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Research Article | Araştırma Makalesi

THE EFFECTS OF ROSEMARY (ROSMARINUS OFFICINALIS L.) EXTRACT ON THE PROLIFERATION AND APOPTOSIS OF A549 AND H1299 HUMAN LUNG CANCER CELLS

BİBERİYE (ROSMARINUS OFFICINALIS L.) EKSTRESİNİN A549 VE H1299 İNSAN AKCİĞER KANSERİ HÜCRELERİNİN ÇOĞALMASI VE APOPTOZU ÜZERİNDEKİ ETKİLERİ

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ABSTRACT

Objective: Apoptosis resistance and increased proliferation rates are characteristics of cancer cells. The anticancer properties of rosemary (*Rosmarinus officinalis* L.) extract (RE) have been demonstrated in a small number of *in vivo* and *in vitro* animal studies; however, no research has investigated the role of RE in human non-small cell lung cancer (NSCLC) A549 and H1299 cells, and its underlying mechanism of action remains unknown. In the current study, we examined the effects of RE on human non-small cell lung cancer cell proliferation, survival, and apoptosis.

Methods: NSCLC cell lines A549 and H1299 were incubated with (2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml, and 12.5 µg/ml) doses of RE for 12, 24, and 48 hours. MTT, Annexin V-PI, and caspase 3/7 assay kit were performed to detect the cell viability, apoptosis and necrosis.

Results: According to MTT analysis, the viability of A549 and H1299 human lung cancer cells was reduced by approximately 49.74% and 47.76%, respectively, for 24 hours by treatment with a dose of 5 µg/ml RE. The results of Annexin V-PI staining and Caspase 3/7 activation showed that RE had a greater effect on inducing cell death and necrosis.

Conclusion: In conclusion, we can say that rosemary extract has both apoptotic and antiproliferative properties on human lung cancer cells. We might propose that additional investigation is necessary to ascertain the therapeutic impacts of rosemary extract.

Keywords: Annexin V-PI, A549, rosemary extract, Caspase 3/7, H1299

ÖZ

Amaç: Apoptoz direnci ve artan proliferasyon oranları kanser hücrelerinin karakteristik özelliğidir. Biberiye (*Rosmarinus officinalis* L.) ekstresinin (BE) antikanser özellikleri, az sayıda *in vivo* ve *in vitro* hayvan çalışmasında gösterilmiştir; ancak BE'nin insandaki Küçük Hücreli Olmayan Akciğer Kanseri (KHDAK) A549 ve H1299 hücrelerindeki rolünü araştıran araştırma yoktur ve bunun altında yatan etki mekanizması belirsizliğini korumaktadır. Bu çalışmada BE'nin insandaki Küçük Hücreli Olmayan Akciğer Kanseri hücre hatlarının çoğalması, canlılığı ve apoptoz üzerindeki etkilerini araştırdık.

Yöntem: KHDAK hücre hatları A549 ve H1299, 12, 24 ve 48 saat süre boyunca (2,5 µg/ml, 5 µg/ml, 7,5 µg/ml, 10 µg/ml ve 12,5 µg/ml) BE dozlarıyla inkübe edildi. Hücre canlılığı, apoptoz ve nekrozu belirlemek için MTT, Annexin V-PI ve kaspaz 3/7 kiti kullanıldı.

Bulgular: MTT analizine göre, A549 ve H1299 insan akciğer kanseri hücrelerinin canlılığı, 5 µg/ml BE doz tedavisiyle 24 saat boyunca yaklaşık sırasıyla %49,74 ve %47,76 oranında azaldı. Annexin V-PI ve Kaspaz 3/7 aktivasyonunun sonuçları, BE'nin hücre ölümünü ve nekrozu indüklemede büyük bir etkiye sahip olduğunu gösterdi.

