European Journal of Biology
Eur J Biol 2024; 83 (1): 50–59
DOI: 10.26650/EurJBiol.2024.1353842

RESEARCH ARTICLE

Induction of Apoptosis through Oxidative Stress Caused by *Rubus tereticaulis* Leaves Extracts in A549 Cells

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ABSTRACT

Objective: Plants have been used for medicinal purposes since the beginning of human history and form the basis of modern medicine, and they are also the source of most chemotherapeutic drugs for cancer treatment. This study aims to investigate for the first time the cytotoxic and apoptotic effects of the active ethanol (RTE) and chloroform (RTC) extracts of *Rubus tereticaulis* leaves in the A549 non-small-cell lung cancer cell line.

Materials and Methods:A549 cells were treated with RTE and RTC individually. The MTT assay was used to quantitatively detect RTE and RTC's cytotoxic effects. The fluorescent signal indicator H2DCF-DA was used to detect cellular reactive oxygen species (ROS) production. Apoptosis was evaluated by fluorescence microscope after acridine orange/ethidium bromide fluorescent staining, annexin V-FITC and immunoblotting analyses, immunofluorescence, and imaging.

Results: Both RTE and RTC induced cytotoxicity in A549 cells in a dose-dependently, which was accompanied with induced ROS accumulation. Both early and late apoptotic cells detected by flow cytometry were increased in the RTE- and RTC-treated cells. In addition, the results show RTC to have higher cytotoxic and apoptotic effects and increased ROS-generation capacity than RTE. Therefore, the polarity of the solvent used to exert the anticancer effect of *R. tereticaulis* leaves is crucial.

Conclusion: This is the first anti-cancer activity study on *R. tereticaulis*. The results suggest *R. tereticaulis* leaves to have an anti-cancer effect on lung cancer cells through ROS-mediated apoptosis and RTC to be an effective therapeutic/adjuvant strategy in cancer treatment.

Keywords: Lung cancer, Anti-cancer effect, Oxidative stress, *Rubus tereticaulis*, Apoptosis

INTRODUCTION

One of the leading causes of cancer-related deaths worldwide is lung cancer. Lung cancer affects 1.8 million people worldwide each year and claims 1.6 million lives. Non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) are the two main kinds of lung cancer and respectively cause approximately 85% and 15% of all newly discovered lung cancers.[1,2]

The best course of treatment for a patient with lung cancer depends on the stage at which they are discovered to have this dreadful disease. It typically entails surgical resection followed by chemotherapy and/or radiotherapy.[3] The main treatments for lung cancer involve chemotherapeutic medications such as paclitaxel, 5-fluorouracil (5-FU), docetaxel, cisplatin, and gemcitabine. The effectiveness of many chemotherapeutic treatments has been significantly reduced in lung cancer due to the emergence of drug resistance strains.[4-6] Additionally, the chemotherapeutic approach to treating lung carcinomas frequently results in serious off-target side effects. In order to replace the widely used chemotherapeutics, a worldwide push has occurred for researching alternative medicines with higher tolerance profiles, such as natural products.

Many cancer cells have a prolonged increase in the creation of intrinsic reactive oxygen species (ROS) during malignant transformation, which sustains the oncogenic phenotype and promotes tumor growth. Redox adaptation enables cancer cells to increase survival and build resistance to anti-cancer medicines by upregulating anti-apoptotic and antioxidant chemicals.[7]

Plants have long been employed for their numerous health

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Submitted: 01.09.2023 • **Revision Requested:** 03.11.2023 • **Last Revision Received:** 26.02.2024 • **Accepted:** 01.03.2024 • **Published Online:** 02.04.2024

advantages. Around 80%-85% of people globally rely on conventional plant-based medicines for their medical requirements. Many plant extracts, isolated chemicals, and their equivalents have been employed as powerful anticancer medications, and research into the medicinal qualities of substances derived from plants has grown in popularity.[8] The antioxidant, anti-cancer, antibacterial, antiviral, and anti-inflammatory effects of phenolic compounds found in plants are well documented.[9] Research on medicinal plants and their polyphenol-rich extracts is crucial. As a result, the need exists for new products with fewer negative effects than those currently in use.

The genus Rubus is a member of the Rosaceae family that has 740 species growing naturally in the world, with 10 taxa in Turkey.[10] *Rubus* species are used internally or externally in traditional folk medicine to treat various diseases.[11] Flavonoids (e.g., quercetin, kaempferol) and phenolic acids (e.g., caffeic acid, chlorogenic acid) have been found in phytochemical investigations of *Rubus* species. The presence of catechins, pectins, carboxylic acids, anthocyanins, vitamin C, and saturated as well as unsaturated fatty acids has also been shown in prior investigations.[12-14] Only two previous studies by some of this team's researchers are found on the phytochemical content and bioactivity (i.e., antioxidant, anti-inflammatory, anticolitis, and wound healing activities) of *Rubus tereticaulis*. One of these studies reported *R. tereticaulis* ethanol extract to contain quinic acid, 5-caffeoylquinic acid, quercetin pentoside, quercetin glucoside, quercetin-3-O-β-D-glucuronide, kaempferol-3-O-β-D-glucuronide, and kaempferol rutinoside. In addition, the total phenol and flavonoid amounts in this species were calculated and revealed to show antioxidant, anti-inflammatory, and anticolitis activity. The compound responsible for these activities has been shown to be quercetin-3-O-β-D-glucuronide, which is the major compound in the extract, along with other phenolic compounds.[14] The second study reported *R. tereticaulis* to show wound healing activity.[15]

The aim of this study is to investigate for the first time the cytotoxic and apoptotic effects of *R. tereticaulis* leaves' active ethanol (RTE) and chloroform (RTC) extracts in the A549 lung cancer cell line.

