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Cytotoxic Effect of Prunus Divaricata Ledeb. Extract on Lung Cancer Cells and Determination of Expression Profiles of Genes in the MAP-Kinase Pathway

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Abstract

Cherry plum (*Prunus divaricata* Ledeb.) is a diploid fruit tree that belongs to the *Rosaceae* family. Anticarcinogenic activity of *Prunus divaricata* Ledeb. methanol extract was examined in lung cancer cell lines A549 and H1299 as well as in healthy cell line Beas-2b in this study. The changes in the expression levels of the most frequently mutated oncogenes in lung cancer, KRAS, EGFR, BRAF, and PIK3CA were investigated as well. In vitro cytotoxicity assays of *P. divaricata* Ledeb. fruit methanol extract was conducted by measuring the percentage of viable cells spectrophotometrically using the cell viability identification kit (CVDK) based on the colorimetric method. The AB 7500 Fast RT-PCR device with Sybr Green Master Mix was used to create gene expression profiles, and the Ct values of all genes were assessed by normalizing the Ct value of the housekeeping gene. The methanol extract of *P. divaricata* Ledeb. fruit was found to have a cytotoxic effect on A549 and H1299 cancer cell lines at 20 mg/ml, but not on the Beas-2b cell line at these concentrations. By reducing proliferation, low KRAS and PIK3CA gene expression caused the cells to undergo apoptosis. The methanol extract of *P. divaricata* Ledeb. fruit has been found to inhibit the development of cancerous cells while promoting the proliferation of healthy cells. We believe it does this through the apoptotic pathway by regulating gene expression levels in oncogenes via the MAP-Kinase pathway.

Keywords: Anticancer, gene expression, *P. divaricata* subs *P. divaricata* Ledeb.

1. INTRODUCTION

Lung cancer is one of the most common types of malignant cancer in the world, with a 5-year survival rate of 10-20% [1]. Beneficial dietary components with pharmacological effects may play a role in the prevention and treatment of a variety of

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diseases. Natural nutritional supplements with anticancer characteristics can be used to boost the efficacy of chemotherapy and reduce the side effects of high-dose treatment brought on by pharmacological treatments. Recent anticancer research draws attention to the potential anticancer activity of plants and wild fruits in in vitro tests. The strong antioxidant effects of phenolic compounds found in plant extracts and a wide range of pharmacological qualities, including anticancer potential, form the basis of this strategy in anti-cancer studies [2]. The *Rosaceae* family of plants is one of the most important plant groups in our country. Its species are used in a wide range of industries, including agriculture, food, cosmetics, perfumes, and landscaping. Prunus L. is one of the *Rosaceae* family's most economically significant genera [3]. The effects of functional food on human health, including Prunus plums, are well-known [4]. With its anticancer, antioxidant, anti-inflammatory, and antihyperlipidemic characteristics, plums have recently been the subject of promising research. Additionally, studies have shown that the polyphenols in plums have qualities that suppress the proliferation of cancerous cells in a number of serious cancers, including breast cancer, hepatocellular carcinoma, gastric cancer, and cervical carcinoma [5]. The most commonly mutated oncogenes in lung cancer are KRAS, EGFR BRAF and PIK3CA genes. Among these genes, KRAS, EGFR, and BRAF are associated with MAPK, a crucial receptor serine/threonine kinase signaling pathway in the growth of cancer [6].

Lung cancer is one of the cancer types with the greatest number of genetic disorders, and its molecular pathogenesis is complicated and heterogeneous. Lung cancer can develop through a variety of mechanisms, including the functional increase of oncogenes or the loss of tumor suppressor gene activity. Purified *P. divaricata* Ledeb. methanol extract was used in this study to examine the cytotoxic effects on lung cancer cells, the molecular mechanism of causing apoptosis, and the expression patterns of mutagenesis oncogenes typically found in lung cancer.

2. MATERIAL AND METHOD

2.1. Sample Collection and Preparation

Fruit samples from *P. divaricata* subs. *P. divaricata* plants collected in Nurhak and Ahırdağı (October 2021) were pureed. The pureed sample was extracted with methanol using a magnetic stirrer (Heidolph MR3001, Sigma-Aldrich). Following filtration, the extracts were concentrated in vacuum at 40°C (Heidolph WB2000).

