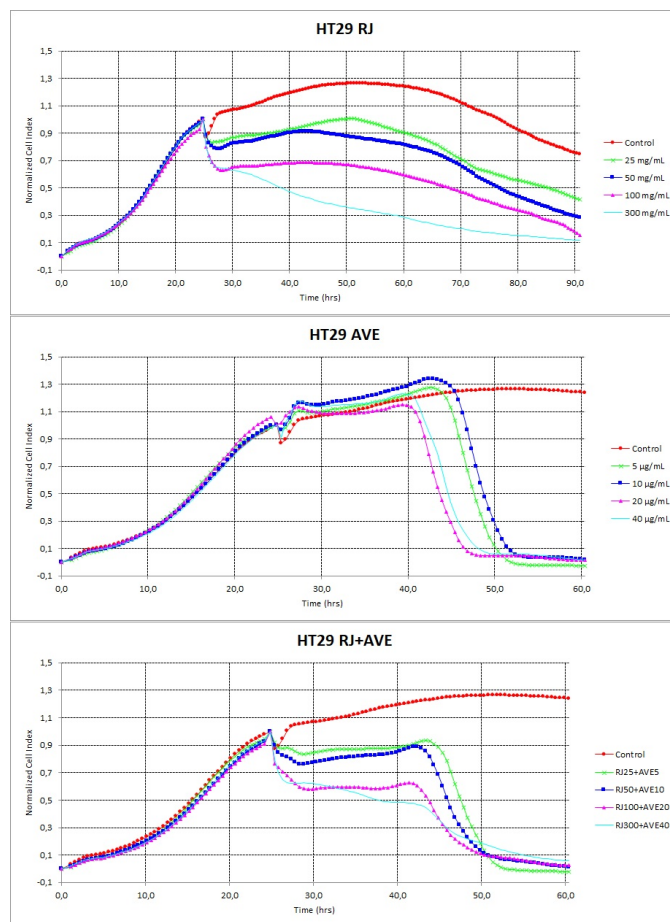
**Figure 1.** Detection of antiproliferative effects of RJ and AVE alone and in combination on A549 cells by RTCA. The effect of concentrations of 25, 50, 100 and 300 mg/mL RJ alone, 5, 10, 20 and 50 µg/mL AVE alone and the combined concentrations of the substances, respectively, on the proliferation of cells was observed ( $p < 0.05$ ).

40 h. after it was applied to the A549 cell and decreased the cell index value. The proliferation of A549 cells began to decrease significantly after 20 h. of treatment with AVE and after 30 h. the cell growth stopped completely. Combined doses of both substances showed an antiproliferative effect 20 h. after treatment. In particular, the combined use of 300 mg/mL RJ and 40 µg/mL AVE compounds clearly highlighted the decrease in proliferation. Furthermore, it was determined that the use of AVE together with RJ showed a higher antiproliferative effect than the antiproliferative effect of AVE alone ( $p < 0.05$ ).

According to the graphs observed in Figure 2, 30 h. after the application of the RJ, it showed influence on HT29 cells and reduced the proliferation of the cells. AVE, on the other hand, showed a marked antiproliferative effect 15 h. after it was applied with increasing concentration and lowered the cell index value. The two agents combined on HT29 cells rapidly reduced proliferation after 20 h.

#### Migration

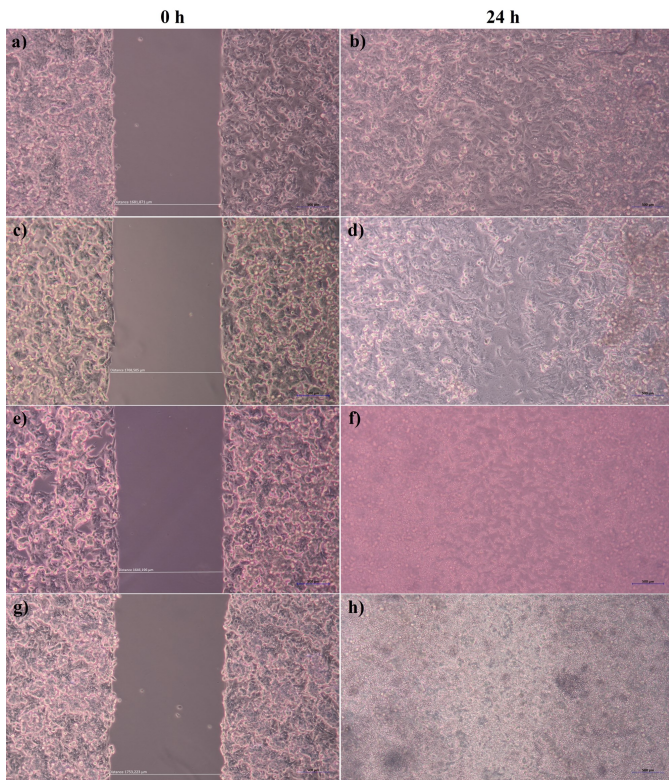
Migration status of cells was observed by applying doses of 100 mg/mL RJ, 20 µg/mL AVE and combination of both



