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PHYTOCHEMICAL CONSTITUENTS AND ANTILEUKEMIC EFFECTS OF *Juniperus oxycedrus* EXTRACT

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Aim. Many genetic and environmental factors can be effective in the process of cancerization. Preventing the progression of leukemia may be possible by controlling the pathways involving mechanisms such as apoptosis and autophagy. When the literature is examined, there are studies showing the effects of various types of juniper on various cancer cell lines, including human chronic myeloid leukemia cells, but the signal pathways in which they act are not fully known. In this study, the anticancer effects of *Juniperus oxycedrus* extract on K-562 human chronic myeloid leukemia cells were investigated.

Method. After the cells were treated with the *Juniperus oxycedrus* extract, cytotoxicity and gene expression analyzes were performed. Changes in the expression of Akt, the member of the PI3K/Akt/mTOR signaling pathway; caspase 3, which is one of the main effective genes in the pathways regulating apoptosis; and the apoptosis suppressor BCL-2 gene, which is an oncogene, were investigated.

Results. According to the MTT test results, *Juniperus oxycedrus* extract showed over approximately 50% cell viability in K-562 cells at all doses. The most appropriate dose of *Juniperus oxycedrus* fruit extract in this research was determined as 50 µg/mL considering cell viability. After the gene expression analysis, it was observed that BCL-2 expression decreased approximately 3.3 times, and caspase 3 expression increased 1.2 times. Although Akt gene expression increased 1.092 times, it was not statistically significant.

Conclusions. Constituents of *Juniperus oxycedrus* plant may have apoptotic effects on chronic myeloid leukemia cells.

Key words: *Juniperus oxycedrus*; leukemia; apoptosis.

Chronic myeloid leukemia (CML) is a hematological disease that develops mainly as a result of a translocation and oncogenic fusion of BCR and ABL1 genes. The abnormal tyrosine kinase activity exhibited by this fusion protein causes activity changes in various signaling pathways with which it interacts. Although the developed tyrosine kinase inhibitors are standard therapeutics used in the treatment of CML, resistance to BCR-ABL tyrosine kinase inhibitors is a clinically unresolved problem [1, 2].

In the field of cancer biology, it is seen that there is an increasing interest in studies investigating the anticancer effects of compounds obtained from plants. It is known that the fruit and essential oils of the juniper tree were used in digestive system disorders in the Middle Ages, the *Juniperus oxycedrus* species was consumed in Turkey and used in different sectors such as medicine, cosmetics and food [3]. In the literature, there are studies examining the anti-inflammatory, antinociceptive, antibacterial and anticandidal effects of the extracts of the juniper plant, which has many species, prepared with various solvents [4, 5]. Huyan et al. stated that *Juniperus sabina* extract exerts an inhibitory

effect on HepG-2 and K-562 cells by reducing cell viability and inducing apoptosis through upregulation of the expression of apoptosis-related genes FasL, caspase 3 and caspase 9 [6].

The aim of the project is to determine the anticancer effects of methanolic extracts prepared from the fruit parts of *J. oxycedrus* plant collected from Niksar district of Tokat, Turkey, in cells where chronic myeloid leukemia can be modeled.

Within the scope of this study, phytochemical analyzes of *J. oxycedrus* plant extract, determination of cytotoxic dose range with MTT (3-(4,5 dimethylhiazol-2yl)-2,5-diphenyl tetrazolium bromide) in K-562 and ECV-304 cells, and gene expression analysis at determined doses were performed. ECV-304 cells were used as control group.

Changes in expression of Akt, BCL-2 and Caspase 3 genes selected as target genes were examined by Real-Time PCR. As a result of the studies, the anticancer effects of *J. oxycedrus* extract on chronic myeloid leukemia cells were investigated through signal pathways.