MATERIALS AND METHODS

Plant Material and Extraction

R. tereticaulis leaves in the flowering period were collected from the Şile region of Türkiye's Istanbul province on June 15, 2016 and identified by Dr. Ahmet Dogan. Voucher specimens were kept in the herbarium of the Marmara University Faculty of Pharmacy (MARE No. 18573). RTE and RTC extracts from the *R. tereticaulis* leaves had been obtained in the previously reported study[14] and stored at 4° C until analysis.

Cell Culture

The A549 cells was purchased from the American Type Culture Collection (ATCC, USA), maintained in DMEM/F12K, and supplemented with 10% fetal bovine serum (FBS), 1% 100 units/mL of penicillin, and 1% 100 µg/mL of streptomycin. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were grown in 75 cm^2 culture bottles.

The study uses the RTE and RTC extracts as experimental groups. Before the cell culture studies, the extracts were dissolved in a 1% dimethyl sulfoxide solution and then diluted with 1X phosphate buffer saline (PBS). The control group is A549 cells exposed to the 0.1% (v/v) dimethyl sulfoxide solution that had not been treated with any extract.

Cell Viability Assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) test (Sigma) was used to assess cellular metabolic activity in a 96-well plate. The A549 cells were plated in wells at a density of 5 x 10^4 cells each, with the cells then treated with increasingly greater concentrations (25-600 µg/mL) of the RTE and RTC samples at 37 °C for 48 h. After incubation, 10 µL of the MTT solution (5 mg/mL in sterile PBS) and 100 mL of fresh DMEM were added to each well and incubated 4 h. The dimethyl sulfoxide (DMSO) solution was then added to 100 µL of a dissolving buffer and incubated for 15 min at room temperature. The optical density was read at 570 nm on a microplate reader to ascertain the MTT reaction in cells (Varioskan Flash Spectral Scanning Multimode Reader, Thermo Scientific). Cell viability was calculated as a percentage of the controls.[16]

Measuring ROS Levels

The fluorescent signal indication 2,7 dichlorodihydrofluorescein diacetate was used to measure intracellular ROS generation (H2DCF-DA). After being oxidized by ROS in the media, the colorless H_2DCF-DA transforms into green luminous dichlorofluorescein (DCF). A fluorescent link is found between elevated ROS levels and the fluorescence that is released.[17]

After 24 h of incubation, 1x10^4 cells seeded on 96 opaque black plates were given RTC and RTE. These were then incubated for 48 h in accordance with the experimental groups. The media were aspirated and given three washings in 1xPBS following incubation. An incubator was filled with 100 µl of 10 µM H2DCF-DA produced in ddH_2O, and the mixture was then incubated at 37 °C. A fluorescence plate reader equipped with an Ex:488nm/Em:525nm laser was used to measure the DCF fluorescence intensity that resulted during incubation (Varioskan Flash Multimode Reader, Thermo Scientific). MTT was compared to the control group with 0.1% DMSO added, and the results were determined using the ratio of ROS:MTT.[16]

Dual Acridine Orange/Ethidium Bromide (AO/EB) Staining

The dyes AO/EB are unique to DNA. McGahon et al. invented the dual AO/EB staining method,[18] which allows one to distinguish among viable, apoptotic (early or late stages), and necrotic cells by combining the differential uptake of fluorescent DNA binding dyes of AO and EB with the morphological feature of the chromatin condensation in the stained nucleus. Both live and nonviable organisms absorb the acridine orange, which then either intercalates into double-stranded nucleic acids (mostly DNA) or binds to single-strand nucleic acids to produce red fluorescence (RNA). Ethidium bromide only enters nonviable cells and intercalates into DNA to produce red fluorescence. A viable cell would therefore have an orange cytoplasm and a uniform bright green nucleus. An early apoptotic cell, whose membranes are still intact but have begun to cleave its DNA, would still have a green nucleus but exhibit bright green patches of condensed chromatin. A late apoptotic cell would exhibit bright orange areas of condensed chromatin in the nucleus (EB predominates over AO). A necrotic cell would have a uniform bright orange nucleus. When stimulated at 480–490 nm by living cells, AO diffuses into dsDNA and distributes green fluorescence. In a nutshell, 6-well plates were seeded with 2×10^5 cells/well and incubated for 24 h. After that, another 24 h of incubation occurred with the addition of RTC and RTE below the IC_{50} values. After being treated with RTC and RTE, the cells were taken out and cleaned with PBS before being stained with a 1:1 mixture of AO/EB (100 µg/mL; CAT: 235474/E8751-Sigma Aldrich, USA). Using a fluorescent microscope, the incidence of apoptotic chromatin condensation was measured and scored for three replicate samples, each containing one hundred cells (Leica DM 1000, Solms, Germany).