2.2. Cell Culture Studies

A549 (Human Lung Epithelial-Associated Cancer Cell), H1299 (Human Non-Small Cell Lung Cancer Cell) cell lines and Beas-2b (Healthy Human Lung Epithelial Cell) cell line obtained from the American Type Culture Collection (ATCC) were used in the current study. DMEM (Gibco) media containing 10% fetal bovine serum (Gibco) and 1% antibiotic (penicillin/streptomycin) was then used to grow and propagate the cells at a temperature of 37°C in an incubator with 5% CO2. For cell viability assays measuring metabolically active cells, the cells were then taken out of the vial containing 0.25% Trypsin/EDTA (Gibco) and planted into 96-well plates with 5x103 cells per well.

2.3. Cell Viability Analysis

Plant extract samples were lyophilized for 24 hours to completely remove the solvents. Following lyophilization, the extracts were diluted in DMSO at a 1:1 ratio to prepare the 1000 mg/ml extraction. The cell culture doses were prepared by dilution with medium at 20 mg/ml, 15 mg/ml, 10 mg/ml, 5 mg/ml, and 1 mg/ml. The incubated cell cultures were aspirated in the sterile cabinet, and 100 µl of adjusted doses was added to

each well. After 24 hours in a 5% CO2 incubator at 37 °C, 10 µl of CVDK was added to each well for cytotoxicity testing. It was incubated for 3-4 hours at 37°C in a 5% CO2 incubator. Colorimetric measurements were taken at 450 nm. The experiment was repeated three times, each at a different time.

2.4. RNA Isolation from Cell Culture

In the cytotoxicity study, the cells were seeded in a 6-well plate with about 80,000 cells per well to establish the variations in the dosage that altered the cell lines' gene expression levels. The predetermined dose was administered to the cells after 24 hours of incubation. The Hibrigen Total RNA Isolation Kit was used to isolate total RNA from the cells after a 24-hour incubation period (Lot: 0322-AK-2180-Türkiye). The experiment was repeated three times, each at a different time.

2.5. Measuring RNA Purity

To measure RNA purity, a ThermoFischer 2000c Nanodrop device was used. The study used samples with 260/280 ratios between around 2.00 and 2.20. To ensure that working conditions were equal, RNA concentrations were changed to 50 ng/ul.

2.6. cDNA (complementary chain) Synthesis

Using the ABM OneScript Plus cDNA Synthesis Kit, whole RNA samples taken from cell lines were used to synthesize cDNA in order to analyze the gene expression profiles (Cat No: G236-Canada). The kit's instructions were followed for the synthesis.

2.7. RT-PCR Protocol

Quantitative Real-time PCR was conducted using Sybr Green Master Mix (abm, Richmond, Canada), (AB 7500 Fast Real-Time PCR, Germany). The reaction conditions were set to include a 10-minute incubation at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The Table 1 supplies the base sequences of the primers used in RT-PCR experiments.

Table 1 The primer sequences used

Primers	Sequence (5'—3')
PIK3CA Forward	5′-GTGGAGGAGCTCTTCAGGGA-3′
PIK3CA -Reverse	5′-AGGCACCCAGGGTGATGCAA-3'
KRAS- Forward	5′-TTGCTTCAGGGTTTCATCCA-3′
KRAS - Reverse	5′-CAGCCTTGAGCACCAGTTTG-3′
BRAF-3- Forward	5'- TGGTGATGAAGGGGTCATTTATG -3'
BRAF -3- Reverse	5'- TTCGGCTTTCCAGTCAGACTC -3'
EGFR- Forward	5'- ATGAGCCGCCTGAGGTTGG -3'
EGFR- Reverse	5'- ATGAGCCGCCTGAGGTTGG -3
GAPDH- Forward (Housekeeping)	5'-AGCCACATCGCTCAGACAC-3'

2.8. Statistical Analysis

GraphPad Prism 8 was used for statistical analysis. The "Student's t test" for group comparison was used to determine the level of significance between different treatment groups compared to the control group. Statistical significance was defined as $p < 0.05$. The mean standard deviation (SD) of three independent experiments is used to represent all data. The absorbance results were converted to percentages, assuming 100% of the cells were viable at 0 concentration of the plant extract, in order to compare the differences in the IC50 values of the various cell types. Two Way Anova

was used after the values had been adjusted, and the column factor (cell types) and interaction values were taken into consideration for the assessment of statistical significance.