Materials and Methods

Preparation of Juniperus oxycedrus Extracts

The plants were collected from Tokat Niksar Akıncı Village in October (Turkey). Identification of species was done by Prof. Dr. Emine Akalın Urusak from İstanbul University, Faculty of Pharmacy. The fruits of the plants were separated from each other and air-dried at room temperature. Fifteen grams of fruit were extracted twice with 150 mL of methanol separately. Extraction was carried out in a shaking hot water bath at 50 °C for 8 hours and then by maceration at room temperature for 16 hours. The amount of extract was determined by taking the first and last weighing of the balloon. The extract was stored at +4 °C to be used in the next experimental stages.

Phytochemical Screening

The *J. oxycedrus* extract was analysed for the presence of the phytochemicals. For the screening of alkaloids, saponins, tannins, glycosides, phenols, carbohydrates, proteins, and flavonoids; the method of Khuda et al. was used [7].

Test for Alkaloids

Hager's test is used for determination of alkaloid presence in the extract. In this test, 3–4 drops of Hager's reagent (picric acid) were added to 2 mL plant extract. The appearance of a yellow precipitate shows the presence of alkaloids.

Test for Saponins

In a test tube, 2 mL plant extract and distilled water was mixed and shaked vigorously for 5 minutes. The formation of a thick foam at the top of the tube reveals the presence of saponins.

Test for Flavonoids

Test for detection of flavonoids was performed by adding a few drops of ferric chloride to 2 mL plant extract. The appearance of blakish-red precipitates confirmes the presence of flavonoids.

Test for Tannins

Alkaline reagent test is used for determination of tannin presence in the extract. In this test, 2 mL of plant extract and 1N sodium hydroxide was mixed thoroughly. The formation of red or yellow precipitates shows the presence of tannins.

Test for Glycosides

Keller Killiani test is performed for determination of glycoside presence in the plant extract. This test was performed by addition of 1 mL of the extract and glacial acetic acid in a test tube. After cooling the mixture 2–3 drops of ferric chloride added to the tube. Finally, 0.5 mL sulfuric acid was added through the sides of the test tube. The formation OD reddish-brown ring at the junction of the two layers confirmed the presence of glycosides.

Test for Carbohydrates

Benedict's test was used for analysis of the carbohydrates in the extract. A few drops of Benedict's Reagent were mixed with the extract, followed by boiling. The formation of reddish-brown precipitates shows the presence of carbohydrates.

Test for Proteins

Xanthoproteic test was performed by the addition of few drops of concentrated nitric acid to about 1 mL of the extract. The appearance of yellow color revealed the presence of proteins in the extract.

Mammalian Cell Culture and Cell Viability Analysis

K-562 (ATCC® CCL-243™) and ECV-304 cells were passaged as suggested by Atasever-Arslan, 2016 [8]. MTT test was performed

for cell viability analysis. *J. oxycedrus* stock extracts were prepared with methanol at concentrations of 50, 20, 10 and 5 µg/mL and made ready for MTT assay. Cells were treated with the extracts for 48 hours. ECV-304 cells incubated with the medium were used as the control group [9]. The cytotoxicity index was calculated by comparing the cytotoxicity values of the extracts with the cytotoxicity values of the control group.

RNA Isolation

Total RNA was isolated from K-562 cells treated with the substance at the concentration determined by the cytotoxicity test for 24 hours, with BIO BASIC Total RNA Miniprep as recommended by the manufacturer.

c-DNA Synthesis and Real-Time PCR

cDNA synthesis from total RNA was performed with OneScript cDNA Synthesis Kit according to the manufacturers protocol. After incubation of K-562 cell line with *J. oxycedrus* extract, changes in expression of Bcl-2, AKT, caspase 3 and β-actin genes were analyzed by Real-Time PCR (qPCR) method using BrightGreen 2X qPCR MasterMix. The primer sequences of the target genes are shown in Table 1. β-actin was used as the reference gene. Gene expression changes were calculated using the 2-ΔΔCt method.

Results and Discussions

Phytochemical Screening Results

The results obtained after the phytochemical evaluation of *J. oxycedrus* extract are shown in Table 2.