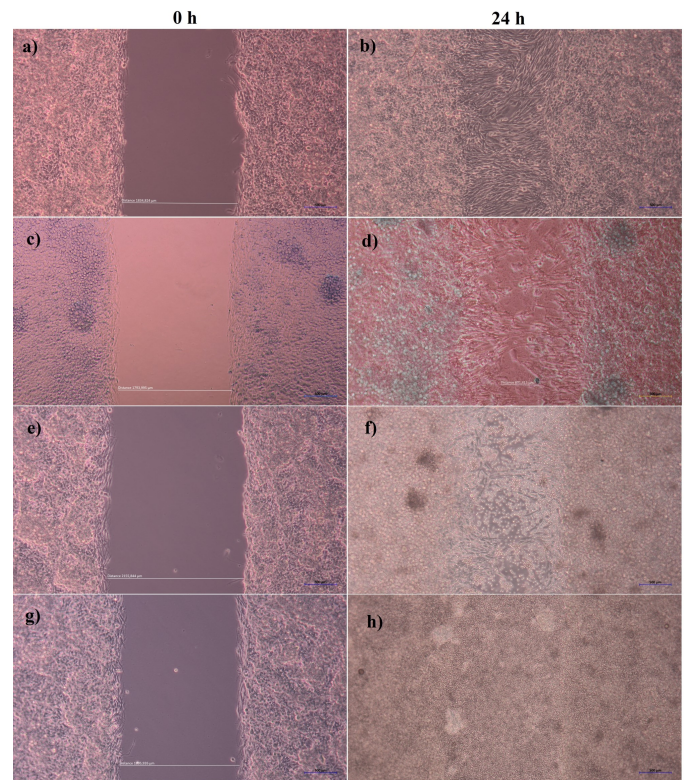
**Figure 2.** Detection of the antiproliferative effects of RJ and AVE alone and in combination on HT29 cells by RTCA. The effect of concentrations of 25, 50, 100 and 300 mg/mL RJ alone, 5, 10, 20 and 50 µg/mL AVE alone and the combined concentrations of the substances, respectively, on the proliferation of cells was observed ( $p < 0.05$ ).

substances detected on A549 and HT29 cell lines. When Figure 3 is examined, 100 mg/mL of RJ applied alone to A549 cells did not show anti-migratory properties after 24 h. and did not prevent the migration of cells. AVE given alone at 20 µg/mL did not stop the migration, but it was observed that it led the cells to apoptosis. The combination of the two substances with the same doses to the cells did not show an anti-migratory effect either.

As seen in Figure 4, 100 mg/mL RJ and 20 µg/mL AVE applied on HT29 cells did not show antimigratory properties alone, but did not prevent cell migration even when both of substance were applied. It was understood that substances cause HT29 colon cancer cells to apoptosis morphologically, but the antimigratory status of the substances was not detected. When all images were evaluated, it was determined that the use of RJ and AVE alone or together did not show antimigratory properties on A549 and HT29 cells.



**Figure 3.** Migration effect of RJ and AVE applied on A549 cells. (a-c-e-g), A549 cells, control (0h); (b), A549 cells, control (24h); (d), 24 hours after treatment of 100 mg/mL RJ to A549 cells; (f), 24 hours after treatment of 20 µg/mL AVE to A549 cells; (h), 24 hours after treatment of 100 mg/mL RJ + 20 µg/mL AVE to A549 cells.