Annexin V-FITC Analysis

For the purpose of detecting apoptosis, the manufacturer's instructions for the Annexin V-FITC staining kit (eBioscience, Thermo Fisher Scientific, USA) were followed. In brief, the A549 cells were seeded onto six-well plates at a density of 1.5 x 10^5 cells per well and left to adhere overnight before being treated for 24 h with IC_{50} doses of RTC and RTE. The trypsin-digested cells were centrifuged for five minutes at 200× rpm. Resuspended in 100 µL Annexin V-FITC labeling solution, the cell pellet was then incubated at 15–20°C for 10–15 min. Immediately after, it was analyzed using flow cytometry (Becton Dickinson, FACS Canto II) at 525 nm for emission and 488 nm for excitation.

Immunoblotting Analysis

The A549 cells were seeded on 6-well plates at 1.5×10^5 per well, and the plates were cultured for a full day. Then, based on their IC_{50} values, they received treatment with RTC and RTE. The NP-40 cell lysis buffer (2 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, and 0.2% NP-40 plus a protease inhibitor cocktail) was used to harvest and prepare the cells after 24 h of incubation. The cells were centrifuged at 14,000× rpm (Beckman Coulter, Krefeld, Germany) for 10 min at 4°C. Next, the cytosolic fraction was made from the final supernatant. The Bradford protein assay method was used to calculate the supernatant's protein concentration. Proteins from cellular supernatants were separated on 8%-10% polyacrylamide gels and then moved using the trans-blot SD semipermeable electrophoretic transfer cell to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked using Tris-HCl-buffered saline with Tween 20 (TBST) and 5% nonfat milk. Following an overnight incubation at 4°C, the primary antibodies caspase-3 (1:1000 dilution), PARP (1:500 dilution) were employed. To standardize protein levels, β-actin (1:2000 dilution) was also blotted for all samples. The membrane was cleaned with TBST and then incubated for an additional hour with secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology, Danvers, MA). Pierce ECL Western staining substrate (Thermo Scientific) was used to visualize immunolabelled proteins, and an imaging system (Vilber Lourmat Sté, Collégien, France) was used to acquire the images.

Immunofluorescence and Imaging

The A549 cells were fixed in 4% buffered formalin after being treated with the RTC and RTE extracts for 48 h. PBS was then used as a washing medium. Cells were permeabilized using 0.3% Triton X-100 in PBS, blocked with goat serum in PBS, and then treated for 1 h at 37°C with caspase-3 (1:100). Alexa fluor was then used as the secondary antibody and allowed to sit at room temperature for 30 min. A Zeiss fluorescence microscope was used to observe the cells following their mounting with 4',6-diamidino-2-phenylindole (DAPI) for nucleus staining.

Statistical Analysis

Statistical analysis was done with GraphPad Prism 8. Data are expressed as mean ± standard deviation. Data were analyzed with the two-way analysis of variance (ANOVA) test. Significance changes with different p values (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

RESULTS

Cell Viability Assessment

The MTT results show a marginally higher viability in the A549 cells treated with RTC and RTE at low dosages (25-50 µg/mL) compared to the control (Figure 1A). This outcome was

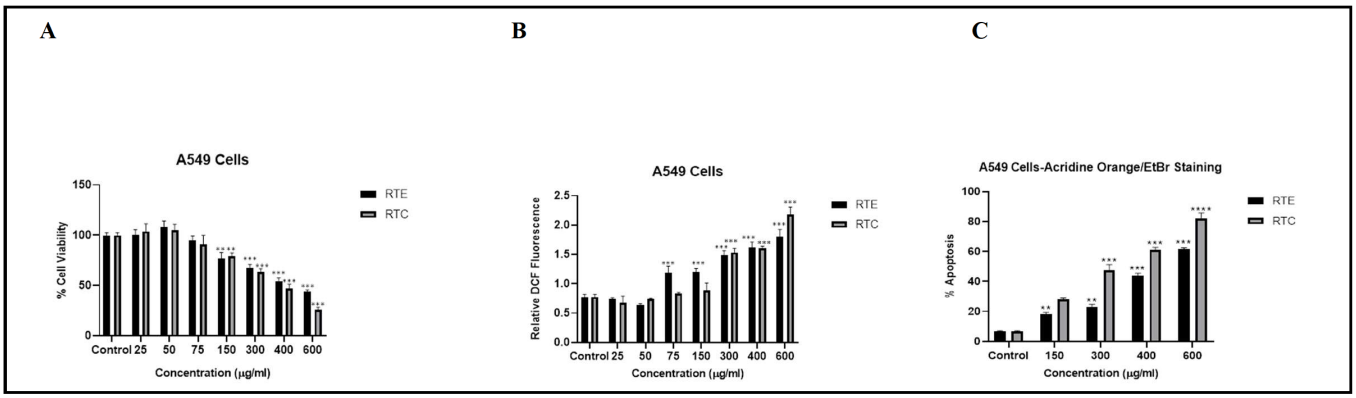

Figure 1. Effect of *R. tereticaulis* leaves' active ethanol (RTE) and chloroform (RTC) extracts on cell viability of A549, change in the amount of intracellular ROS and apoptotic effects in the cell. **(A)** Effects of RTC and RTE on A549 cell viability in a dose-dependent manner (**p<0.01 and *** p<0.001). **(B)** Dose-dependent effects of RTC and RTE on the amount of intracellular ROS in A549 cells (*** p<0.001). **(C)** Dose-dependent apoptotic effects of RTC and RTE in A549 cells (**p<0.01, *** p<0.001 and ****p<0.0001).

anticipated. When comparing the cells to those in the control group, a statistically significant decline was seen in the viability of the A549 cells that had been treated with 150, 300, 400 and 600 µg/mL each of RTC and RTE ($p < 0.001$). RTC was found to be more cytotoxic than RTE against A549 cells. The RTC and RTE extracts inhibited proliferation of the A549 cells at the IC_{50} values of 395 and 480 µg/mL, respectively.

