

Apoptosis-related gene modulation by *Juglans regia* leaf extract in breast and colon cancer cells

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Abstract: *Juglans regia* L. (walnut) leaves are rich in phenolic compounds, flavonoids, and terpenoids known for their antioxidant and pharmacological activities. Although *J. regia* has been widely used in traditional medicine, its potential anticancer mechanisms on different human cell lines remain to be fully elucidated. This study aimed to investigate the cytotoxic and apoptotic effects of ethanol extracts of *J. regia* leaves on human breast adenocarcinoma (MDA-MB-231), human colorectal adenocarcinoma (HT-29), and human keratinocyte (HaCaT) cell lines. Dried *J. regia* leaves collected from Balıkesir, Türkiye, were extracted with ethanol using the Soxhlet method. Cytotoxicity was evaluated by MTT assay in a dose- and time-dependent manner (100–50 µg/µL concentrations; 24, 48, and 72 h). Gene expression analysis of apoptosis-related *Bax*, *Bcl-2*, and cell cycle regulator *p27* was performed by quantitative real-time PCR (qRT-PCR). The ethanol extract of *J. regia* leaves exhibited a significant antiproliferative effect on MDA-MB-231 and HT-29 cells, while it showed no cytotoxicity on HaCaT cells. The effective concentration was determined as 100 µg/µL. qRT-PCR analysis revealed upregulation of pro-apoptotic *Bax* and *p27* genes and downregulation of anti-apoptotic *Bcl-2* in MDA-MB-231 and HT-29 cells in a time-dependent manner, suggesting induction of apoptosis and cell cycle arrest. These findings demonstrate that ethanol extracts of *J. regia* leaves possess potent anticancer and antiproliferative activities, likely mediated through the modulation of apoptotic pathways and regulation of the mitotic phase. *J. regia* may represent a promising natural source for developing novel anticancer agents.

INTRODUCTION

The genus *Juglans* (Juglandaceae) comprises numerous species distributed worldwide. *Juglans regia* L. (the walnut tree) is a well-known member that thrives in temperate regions and is commercially cultivated across Southern Europe, North Africa, Asia, the United States, and western South America (Oliveira *et al.*, 2008). Secondary metabolites such as terpenes, phenolic acids, flavonoids, and organic acids found in *J. regia* leaves show anthelmintic, antibacterial, antifungal, astringent, and hypoglycemic properties (Żurek *et al.*, 2023). Due to their high radical-scavenging potential, they possess high antioxidant properties. This property reduces oxidative stress in many health problems, thus demonstrating cell-protective properties. Exogenously acting antioxidants enhance the body's antioxidant regulation, maintaining the

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redox balance of living systems and supporting organ and cell health (Carocho & Ferreira, 2013). In this context, walnut leaf extract is used in traditional medicine for antidiarrheal, anti-inflammatory, hyperhidrosis, and gastrointestinal system treatment (Nahrstedt *et al.*, 1981; Pereira *et al.*, 2007; Pires *et al.*, 2018).

Cancer is a complex, multifactorial disease that requires a comprehensive approach for its prevention, control, and treatment. It is the second leading cause of death worldwide, accounting for a substantial proportion of global mortality (Marimuthu, 2008). According to the World Cancer Report, cancer incidence continues to rise at an alarming rate across the globe. Since ancient times, plants have been employed for the prevention and treatment of various human diseases, including cancer. *J. regia* has been reported in ethnomedicine as a plant with notable anticancer potential. Previous studies have demonstrated that *J. regia* leaf extracts exhibit antiproliferative activity against several cancer cell lines. Experiments on human kidney cancer cell lines (A-498 and 769-P) and the colon cancer cell line Caco-2 have shown that methanolic extracts of walnut leaves possess marked antiproliferative effects, likely attributed to their phenolic constituents (Carvalho *et al.*, 2010). Similarly, Yang and colleagues reported that walnut seed extract exerts strong antiproliferative effects on human HepG2 liver and Caco-2 colon cancer cells (Yang *et al.*, 2009). Furthermore, Żurek *et al.* (2023) demonstrated that methanolic extracts of *J. regia* leaves display potent cytotoxicity against colon adenocarcinoma (DLD-1 and Caco-2) and breast adenocarcinoma (MCF-7) cells, among others commonly observed in human cancers.