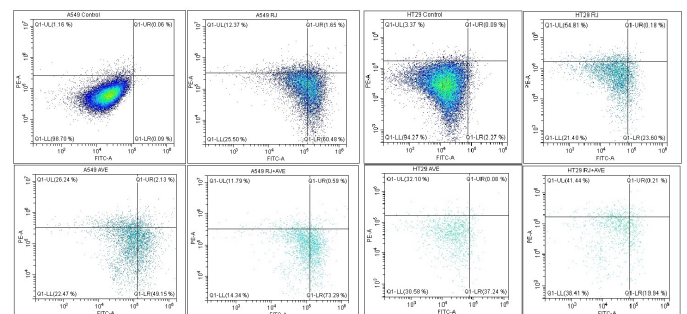


**Figure 4.** Migration effect of RJ and AVE applied on HT29 cells. (a-c-e-g), HT29 cells, control (0h); (b), HT29 cells, control (24h); (d), 24 hours after treatment of 100 mg/mL RJ to HT29 cells; (f), 24 hours after treatment of 20 µg/mL AVE to HT29 cells; (h), 24 hours after treatment of 100 mg/mL RJ + 20 µg/mL AVE to HT29 cells.

#### Flow cytometric determination of apoptosis in A549 and HT29

The apoptotic effect of the RJ and AVE, on the cells was evaluated in flow cytometry using the Annexin V-FITC staining method. As a result of flow cytometry analysis, viability, early apoptosis, late apoptosis and necrosis rates were determined 24 h. after the interaction of cells with substances. As seen in Figure 5, RJ was found to cause early apoptosis in 12.37% and necrosis in 60.48% of A549 cells. It was determined that while the AVE caused the cell to early apoptosis by 26.24% and necrosis by 49.15%, the combined use of the two substances decreased the rate of early apoptosis and increased necrosis. After examining the interaction of HT29 cells with RJ and AVE in flow cytometry, it was determined that RJ alone led HT29 cells to early apoptosis at a rate of 54.81% and necrosis at a rate of 23.60%, while AVE alone led cells to early apoptosis at a rate of 37.24%. The combined use of the two substances on cells increased the rate of early apoptosis to 41.44% and reduced the rate of necrosis to 19.94%. Therefore, the combined use of RJ and AVE reduced the necrotic rate caused by AVE, revealing the therapeutic effect of RJ.

When all flow cytometry analysis results were evaluated, the combined use of the two substances in A549 cells decreased the rate of early apoptosis compared to the use of RJ alone and AVE alone. When evaluated in the case of



**Figure 5.** Detection of apoptotic rates of cells analyzed in flow cytometry by staining with Annexin V-FITC 24 hours after treatment of A549 and HT29 cells with RJ, AVE and RJ+AVE ( $p < 0.05$ ).

necrosis, the combined use of substances increased the rate of necrosis considerably. Considering the apoptotic process of the interactions of HT29 cells with the substances, the combination of RJ and AVE increased early apoptosis compared to the AVE application alone. However, the combination of the two substances also reduced the rate of necrosis. It has been observed that the combined use of RJ and AVE may have curative properties on HT29 cells.

## Discussion

The remarkable chemical diversity in nature makes natural products a reservoir of bioactive compounds with therapeutic potential. Aloe vera, one of these natural products, has various biological activities, including antibacterial, anti-inflammatory, antiviral, antimicrobial and anticancer effects, as well as some side effects [27]. Although AVE and RJ alone have been shown to have anticancer activity [28, 29, 30, 31], the combination of AVE with RJ has not been evaluated in previous studies. The aim of this study is to determine the synergistic effect of the combination of AVE and RJ on A549 and HT29 cells. AVE is a medicinal plant known for its antidiabetic, anti-inflammatory, antibacterial and anticancer properties, and many of these medicinal properties have been attributed to the gel inside the leaf. However, these biological activities are thought to be due to the synergistic effect of all components rather than a single component in the gel. However, recent in vitro and in vivo studies have raised concerns about whether AVE prevents tumorigenesis due to its side effects. The use of AVE together with RJ can reduce the toxic effect of AVE while increasing its anticancer activity. In this study, AVE and RJ were applied together to non-small cell lung cancer and colorectal cancer cells and it was determined that the combination of AVE-RJ showed more effective antiproliferative activity in cells, induced early apoptosis and reduced the toxic effect. As shown in our research, RJ is a potent natural product that can reduce the toxic effects of AVE and may provide significant protection against AVE-induced necrotic cell death. Further research might explore the impact of RJ and AVE combination on other apoptotic and anti-apoptotic pathways in lung cancer and colorectal cancer.

## Conclusion

When all the results and findings were evaluated, it was understood that the RJ and AVE had anti-proliferative effects, but it was also observed that the compounds led the cells to early apoptosis. The combined use of RJ and AVE has been found to have a greater therapeutic effect on HT29 colon cancer cells than on A549 lung cancer cells.

## Ethical approval

It is a study that does not require an ethics committee.

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