ROS Generation Assessment

RTC and RTE had slight antioxidative effects on ROS levels in the A549 cells when compared to the control at the same low dosages (25–50 µg/mL). RTC and RTE demonstrated a prooxidant effect at high concentrations (50-600 µg/mL), whereas they demonstrated an antioxidant effect at low doses (25-50 µg/mL; Figure 1B). While a statistically significant increase had occurred in the quantity of intracellular ROS in the A549 cells treated with 75 µg/mL and 150 µg/mL of RTE compared to the control group ($p < 0.001$), no such statistically significant increase was seen in the A549 cells treated with the same doses of RTC (Figure 1B). When comparted to the control group, the A549 cells treated with 300, 400, and 600 µg/mL each of RTC and RTE showed a statistically significant increase in the amount of intracellular ROS ($p < 0.001$). In particular, the amount of intracellular ROS in cells treated with 600 µg/mL of RTC dramatically increased in comparison to the levels of intracellular ROS in the control cells and those treated with RTE.

Apoptosis Assessment

As a result of apoptosis, many cellular structures and organelles are damaged or destroyed. The study has evaluated the apoptotic effects of RTC and RTE using different methods such as AO/EB double staining and Annexin V-FITC. The A549 cells treated with different concentrations (150-600 µg/mL) of RTC and RTE showed the morphological features of apoptosis. According to the dual AO/EB staining, increasing the RTC and RTE doses decreased the live cell rates and increased apoptotic cell rates (Figures 1C, 2 and 3). In particular, the live cell rates of A549 cells treated with 400 and 600 µg/mL of RTC decreased more and the apoptotic cell rates increased more compared to the other groups ($p < 0.0001$; Figure 3). Additionally, the annexin V-FITC results support the AO/EB data (Figure 4).

Western Blotting Results

Increased levels of cleaved caspase-3 and cleaved PARP were detected in the A549 cells after 48 h of RTC and RTE treatment (Figure 5). RTC was determined to have a stronger apoptotic effect compared to RTE ($p < 0.001$; Figures 5B and 5C).

Direct Immunofluorescence Results

In order to investigate the apoptotic effect of *Rubus* extracts on A549 cells, this study exposed the A549 cells to various doses (400 and 600 µg/mL) of RTE and RTC extracts for 48 h. Caspase-3 levels of the cells were analyzed by immunofluorescence staining. While apoptosis in the cells increased significantly compared to the control group after applying 600 µg/mL of RTC, no significant difference was observed in the group that was applied RTE (Figures 6 and 7).

DISCUSSION

Many chemotherapy therapies no longer work as well as they once did because of the development of drug resistance in

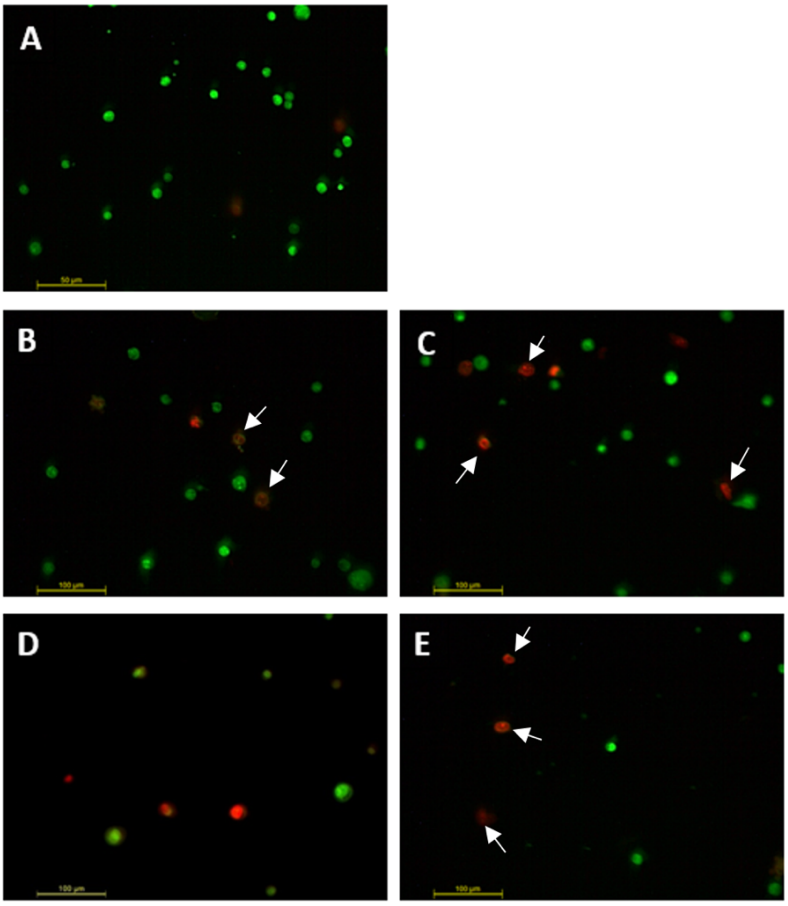

Figure 2. Detection of dose-dependent apoptotic effects of *R. tereticaulis* leaves' active chloroform (RTC) extracts in A549 cells with AO/EB dye. **A)** Control, **B)** 150 µg/mL, **C)** 300 µg/mL, **D)** 400 µg/mL, **E)** 600 µg/mL. Magnification: 20X. Arrows indicated the apoptotic cells.