Sonuç: Sonuç olarak biberiye ekstresinin insan akciğer kanseri hücreleri üzerinde hem apoptotik hem de antiproliferatif özelliklere sahip olduğunu söyleyebiliriz. Biberiye ekstresinin terapötik etkilerini belirlemek için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: Annexin V-PI, A549, biberiye ekstresi, Caspase 3/7, H1299

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Introduction

The majority of cancer-related fatalities are caused by lung cancer.[1] Lung cancer includes non–small-cell lung cancer (NSCLC; approximately 85%) and small-cell lung cancer (SCLC; approximately 15% of all lung cancers).[2] Adenocarcinoma, large cell carcinoma, and squamous cell carcinoma are the three primary subtypes of NSCLC.[3] Despite the use of powerful chemo- and radiation therapy, less than 20% of people with NSCLC survive for five years. This disease is particularly aggressive. Research into novel therapeutic options for this aggressive kind of cancer is required because the resistance of NSCLC to existing medicines is a rising concern.[4]

According to Hanahan and Weinberg, cancer has six distinct characteristics: it continues to exhibit proliferative signals, avoids growth suppressors, resists cell death, permits replicative immortality, stimulates angiogenesis, and initiates invasion and metastasis.[5]

Plant-based natural materials have been screened to find numerous potential medicinal compounds. Certain medications, such as etoposide, which is derived from the mandrake plant and Queen Anne's lace, and docetaxel and paclitaxel, which are derived from the bark and wood of the Nyssaceae tree, are presently being effectively used in the treatment of cancer.[6]

The Mediterranean region is home to the plant *Rosmarinus officinalis* L., a member of the Lamiaceae (mint) family with a diversity of medicinal and culinary uses. The primary polyphenols present in rosemary extract (RE) are rosmarinic acid (RA), carnosol (CN), and carnosic acid (CA).[7]

Diterpenic compounds of the plant are recognized to have a broad range of biological activities, containing antifungal,[8] antibacterial,[9] anticancer,[10] antioxidant,[11] antiangiogenic,[12] anti-inflammatory,[13] and chemoprotective.[14] Additionally, rosmarinic acid has a broad range of biological properties, the most notable of which are anti-inflammatory,[15] anti-oxidative,[16, 17] anti-apoptotic,[18] antifibrotic,[19] and neuroprotective.[20]

Among other health advantages, plants high in polyphenols have drawn a lot of interest for their anticancer capabilities. The investigation of novel and potentially useful mechanisms of action, in addition to new chemical classes of anticancer drugs, is possible through the study of natural products.[21]

It has been discovered that RE and a few of its polyphenol constituents, such as CA, RA, and CN, have strong anticancer properties. Research employing cancer cells from the breast,[22-24] pancreas,[25] prostate,[26] and liver[24,27] has demonstrated that treatment with RE inhibits the growth and viability of cancer cells and induces apoptosis.

A small number of research have investigated how RE administration affects tumor growth *in vivo* in animals. Inhibitory effects have been reported in myeloid leukemia, colon, prostate, and skin cancer models.[26, 28-31]

In this study, we aim to investigate the effects of rosemary extract on human non-small cell lung cancer (NSCLC) A549 and H1299 cell proliferation, survival, and apoptosis.

Methods

Plant Material

The aerial parts of *Rosmarinus officinalis* were used in the study. Plant samples were collected during the flowering stage from Türkiye. Plant samples were kept in the shadow and dried. A 70% ethanolic extract was prepared from the plant material by sonication. The extract was then filtered and concentrated. The dried extract was weighed and stored at +4°C until further study.

Cell Culture

Human non-small-cell lung cancer cell lines H1299 (CRL-5803) and A549 (CCL-185) were provided by the ATCC (Manassas, VA, USA). The A549 and H1299 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, USA) and RPMI-1640 medium (Thermo Fisher Scientific, USA), respectively. The cultures were then placed in an incubator at 37°C in 5% CO_2 and supplemented with 1% penicillin- (Capricorn Scientific, Germany) and 10% fetal bovine serum (Capricorn Scientific, Germany). The cells were placed in ninety-six-well plates and six-well plates for cell viability and flow cytometry, respectively, after being trypsinized with 0.25% trypsin at 80-90% confluence.