Cytotoxicity Analysis Results

The cytotoxic effect of *J. oxycedrus* microalgae fruit extract against K562 cells was investigated using the MTT cytotoxicity test. Non-cancer ECV304 cells were used as controls. It showed that 50 µg/mL concentration had the highest cytotoxic effect against K562 and ECV304 cells at a rate of 49±7.23% and 64±2.62 respectively. Other concentration of the extract (20, 10 and 5 µg/ mL) showed 28±1.26%, 24±3.26% and %0±2.83 cytotoxicity against K562 cells, respectively and 39±12.97%, 4±1.69% and 17±19.84%. The most appropriate dose of *J. oxycedrus* fruit extract for all experiments in this research was determined as 50 µg/mL considering cell viability (Fig. 1).

Real-Time PCR Results

After determining the appropriate dose (50 µg/mL) K-562 cells were incubated with the extract for 24 hours, RNA isolation was performed. cDNA was synthesized from the isolated total RNA. qPCR experiments were carried out using the obtained cDNA and specific primers. The primers used are BCL-2 and Caspase 3, which are involved in signaling pathways associated with apoptosis, and Akt, which is a member of the PI3K/Akt/mTOR pathway.

Akt is an important signaling molecule in tumor formation and progression. It is involved in cell proliferation and survival. A 1.092-fold change in gene expression was observed in K-562 cells incubated with *J. oxycedrus* fruit extract, close to control (Fig. 2).

BCL-2, the main regulator of the intrinsic pathway of apoptosis, is an antiapoptotic gene and is responsible for suppressing apoptosis. As a result of the experiments, it was observed that *J. oxycedrus* fruit extract decreased BCL-2 gene expression by 0.208 times in K-562 cells (Fig. 3).

Caspase 3 is one of the effector caspases located at the junction of the intrinsic and extrinsic apoptotic signaling pathways. It plays a role in apoptosis by being induced by

Table 1. **Primer Sequences of the Target Genes**

Target Gene	Primer Sequence
Bcl-2	Forward: 5′-GGTGGGGTCATGTGTGTGG-3′ Reverse: 5′-CGGTTCAGGTACTCAGTCATCC-3′
AKT1	Forward: 5′-AGCTCAGCCCACCCTTCAA-3′ Reverse: 5′-GCTGTCCACACACTCCATGCT-3′
Caspase 3	Forward: 5′-CAAACTTTTTCAGAGGGGATCG-3′ Reverse: 5′-GCATACTGTTTCAGCATGGCAC-3′
β-aktin	Forward: 5′-GGACATCCGCAAAGACCTGTA-3′ Reverse: 5′-ACATCTGCTGGAAGGTGGACA-3′

Table 2. **Phytochemical Screening Test Results of *Juniperus oxycedrus* extract**

Phyto-chemicals	Chemical Tests	Results
		Juniperus oxycedrus
Alkaloids	Hager's test	–
Saponins	Foam test	–
Flavonoids	General Test	+
Tannins	Alkaline Reagent Test	+
Glycosides	Keller Killiani Test	+
Carbo-hydrates	Benedict's Test	+**
Proteins	Xanthoproteic Test	N

** Green (0,1–0,5 % sugar), N: Not detected

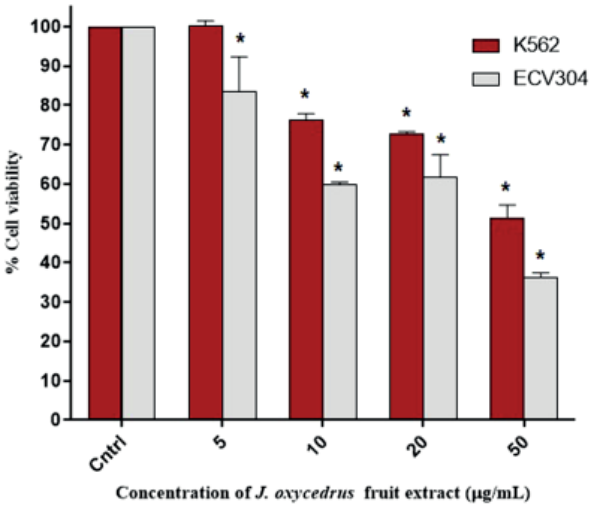

Fig. 1. **Cytotoxic effects of *J. oxycedrus* fruit extract against K562 and ECV304 cells**
The control was accepted as 100% and considered as baseline, then the amount of decrease in cell viability was shown at 5, 10,20, and 50 µg/mL, respectively (* $P < 0.05$)

various members of the BCL-2 family. While *J. oxycedrus* fruit extract decreased BCL-2 expression, a 1.218-fold increase in Caspase 3 expression was observed (Fig. 4).