lung cancer.[4-6] Furthermore, the chemotherapeutic method of treating lung carcinomas frequently results in substantial off-target adverse effects. The trend toward medications made from plants has begun due to their lack of negative side effects and their high levels of phenolic compounds (e.g., flavonoids, phenolic acids).[19] This study focused on the anticancer activity of *R. tereticaulis*, which a previous study on lung cancer had shown to have high phenolic and flavonoid content.[14]

The cytotoxic effect of the ethanol and chloroform extracts of *R. tereticaulis* on human lung cancer cells was investigated by MTT assay after 48 h of incubation with RTE and RTC extracts; 50% of the cells were inhibited at the concentrations of 395 and 480 µg/mL for the respective RTC and RTE extracts. No study in the literature has been found regarding the anticancer activity of *R. tereticaulis*, not only against lung cancer cell line but also against any other cancer line. However, well-known *Rubus* species such as *R. coreanum, R. adenotrichos, R. occidentalis,* and *R. fairholmianus* have all shown anti-cancer effects in previous studies. Two different studies have shown *R. coreanum* ethanolic extracts and *R. coreanus* to have cytotoxic effect on HT-29 colon cancer cells.[20,21] In addition, *R. occidentalis* has been shown to have activity against a number of cancer types such as esophageal, colorectal, epidermal, and breast cancer in both *in vitro* and *in vivo* studies.[22] These results are in line with those of the current study and confirm the anticancer activity of the *Rubus* species. Although many studies are found to have reported the cytotoxic effects of the *Rubus* species, the cellular mechanisms underlying their effects have not been sufficiently elucidated. This study presents for the first time the half-maximal inhibitory concentrations of the ethanol and chloroform extracts of *R. tereticaulis* on A549 cells.

During malignant transformation, many cancer cells produce an excessive amount of intrinsic ROS, which encourages tumor growth.[20] Cancer cells can boost their survival rate and develop resistance to anti-cancer medications as a result of redox adaptation, which controls anti-apoptotic and antioxidant molecules.[7] Additionally, a growing body of proof is found showing ROS to play a crucial role in both other cells and inflammatory cells when inducing apoptosis.[23] Natural antioxidants at high doses are well-known for exhibiting pro-oxidant activity and to produce ROS via the Fenton reaction. Higher concentrations of antioxidant molecules, particularly in cancer cells, induce DNA damage and apoptosis through their pro-oxidant activity.[24] One previous study found bioactive compounds iso-

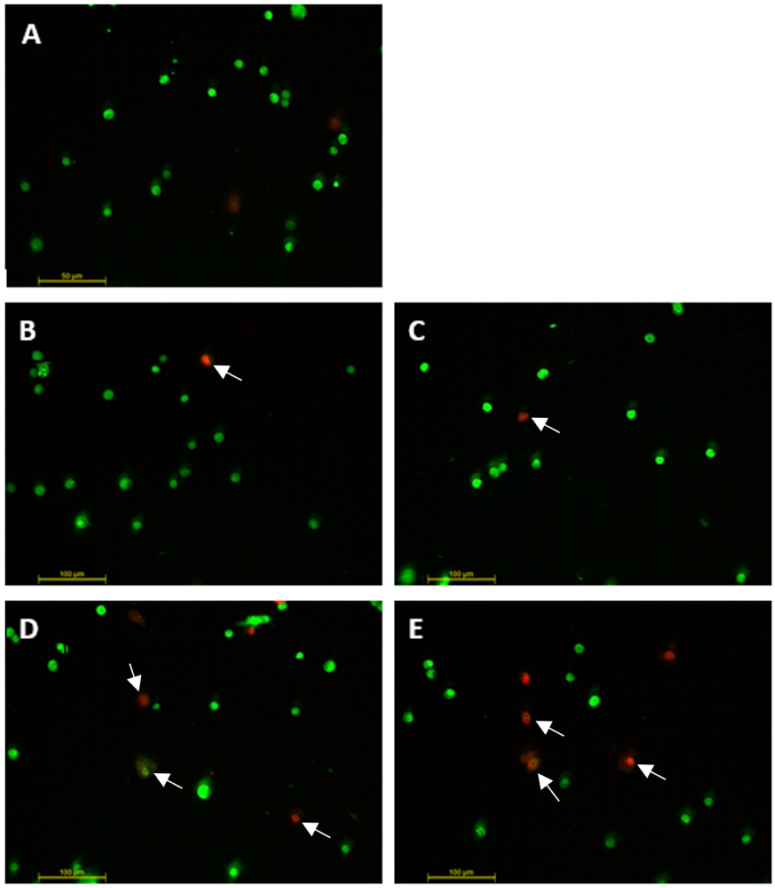

Figure 3. Detection of dose-dependent apoptotic effects of *R. tereticaulis* leaves' active ethanol (RTE) extracts in A549 cells with AO/EB dye. **A)** Control, **B)** 150 µg/mL, **C)** 300 µg/mL, **D)** 400 µg/mL, **E)** 600 µg/mL. Magnification: 20X. Arrows indicated the apoptotic cells.