An Assessment of Cell Viability Using the MTT Assay

The effects of various doses (2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml, and 12.5 µg/ml) of rosemary extract [32] on human lung cancer cells H1299 and A549 were assessed over 12, 24, and 48 hours using the MTT cell viability test. H1299 and A549 cell lines ($5x10^3$ cells/well) were seeded into 96-well plates including medium and incubated overnight. After incubation, RE at different concentrations was diluted in cell culture medium and added to the dishes. Plates were incubated for 12, 24, and 48 hours at 37 °C in 5% CO_2. The fresh medium and 15 µl of MTT solutions were added and incubated for four hours under the same circumstances. Then, 100 µl isopropanol–HCl to dissolve the formed dark blue formazan crystals was applied and incubated for half an hour in a dark condition. The untreated cells served as the control. Epoch Microplate Reader (Winooski, USA) was used to read the wells at 570 nm. Each assay was performed three times. We used GraphPad Prism software (San Diego, CA, USA) to analyze the data and determine the inhibitor doses (IC_{50}) required to achieve 50% inhibition of cell viability.

Flow Cytometry

Annexin V-PI Analysis

A549 and H1299 cells adhered to the plate were detached with trypsin-EDTA (Capricorn Scientific, Germany). Then, all cells were collected, and washed with phosphate-buffered saline (PBS), and the concentration was set to 1 x 10^5 cells in 100 µl. The cell

solution was put into 12 x 75 mm polystyrene tubes, and 1X Annexin Binding Buffer, 5 µl of Annexin V-FITC, and propidium iodide (PI) were added. After an incubation period of 15 minutes at rt, the cells were examined using an ACEA NovoCyte (USA) flow cytometry device.

Caspase 3/7 Activity Assay

In 6-well plates, H1299 and A549 cell lines were first seeded at a density of 5 x 10^5 cells per well and left to culture for the entire night. After that, the cells were treated with rosemary extract and incubated at 37 °C for 24 hours. After incubation, cells were collected in 0.5 ml of warm medium and incubated with a caspase 3/7 detection reagent for 1 hour at 37 °C. The cells were then rinsed and suspended in 0.5 milliliters of assay buffer. In the cells, caspase 3/7 activity was assessed using the NovoCyte D3000 flow cytometry instrument (USA). The process was used to determine the activation levels of caspases 3/7, which are indicators of the induction of apoptosis in the treated cells.

Statistical Analysis

The statistical analysis was assessed using version 8.4.2 of GraphPad Prism (San Diego, CA, USA). Two-way ANOVA and Tukey's test for the multiple comparisons of means were used to assess the significance of the data between the groups. The statistical significance level was accepted as $p < 0.05$.

Results

Cytotoxic Effects of RE on A549 and H1299 Human Lung Cancer Cells

The effects of rosemary extract on cell viability at various concentrations and durations are demonstrated in Figure 1. Rosemary extract was treated at 0, 2.5, 5, 7.5, 10, and 12.5 µg/ml doses for H1299 and A549 human lung cancer cells. The incubation times were 12, 24, and 48 h. As shown in Figure 1 rosemary extract demonstrated potent cytotoxicity against human lung cancer cell lines, A549 and H1299 with IC_{50} being at 5.041 µg/ml and 4.151 µg/ml respectively for 24 h (Figure 1). Furthermore, according to MTT analysis, the viability of A549 and H1299 human lung cancer cells was reduced by approximately 49.74% and 47.76%, respectively, for 24 hours by treatment with a dose of 5 µg/ml RE.

The Impact of RE on Cell Viability, Apoptosis, and Necrosis in A549 and H1299 Cells

The Annexin V-PI assay was carried out following rosemary extract administration to determine which stage of apoptosis is mostly caused by the extract. A549 and H1299 human cell lines were treated with 4 µg/ml and 5 µg/ml rosemary extract for 24 hours. As a control, untreated cells were used. It was found in both A549 and H1299 cell lines treated with 5 µg/ml RE, reduced cell viability, induced apoptosis and necrosis compared to the control and 4 µg/ml RE dose (Figure 2).