3. RESULTS AND DISCUSSION

3.1. Anti-proliferative effect of *Prunus divaricata* Ledeb. on A549 cells

The CVDK cell viability assay was used to evaluate the ability of *Prunus divaricata* Ledeb. extract to inhibit the proliferation of the A549 cell line. Figure 1 illustrates the finding that *Prunus divaricata* Ledeb. extract inhibited lung cancer cell viability in a time- and dose-dependent manner. Additionally, the extract of *Prunus divaricata* Ledeb.'s IC50 value in A549 cells was shown to be 9.37 mg/ml. Furthermore, it was shown that the lowest amount of cell viability on A549 cells was achieved by applying 20 mg/ml of *Prunus divaricata* Ledeb. extract.

3.2. Anti-proliferative effect of *Prunus divaricata* Ledeb. on H1299 cells

The CVDK cell viability assay was used to evaluate the ability of *Prunus divaricata* Ledeb. extract to inhibit the proliferation of the H1299 cell line. Figure 1 illustrates that *Prunus divaricata* Ledeb. extract suppressed lung cancer cell viability in a time- and dose-dependent manner. In H1299 cells, the extract of *Prunus divaricata* Ledeb., has an IC50 value of 9.54 mg/ml. Moreover, it was found that the lowest amount of cell survival on H1299 cells was achieved by applying 20 mg/ml of *Prunus divaricata* Ledeb. extract.

3.3. Anti-proliferative effect of *Prunus divaricata* Ledeb. on Beas-2B cells

The CVDK cell viability assay was used to determine the ability of *Prunus divaricata* Ledeb. extract to inhibit proliferation of the Beas-2B cell line. The cell viability of healthy lung cells was reduced by the *Prunus divaricata* Ledeb. extract in a time- and dose-dependent way, initially steady and then dose-dependently, as shown in Figure 1. Additionally, 17.58 mg/ml was shown to be the IC50 value for *Prunus divaricata* Ledeb. extract in Beas-2B cells. Furthermore, the application of 10 mg/ml *Prunus divaricata* Ledeb. extract was found to be the closest dose to 100% in cell viability on Beas-2B cells.

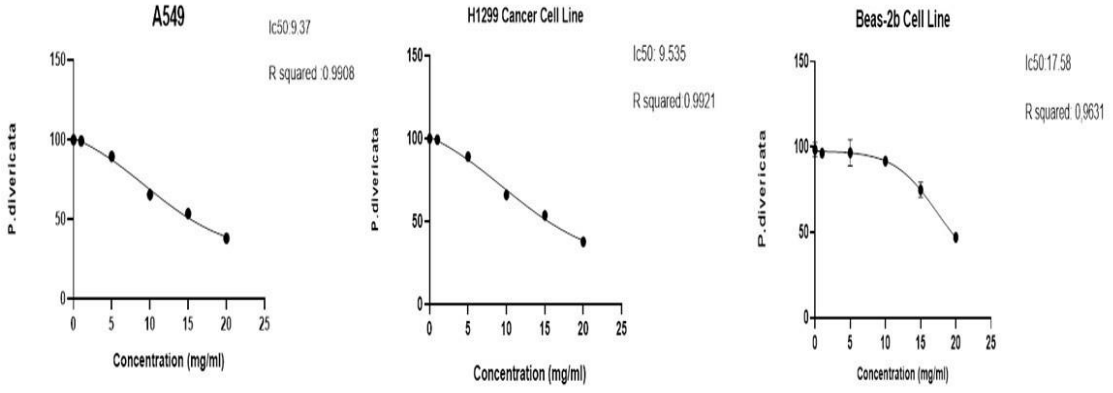

Figure 1 Concentration dependent cell viability levels

3.4. Results of oncogene gene expression analyzes by RT-PCR in A549, H1299 and Beas-2B cells

By adding 20 mg/ml *Prunus divaricata* Ledeb. extract to all cell lines, the levels of PIK3CK, EGFR, KRAS, and BRAF oncogenes on the MAPK pathway were compared with those of cancer cells and healthy control cells. Moreover, untreated healthy lung cells and cancerous cell lines were contrasted. Comparing cells treated with *Prunus divaricata* to untreated cells revealed that BRAF gene expression decreased in A549 and H1299 lung cancer cells while increasing in Beas-2B cells (Figure 2).