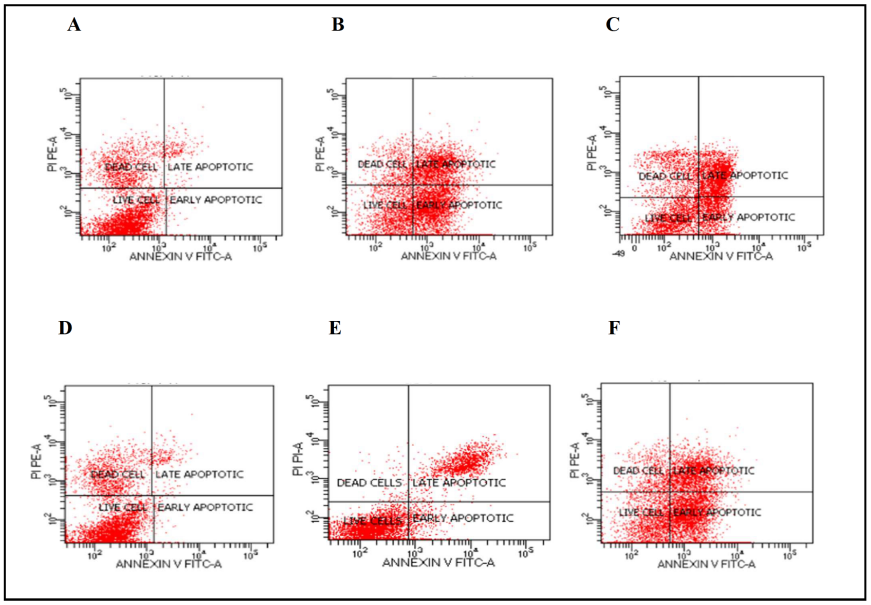

Figure 4. Annexin-V-FITC dual staining to assess the apoptotic activity of *R. tereticaulis* leaves' active ethanol (RTE) and chloroform (RTC) extracts on A549 cells. Cells were treated with different concentrations RTC and RTE for 24 h, stained with Annexin- V/PI, and measured by a flow cytometry. **A)** RTC- Control, **B)** RTC- 300 µg/mL, **C)** RTC- 600 µg/mL, **D)** RTE- Control, **E)** RTE- 300 µg/mL, **F)** RTE- 600 µg/mL.

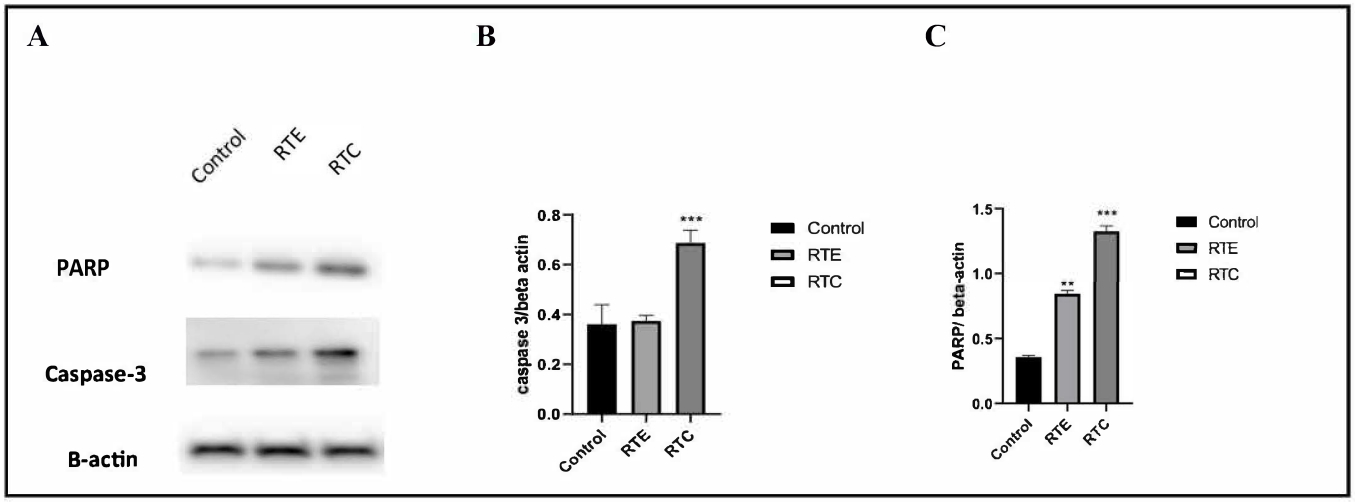

Figure 5. Examination of apoptotic proteins after 24-hour *R. tereticaulis* leaves' active ethanol (RTE) and chloroform (RTC) extracts application to A549 cells. **A)** Immunopositive bands for caspase-3 and PARP, **B)** Graphic indicates cleaved PARP normalization, **C)** Graphic indicates cleaved caspase-3 normalization. **p<0.01 and *** p<0.001.