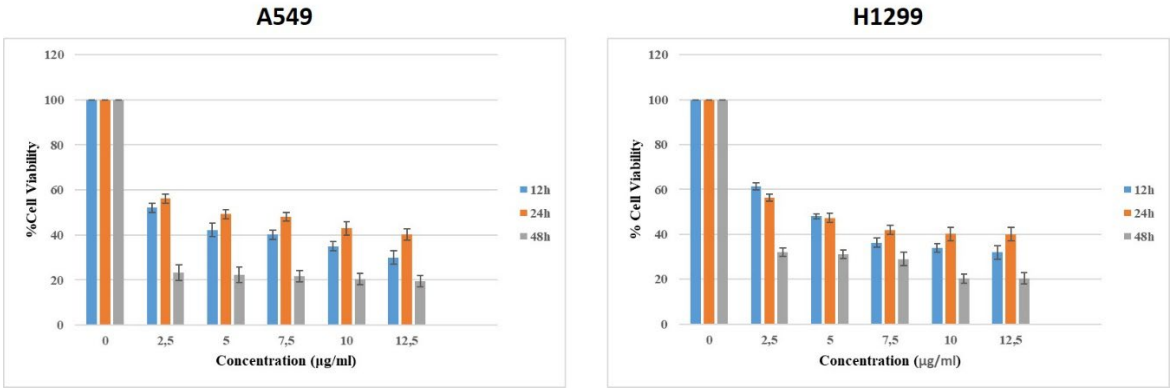

Figure 1. The effect of different concentrations of rosemary extract on the cell viability of A549 and H1299 human lung cancer cells. RE reduces cell viability in A549 and H1299 human lung cancer cells. All experiments were performed three times.

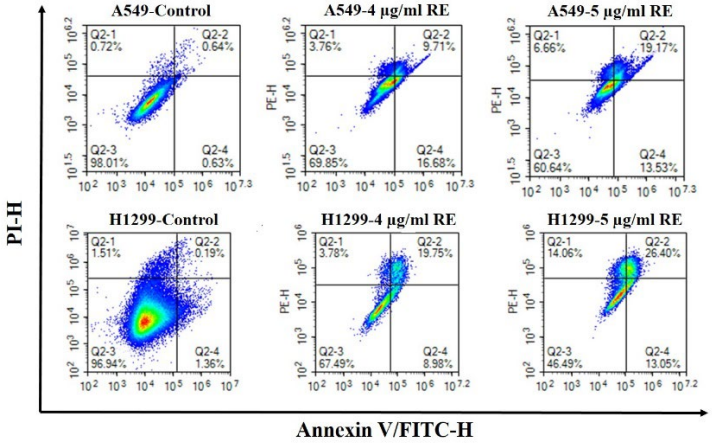

Figure 2. The effect of RE on apoptosis, and cell viability in A549 and H1299 lung cancer cells. The percentages of early and late apoptotic cells, necrotic cells, and cell viability were assessed by flow cytometry. Annexin V-PI staining was applied following RE treatment in A549 and H1299 lung cancer cell lines

Percentages of necrotic cells, late and early apoptotic cells, and cell viability were assessed by flow cytometry. Annexin V-PI staining was performed after RE treatment at 4 µg/ml and 5 µg/ml in H1299 and A549 cells. As a control, untreated cells were used. The four groups (Q2-1, Q2-2, Q2-3, and Q2-4) represent necrosis (Annexin V-negative/PI-positive), late apoptosis (Annexin V-positive/PI-positive), cell viability (Annexin V-negative/PI-negative), and early apoptosis (Annexin V-positive/PI-negative) that are identified by flow cytometry.

In comparison with the control group, it was determined that cell viability gradually reduced as the dose increased and there was a decrease, especially in the groups given 5 µg/ml RE both in A549 and H1299 cell lines**.** Furthermore, the apoptotic and necrotic cells were increased with the treatment of 5 µg/ml RE compared to the control and 4 µg/ml dose of RE (Figure 3).

Assessment of Caspase 3/7 assay

One accurate indicator of apoptosis is the activation of caspase 3/7. This assay measures caspase 3/7 activity to determine the composition and percentage of cells in various phases of apoptosis. A549 and H1299 lung cancer cell lines, including both 4 µg/ml and 5 µg/ml RE treated and untreated control cells, were subjected to caspase 3/7 assessment.

Similar to Annexin V results especially at doses of 4 µg/ml and 5µg/ml rosemary extract increased caspase activation compared to the control group at 24 hours in A549 and H1299 cells. (Figure 4).