Acute myeloid leukemia is the most common among adult leukemia and has the lowest survival rate. Genetic mutations are found in 97% of cases. Although the cause that induces the mutations is unclear, exposure to carcinogenic agents are risk factors [10].

In chronic myeloid leukemia, oncogenic fusion of BCR-ABL1 genes occurs as a result of translocation between chromosomes 9 and 22. The abnormal tyrosine kinase activity

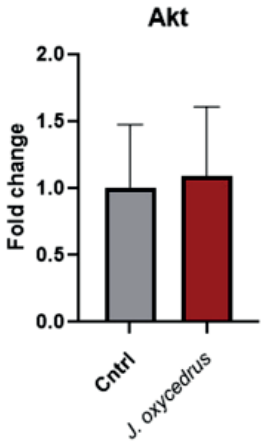

Fig. 2. **Effect of *J. oxycedrus* fruit extract on Akt gene expression**
(* $P > 0.05$)

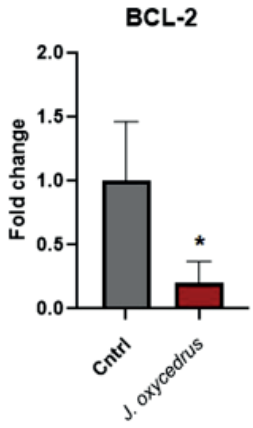

Fig. 3. **Effect of *J. oxycedrus* fruit extract on BCL-2 gene expression**
(* $P < 0.05$)

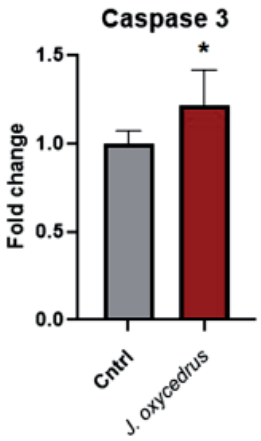

Fig. 4. **Effect of *J. oxycedrus* fruit extract on Caspase 3 gene expression**
(* $P < 0.05$)

exhibited by the BCR-ABL1 fusion protein leads to uncontrolled proliferation of cells and malignancy of pluripotent stem cells by activation of Ras/Raf/mitogen-activated protein kinase (MAPK) pathways [11]. In the treatment of the disease, the use of tyrosine kinase inhibitors, which block the ATP binding site of ABL1, is generally preferred. However, when drug resistance or drug sensitivity is observed in patients, additional therapies are needed [12]. In addition, due to the side effects of anticancer drugs, it is important to elucidate the anticancer activities of various natural products to support the treatment and to determine the supportive properties of chemotherapy [11, 12].

The bioactive metabolites contained in the juniper plant, which has many species, have been used in public health since ancient times. In one of the studies examining the effects of various cytotoxic juniper extracts with high antiproliferative effect, high efficiency activity was observed in K-562 human chronic myeloid leukemia cells. Studies show that juniper species, which can perform efficient biosynthesis of antiproliferative agents, are a natural source of drug precursors in the pharmaceutical industry [13].

In the scope of this study, the phytochemical structure and cytotoxic effect of *J. oxycedrus* extract was investigated; and the changes in the expressions of Akt1, the member of the PI3K/Akt/mTOR signaling pathway, caspase 3, which is one of the main effective genes in the pathways regulating apoptosis, and the apoptosis suppressor BCL-2 gene, which is an oncogene, were investigated.