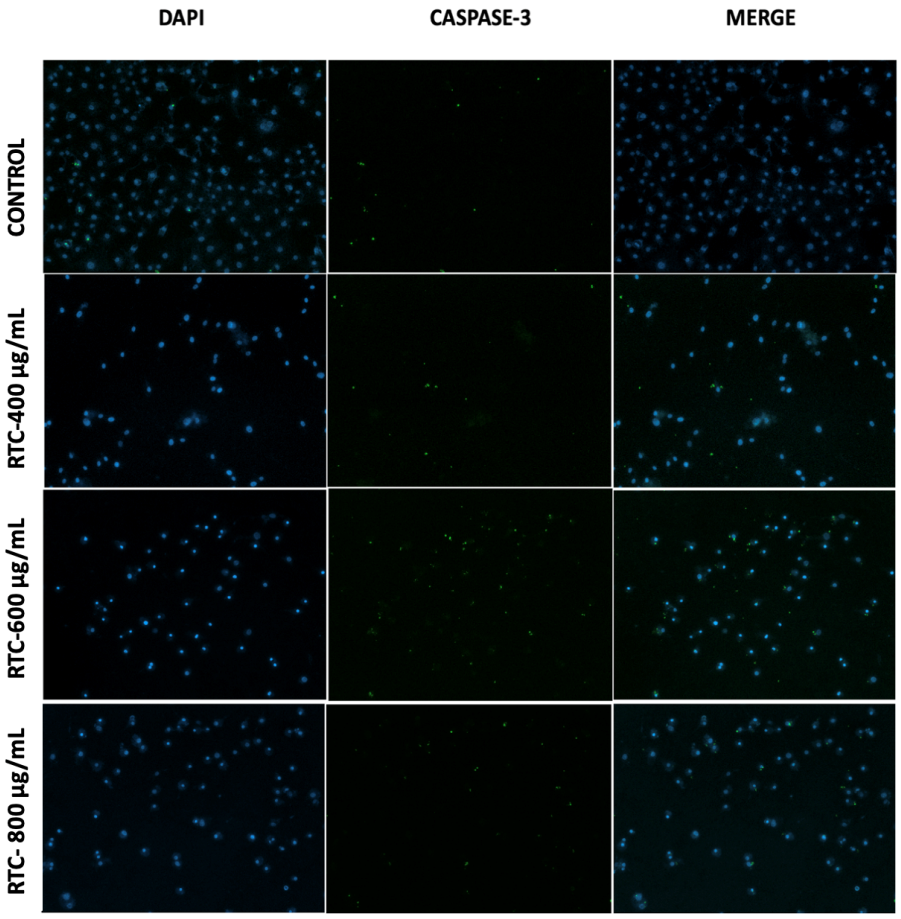

Figure 6. Fluorescence microscope images of A549 cells which were subjected to immunofluorescence detection (10x) after *R. tereticaulis* leaves' active chloroform (RTC) extracts.

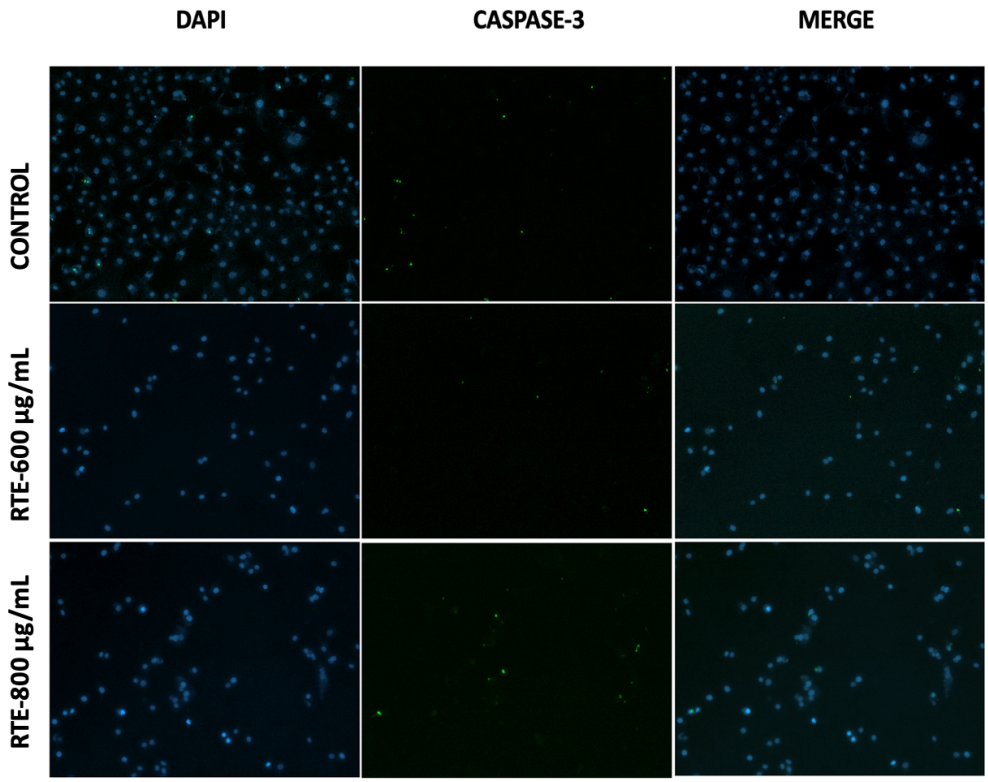

Figure 7. Fluorescence microscope images of A549 cells which were subjected to immunofluorescence detection (10x) after *R. tereticaulis* leaves' active ethanol (RTE) extracts.