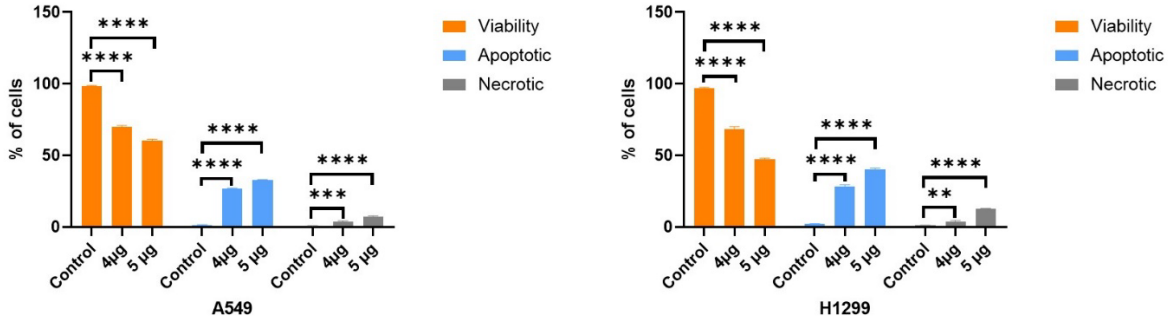

Figure 3. RE stimulates apoptosis in A549 and H1299 cell lines. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

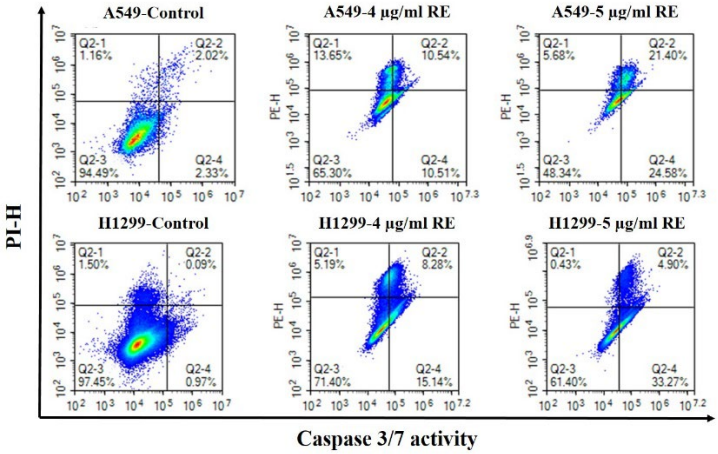

Figure 4. Caspase 3/7 activation as an apoptotic marker in human lung cancer cell lines A549 and H1299 Caspase 3/7 activities in these cells were evaluated by flow cytometry.

Compared to control group, it was determined that apoptosis or caspase 3/7 activation was increased according to doses. It was determined that 5 µg/ml rosemary extract treatment has the highest caspase 3/7 activation in both H1299 and A549 cells (Figure 5).

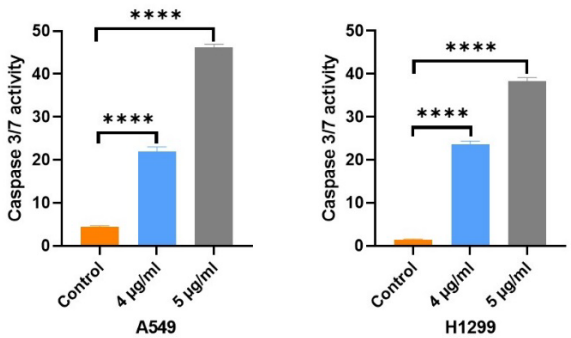

Figure 5. Rosemary affects caspase 3/7 activities in A549 and H1299 cells. RE affects caspase 3/7 activities significantly in two cancer cell lines. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

Discussion

Plants are the source of a number of drugs used in the treatment of cancer.[33,34] High polyphenolic plant-derived extracts have also demonstrated anticancer properties, such as green tea,[35] rosemary extract,[36] and specific polyphenols like quercetin,[37] oleuropein,[38] etc.