As a result of the MTT tests, it was observed that the extracts obtained from the fruit parts of the *J. oxycedrus* plant showed over approximately 50% cell viability in K-562 cells at all doses. It is seen that the viability of K-562 cells decreases as the dose increases in the fruit extract. The viability of ECV-304 cells is also very low at a dose of 50 µg/mL, where the fruit extract has the highest lethality. ECV-304 cells are intravascular endothelial cell line. Angiogenesis plays an important role in metastasis [14]. The fact that the selected 50 µg/mL dose in these cells is cytotoxic indicates that the extract reduces metastasis and vascularization in the cells. This 50 µg/mL dose was chosen for the further experimental stages in order to investigate the intracellular signaling mechanisms.

After the gene expression analysis, it was observed that Akt expression increased 1.092 times (not statistically significant, $P > 0.05$), BCL-2 expression decreased approximately 3.3 times, and caspase 3 expression increased 1.2 times.

Akt1 is an important signaling molecule in tumor formation and progression. Dysregulation of Akt causes diseases that are difficult to treat clinically, such as diabetes, neurological and cardiovascular diseases, including cancer. Akt1 is widely found in tissues and is involved in cell proliferation and survival [15]. Its overactivation may mediate cellular events that promote tumorigenesis through its downstream effectors. Therefore, it is one of the most overactivated protein kinases in human cancers [16].

BCL-2 is a proapoptotic gene and suppresses elements that induce apoptosis. The decrease in its expression may pave the way for cancer cells to turn to apoptotic pathways. Caspase 3 is one of the essential elements of the apoptotic pathway. It is a determinant gene in the pathway to apoptosis when activated by other apoptotic signals [17]. A 1.2-fold increase in its expression may produce a similar effect to the decrease in BCL-2, resulting in apoptosis and programmed death of cells.

The obtained results revealed that the compounds contained in the *J. oxycedrus* plant were considered worthy of investigation in terms of their anticancer effects on chronic myeloid leukemia cells, but more research is still needed.

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Conflict of Interest

Authors declare that there is no conflict of interest.

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ФІТОХІМІЧНІ СКЛАДОВІ ТА ПРОТИЛЕЙКЕМІЧНІ ВЛАСТИВОСТІ ЕКСТРАКТУ *Juniperus oxycedrus*

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Мета. Багато генетичних факторів і факторів навколишнього середовища можуть бути ефективними в процесі виявлення та лікування онкологічних захворювань. Запобігти прогресуванню лейкемії можна, контролюючи шляхи, що включають такі механізми, як апоптоз і аутофагія. Є дослідження, які демонструють вплив різних видів ялівцю на різні лінії ракових клітин, включаючи клітини хронічного мієлоїдного лейкозу людини, але сигнальні шляхи, за якими вони діють, не повністю відомі. У цій роботі було досліджено протипухлинну дію екстракту *Juniperus oxycedrus* на клітини хронічного мієлоїдного лейкозу людини К-562.

Матеріали й методи. Після обробки клітин екстрактом *Juniperus oxycedrus* було проведено аналіз цитотоксичності та експресії генів. Зміни в експресії Akt, члена сигнального шляху PI3K/Akt/mTOR; каспаза 3, яка є одним з основних ефективних генів у шляхах регуляції апоптозу; і досліджено ген супресора апоптозу BCL-2, який є онкогеном.

Результати. Згідно з результатами тесту MTT, екстракт ялівцю оксицедрового показав приблизно 50% життєздатності клітин К-562 у всіх дозах. Найбільш відповідна доза екстракту плодів *Juniperus oxycedrus* у цьому дослідженні була визначена як 50 мкг/мл з урахуванням життєздатності клітин. Після аналізу експресії генів було виявлено, що експресія BCL-2 зменшилася приблизно в 3,3 рази, а експресія каспази 3 зросла в 1,2 рази. Хоча експресія гену Akt зросла в 1,092 рази, вона не була статистично значущою.

Висновки. Компоненти рослини *Juniperus oxycedrus* можуть мати апоптотичний ефект на клітини хронічного мієлоїдного лейкозу.

Ключові слова: *Juniperus oxycedrus*; лейкоз; апоптоз.