lated from *R. fairholmianus* to exert anti-cancer effects by increasing ROS production in MCF-7 breast cancer cells.[25] The current study has shown for the first time the cytotoxic effect of RTC and RTE treatment on A549 cells to be dependent on their pro-oxidant properties. According to this investigation's findings, RTE and RTC treatment of A549 cells has resulted in a decrease in the amount of ROS at lower concentrations while resulting in an increase at higher concentrations.

To date, very few studies have been conducted to elucidate the apoptotic effect of the *Rubus* species on cancer cells; nor has research yet been conducted to elucidate the apoptotic mechanism of *R. tereticaulis* in either cancer or normal cells. During apoptosis, biochemical events occur such as caspase activation in the cell and DNA and protein degradation, as well as some changes to membrane structure. Caspases in particular, which are activated during the caspase phase, take part in breaking down many proteins, nuclei, and cytoskeletons.[26,27] There are some studies that have shown the *Rubus* species' effect on apoptosis signal transduction pathways, but these are limited.[21,25,28] In fact, no studies are found based on *R. tereticaulis.* The present study has reported the treatment of around IC_{50} doses of the RTE and RTC extracts to induce apoptosis significantly compared to the non-treatment group. Morphological, biochemical, and molecular changes related to apoptosis in cells can be measured by different methods. This study analyzed apoptosis using dual AO/EB staining, annexin V-FITC and immunoblotting analyses, immunofluorescence, and imaging. Apoptotic, necrotic, and living cells can be distinguished by these methods. The results of the current study reveal RTC and RTE at IC_{50} doses to increase apoptosis in A549 cancer cells, with RTC inducing more apoptosis than RTE. Consequently, the mechanisms underlying the cytotoxic and apoptotic activity of the different *Rubus* extracts have been clarified as a result of the evidence for the generation of intracellular ROS and apoptosis. Further studies should be conducted to elucidate the signal transduction pathways underlying these impacts. Additionally, RTC may have had a slightly stronger apoptotic and ROS generative impact than RTE due to being prepared with a different solvent. This may also be due to RTC containing more apolar compounds with cytotoxic activity than RTE.[29]

Recent data have shown inflammation to be closely associated with tumors and to be a critical component of cancer progression.[30] Epidemiologic evidence has also revealed approximately 25% of all human cancers worldwide to be associated with chronic inflammation, chronic infection, or both.[31] The use of anti-inflammatory agents can improve patients' prognoses by reducing the incidence and recurrence of various cancers.[32] Naturally derived agents with anti-inflammatory activity have also been reported to have anti-cancer activity.[33] Therefore, conducting research is important on medicinal plants

whose anti-inflammatory and antioxidant activity have already been investigated. Previous studies have revealed RTE and RTC to show antioxidant and anti-inflammatory activity due to their phenolic and flavonoid contents. In addition, the major compound of RTE that has antioxidant and anti-inflammatory activity was revealed to be quercetin-3-O-β-D-glucuronide.[14] Some studies have suggested that natural phenolic compounds with pro-oxidant and anti-inflammatory properties may be promising anticancer agents against lung cancer.[34,35] Thus, the phenolic compounds in RTE and RTC that have pro-oxidant and anti-inflammatory activity may be responsible for the anticancer activity. In addition, quercetin glucuronides have been shown to have cell cycle arresting and apoptosis-inducing effects against the human lung cancer cell line NCI-H209.[36] Therefore, one can argue that quercetin-3-O-β-D-glucuronide, which has been shown to have antioxidant and anti-inflammatory activity in previous studies and is the major compound in RTE, is also significantly responsible for RTE's anticancer activity.

CONCLUSION

The present results have demonstrated *R. tereticaulis* extracts, especially RTC, to be able to be a potential therapeutic agent for human lung cancer treatment. However, *in vivo* studies are needed to fully demonstrate RTC's anticancer effect against lung cancer.

Acknowledgments: The authors would like to thank Dr. Ahmet Doğan for his help in identification of the plant material.

Ethics Committee Approval: Ethics committee approval is not required for the study.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.N.O., E.D., A.S., A.K.; Data Acquisition- G.N.O., E.D., A.S.; Data Analysis/Interpretation- G.N.O., E.D., A.S., A.K.; Drafting Manuscript- G.N.O., E.D.; Critical Revision of Manuscript- G.N.O., E.D., A.S., A.K.; Final Approval and Accountability- G.N.O., E.D., A.S., A.K.

Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: Authors declared no financial support.

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How to cite this article

Oter GN, Durmus E, Sen A, Kocyigit A. Induction of Apoptosis through Oxidative Stress Caused by *Rubus tereticaulis* Leaves Extracts in A549 Cells. Eur J Biol 2024; 83(1): 50–59. DOI:10.26650/EurJBiol.2024.1353842