Following foodomics techniques, the antiproliferative action of polyphenol-rich extracts from rosemary has been shown in a variety of cancer cell lines in recent years.[39]

Rosemary's phytochemical components, which include phenolic acids (e.g., chrologenic acid caffeic acid, rosmarinic acid, gallic acid), triterpenes (e.g., oleanolic acid, ursolic acid, betulinic acid), and flavonoids (e.g., salvigenin, genkwanin, apigenin, scutellarein, cirsimaritin) are responsible for these health-promoting qualities.[40] The anti-neurodegenerative, antioxidant, antibacterial, anti-inflammatory, hypolipidemic, hypoglycemic, hypotensive, antiatherosclerotic, anticancer, and antimutagenic qualities of rosemary have been shown in both *in vivo* and *in vitro* studies.[41]

Rosemary (*Rosmarinus officinalis*) is a well-liked herb in both culinary and traditional medicine. It possesses pharmacologic properties for cancer treatment and chemoprevention, according to recent studies. Tai et al. assessed whether RE and its three primary active ingredients carnasol (CS), rosmarinic acid (RA), and carnosic acid (CA) could increase the antiproliferation activity of cisplatin (CDDP), and assess the antiproliferative activity of RE against human ovarian cancer cells. Utilizing human ovarian cancer A2780 and its CDDP-resistant daughter cell line A2780CP70, they demonstrated that RE had strong antiproliferation action, with IC_{50} (50 percent inhibitory concentration) measured at 1/1000 and 1/400 dilutions, respectively. With CDDP, RE improved the antiproliferation effect on A2780 and A2780CP70 cell lines. They have shown that by altering the cell cycle at several stages, RE suppressed the growth of ovarian cancer cells.[42]

The antiproliferative ability of rosemary cell lines against human HT-29 colorectal cancer cell line has been examined by Urquiza-López et al. Three rosemary cell line cultures were established: green (RoG), yellow (RoY), and white (RoW). Cell aggregates were sorted based on color. After 48 hours of treatment with the RoW extract (IC_{50} of 49.63 µg/ml), the antiproliferative activity test against HT-29 colon cancer cells using the MTT assay showed that the viability of the HT-29 cells was impaired.[43]

The most prevalent cancer diagnosed in men in North America is prostate cancer, which is usually categorized as androgen receptor positive or negative based on androgen receptor (AR) expression. Hormone therapy is a treatment option for AR-positive prostate cancer; however, AR-negative prostate cancer is aggressive and has no response to hormone therapy. The research in the literature has indicated that RE possesses anti-inflammatory, anti-cancer, and antioxidant properties. Jaglanian et al. discovered that administering RE to the androgen-insensitive PC-3 prostate cancer cell led to a notable suppression of Akt, mTOR signaling, migration, proliferation, and survival. These results imply that RE has strong anti-prostate cancer properties. Aside from cell proliferation, RE treatment resulted in a dose-dependent inhibition of cell survival with IC_{50} values of 4.17 µg/ml and 2.43 µg/ml for 22RV1 and PC-3 cell lines, respectively. [44]

The most prevalent malignancy in women to be diagnosed is breast cancer. Chemotherapy agents have been established in part because of compounds originating from plants. The impacts of rosemary extract on TN MDA-MB-231 cells survival/apoptosis, proliferation, mTOR, and Akt signaling were investigated by Jaglanian and Tsiani. In a dose-dependent manner, RE impeded the proliferation and survival of the MDA-MB-231 cells. Moreover, RE promoted the cleavage of PARP, a hallmark of apoptosis, and reduced the phosphorylation/activation of Akt and mTOR. According to their research, RE affects important signaling molecules contained in cell proliferation and survival and possesses strong anticancer capabilities against TN breast cancer.[45]

The spread of melanoma skin tumors is fast expanding worldwide, and their high resistance to cytotoxic agents contributes to their malignancy. Therefore, novel cytotoxic medication treatments would be highly beneficial in improving the prognosis of melanoma. The impact of a rosemary hydroalcoholic extract on the survival of the human melanoma A375 cell was examined by Cattaneo et al. Using MTT and Trypan blue tests, the impact of the crude extract or purified components on the growth of cancer cells was examined. Cell growth was hindered by rosemary extract in a time- and dose-dependent manner. Extract dilutions at ratios of 1:120, 1:240, and 1:480 significantly decreased cellular metabolic activity. The anti-proliferative impact was visible as early as 24 hours and was strengthened at 48 and 72 hours. After a 72-hour incubation period, the estimated IC_{50} was 1:480. [46]

As mentioned above, there is some research in the literature about the anticancer effects of rosemary extract on several cancer cell lines however, there was a limited study focused on lung cancer cells. In the current study, we examined the possible effects of RE on human lung cancer cell lines A549 and H1299. Firstly to select the inhibition concentrations of rosemary extract, an MTT assay was used to verify the cell survival of H1299 and A549 human lung cancer cells after being treated with 2.5, 5, 7.5, 10, and 12.5 µg/ml of rosemary extract. The durations of incubation were 12, 24, and 48 hours. The strong cytotoxicity of rosemary extracts against the human lung cancer cell lines A549 and H1299 with IC_{50} values of 5.041 µg/ml and 4.151 µg/ml, respectively, over a 24-hour period.