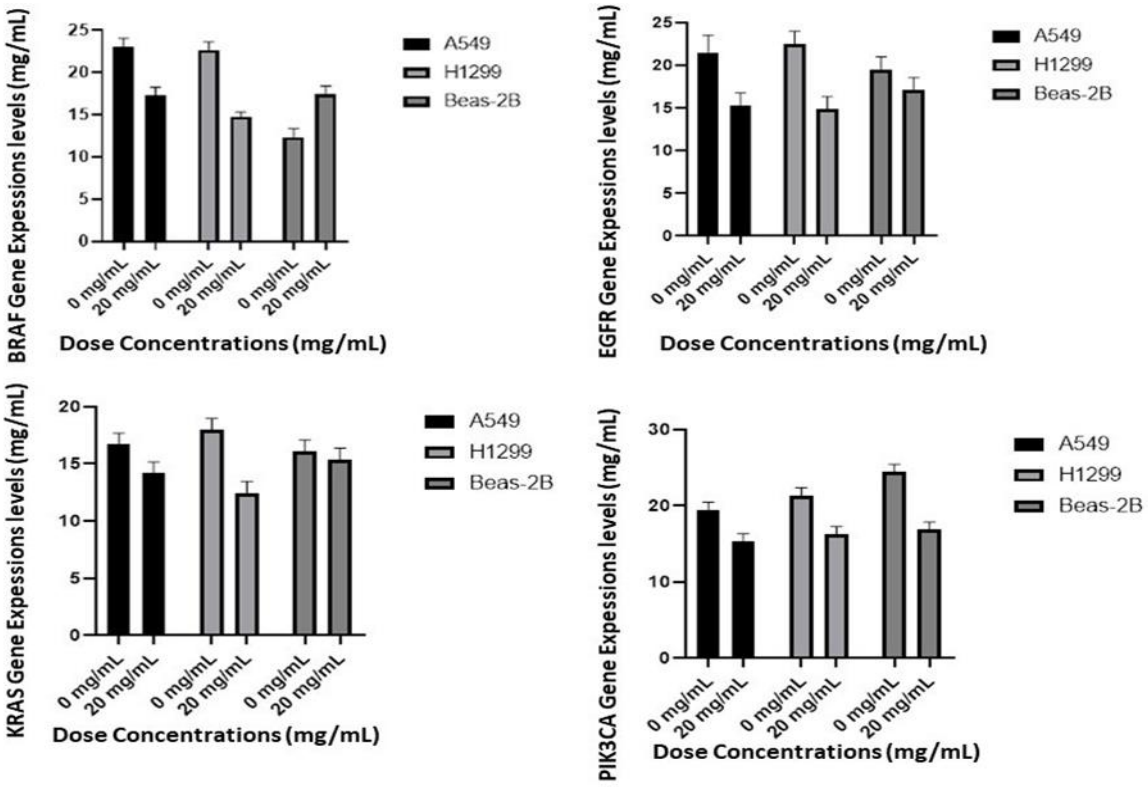

Figure 2 Gene expressions levels

When A549 and H1299 lung cancer cells treated with *Prunus divaricata* Ledeb. were contrasted with untreated cells, the levels of EGFR and KRAS gene expression were shown to be lower in the treated cells. EGFR and KRAS gene expression were shown to be lower in the treated cancer cell lines A549 and H1299 than in healthy Beas-2B cells after *Prunus divaricata* Ledeb. Treatment.

When PIK3CA gene expression was evaluated, A549 and H1299 lung cancer cells as well as Beas-2B healthy lung cells showed lower PIK3CA gene expression when compared to cells that had not been treated with Prunus divaricata Ledeb. In comparison to healthy Beas-2B cells left untreated, the expression levels of the PIK3CA gene were significantly reduced after the application of Prunus divaricata Ledeb. in the cancer cell lines A549 and H1299.

Although lung cancer most frequently affects men, it is the fifth most common cancer in women. The use of traditional and alternative cancer therapeutic approaches has recently gained substantial scientific attention and being researched in medical practice Application of phenolic substances is widespread in certain basic industries, such as food, health, and medicine. These substances contain natural antioxidants as part of the human diet [7]. The cytotoxic,

antioxidant, and anticarcinogenic properties of several plant extracts are currently being studied in in vitro experiments. Prunus plums have been reported to be used as alternative treatment support products in a variety of health problems including hypertension, diabetes, and gastrointestinal disorders, and it is one of the promising plant-based therapeutics. However, the cytotoxicity of fruit extracts from some of its species against cancer cells has not been thoroughly investigated [8]. To our knowledge, our research is the first study to address the gaps in the literature by examining the effects of *Prunus divaricata* Ledeb. fruit extract, subtype of Prunus, on lung cancer cells.