Apoptosis is a normal physiological mechanism of cell death that removes undesired cells while preserving tissue equilibrium. Additionally, pathological circumstances and extreme stress can cause it to happen.[47]

Two natural extracts, high in carnosic acid and rich in curcuminoid compounds, turmeric root extract (TE) and rosemary leaf extract (RE) were tested *in vitro* by using Annexin V and caspase 3/7 experimental protocols. Using the identical extract quantities and experimental setup, the researchers discovered that TE alone was a more potent cell therapy than RE alone. Caspase 3/7 activation and Annexin V staining demonstrated that TE had a higher effect on triggering cell apoptosis and a similar result was obtained from the combination treatment with just half the concentration of each extract.[48]

To ascertain which stage of apoptosis the rosemary extract mostly causes, the Annexin V assay and caspase 3/7 assays were performed after the extract was administered. It was discovered that A549 cells treated with 5 µg/ml rosemary extract induced apoptosis and necrosis also reduced cell viability. In the current study, we found a dose-dependent inhibition of A549 and H1299 human lung cancer cell proliferation with rosemary extract treatment.

It has been reported that rosemary extract decreases Akt/mTOR/p70S6K activation and inhibits the survival and proliferation of A549 human lung cancer cells. [21] Similarly an experimental study has shown that inhibition of non-small cell lung cancer survival and proliferation by rosemary extract is related to the activation of AMPK and ERK.[49] In the present study, we observed that rosemary extract has both apoptotic and antiproliferative properties on human lung cancer cells. Further research is required in the future to completely elucidate the molecular mechanisms.

In an experimental study, MG-63 bone osteosarcoma cell line viability was importantly reduced with increasing concentration of analyzed extract (beyond 300 µg/mL for rosemary dry extract). Their findings indicated that apoptosis is one of the basic mechanisms included in the cytotoxic properties of the analyzed extract.[50] In another study in CT-26 mouse colorectal cancer cells, the authors indicated that ginger and rosemary could induce cell death by early apoptosis.[51] In the study by Jang et al., the findings demonstrated that rosmarinic acid (RA) mainly boosted the number of cells in late apoptosis and necrosis, while suberoylanilide hydroxamic acid (SAHA) mainly boosted the number of cells in early apoptosis in the DU-145 cell line. Also, in the PC-3 cell line, RA boosted the number of early apoptotic cells, and SAHA boosted the number of late apoptotic cells compared to NC (DMSO).[52] Likewise the literature, we also examined the effects of rosemary extract on H1299 and A549 human lung cancer cells and found the early and late apoptotic index by using flow cytometry.

In summary, we may say that rosemary extract has both antiproliferative and apoptotic effects on human lung cancer cells. More research is needed to fully understand the medicinal potential of rosemary extract. Furthermore, the effect of RE noticed in human lung cancer cells brings out the possibility of using rosemary as a supplement/addition to existing treatments in high-grade tumors, which have limited medical treatment but can be treated with surgical intervention. We suggest that further research (such as *in vivo* studies) is needed to evaluate rosemary extract's therapeutic effects.

Compliance with Ethical Standards

No ethical approval was required, as this was an *in vitro* cell line study.

Conflict of Interest

There are no relevant conflicts of interest for the authors of this article.

Author Contribution

EA, BK, AFC: Study idea, study design, hypothesis; EA, BK: Literature Search; EA, TOS, SAE: Material preparation, data collection, and analysis; EA, TOS: Cell culture experiments; EA, BK, TOS: Writing the first draft of the article; EA, SAE: Reviewing and Editing. All the authors read and approved the final manuscript.

Financial Disclosure

Financial disclosure none.

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