Cell proliferation involves doubling in size and cell division by DNA replication in the mitotic cell cycle. Cancer-related abnormalities in cell cycle checkpoints and genetic changes in regulatory mechanisms that control molecular signaling pathways are the two main causes of hyperproliferative states [9]. *Prunus divaricata* Ledeb. contains polyphenols that may prevent the onset, growth, and progression of cancer by altering multiple cell-signaling pathways to cause apoptosis and cell cycle arrest. Polyphenols' anticancer molecular mechanism may be too complex to fully understand [10]. In this work, *Prunus divaricata* Ledeb. fruit extract was used to treat two different cancer cell lines and one healthy cell line from the lung tissue. Our findings on cell proliferation indicated that the administration of 20 mg/ml *Prunus divaricata* Ledeb. extract resulted in the lowest amount of cell viability on A549 and H1299 cancer cells. It was discovered that treatment with 10 mg/ml *Prunus divaricata*Ledeb. extract resulted in cell viability that was close to 100% in Beas-2B cells. These findings demonstrated that extract from *Prunus divaricata* Ledeb. reduced lung cancer cell proliferation.

GTP activation is the basis of the MAPK pathway. The extracellular region of a receptor from the receptor tyrosine kinase family will attach to a ligand, such as the epidermal growth factor (EGFR), to initiate the MAP-Kinase pathway. EGFR, which is found in the pathways that trigger tumorigenesis, is considered as a target molecule. A high degree of sensitivity to tyrosine kinase inhibitors develops as a result of EGFR mutations in lung cancer [11]. Less than 10% of squamous cell carcinomas and 20% to 25% of lung adenocarcinomas have the KRAS oncogene. KRAS mutations are crucial in ruling out variants of the EGFR, BRAF, HER2, and many other oncogenic drivers, such as ALK and ROS rearrangements [12]. The results of our study showed that the treatment of A549 and H1299 lung cancer cells with *Prunus divaricata* Ledeb. extract reduced the expression of the genes EGFR and KRAS. EGFR and KRAS gene expressions were shown to be lower after *Prunus divaricata* Ledeb. treatment in cancer cell lines A549 and H1299 compared to healthy Beas-2B cells. Our findings are consistent with previous research. It is advised to review clinical and laboratory findings concurrently in individuals with KRAS variants since a specific treatment for KRAS variations has not yet been identified despite the use of a variety of drugs to inhibit the variations.

Approximately 6-8% of non-small cell adenocarcinomas have BRAF mutations. Lung cancer patients with BRAF activating mutations have a different carcinogenic gene that is not controlled by EGFR. BRAF variants activate MAPK2 and MAPK3 to cause cancer. BRAF mutations are present in 1-3% of lung adenocarcinomas, despite being common in malignant melanomas [13]. When we compared the BRAF gene expression between the cells treated with *Prunus divaricata* Ledeb. extract and untreated cells, we found that BRAF gene expression decreased in A549 and H1299 lung cancer cells while significantly increased in Beas-2B cells.

Mutations in the PIK3CA gene disrupt the activation of an important PI3K pathway in cell proliferation, leading to abnormal function. By regulating and amplifying signals critical for cellular survival and proliferation, co-occurring KRAS and PIK3CA mutations cause cancer. According to the research, 4% of lung cancers have PIK3CA mutations [14]. In a study of lung cancer patients, PIK3CA and KRAS variants were found to be associated with a poor prognosis and shorter survival [15].According to the results of our study, PIK3CA gene expression was decreased in A549 and H1299 lung cancer cells and Beas-2B healthy lung cells treated with and without *Prunus divaricata* Ledeb. extract. PIK3CA gene expression levels were significantly lower in A549 and H1299 cancer cell lines after application of *Prunus divaricata* Ledeb. compared to healthy Beas-2B cells without treatment.

4. CONCLUSION

In conclusion, the genes KRAS, BRAF, PIK3CA, and EGFR are crucial for the development and differentiation of lung cells. Participating in autophosphorylation pathways and promoting carcinogenesis are both critical stages in the development of cancer. Each of them is a therapeutic target gene due to their frequent involvements in lung cancer, particularly KRAS, and their resistance to treatment. In addition to the genes evaluated in regular analyses, KRAS, BRAF, PIK3CA, and EGFR genes should be further studied in order to better understand the molecular pathogenesis of lung cancer and to improve survival.

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Authors' Contribution
The authors contributed equally to the study.

The Declaration of Conflict of Interest/ Common Interest
No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval
This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics
The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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