In the present study, the cytotoxic effects of *J. regia* leaf ethanol extract were evaluated on HaCaT, HT-29, and MDA-MB-231 cell lines. Dose- and time-dependent MTT assays were conducted to determine both cytotoxicity and the effective inhibitory dose. Based on these findings, the time-dependent expression levels of the apoptosis-related genes *Bax*, *Bcl-2*, and *p27* were analyzed in HaCaT, MDA-MB-231, and HT-29 cell lines.

METHOD

Samples and Extraction Procedure

J. regia L. leaves were collected in June 2025 from the Susurluk region of Balıkesir, Türkiye, at the following coordinates: 39°56'52' N, 28°10'05' E, 100 m, 28.10.2025 (GT 5201). To prevent degradation of phenolic substances in the plant, the collected plant samples were washed with sterile distilled water and dried at room temperature in the dark. The dried plant samples were dried in a hot air oven at 36 °C for 3 days. After the drying process, the leaves were ground into powder and stored at -20 °C until use.

For extraction, 7 g of powdered *J. regia* (JR) leaf material was subjected to Soxhlet extraction using ethanol of analytical grade as the solvent. Extraction was carried out at a constant temperature of 60 °C for 5 h to ensure complete extraction of bioactive molecules. The solvent in the crude extract collected in the collection vessel was removed under pressure using a rotary evaporator (Heidolph Instruments GmbH). The extract was concentrated. The dried extract was weighed, transferred into an airtight container, and stored at -20 °C for subsequent analyses (Elouafy *et al.*, 2022).

MTT Assay for the Determination of Cytotoxic and Antiproliferative Activity

This study used the MDA-MB-231 (human breast adenocarcinoma), HaCaT (human primary keratinocyte), and HT-29 (human colorectal adenocarcinoma) cell lines. The cell lines used in this study were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS). Cells were incubated in cell growth dishes in a humidified incubator containing 5% CO₂ and 95% air at a constant temperature of 37 °C. When the desired cell population was reached, cells were removed from the flask using trypsin, and the total cell number was counted. The cell suspension was distributed into 96-well microplates at 5×10^3 cells per well. The microplates were incubated in the incubator for 24 hours to allow the cells to adhere to the flask. The crude ethanol extract was dissolved in dimethyl sulfoxide (DMSO)

to obtain a 50 mg/mL master stock solution. After the incubation period was completed, the extract was added to the experimental groups at a final concentration of 50, 100, 200, 400, 600, 800, and 1000 µg/µL. The final DMSO concentration used in all wells did not exceed 1%. DMSO was also added to the control groups at the same rate. After application, the plates were incubated for 24, 48, and 72 hours. The MTT assay was performed to determine the proliferative effects of the experimental and control groups. MTT solution was added to the groups that completed the incubation period and incubated for 4 hours. The formazan crystals formed at the end of this period were dissolved in isopropanol containing 0.004 M HCl, and their absorbance at 550 nm was measured. The experimental and control groups were compared to determine the cytotoxic activity of the extract in a dose- and time-dependent manner. The experiment was carried out in 3 repetitions. The results were evaluated statistically with One Way Anova in GraphPad Prism 8 program ($p < 0.05^*$ was considered significant) (Türkoğlu *et al.*, 2024).

Transcriptional Quantification of Key Apoptotic Markers via qRT-PCR

To evaluate the effect of JR on apoptotic gene expression, cells grown in cell culture were seeded in individual cell growth dishes at a density of 2×10^6 cells. JR ethanol extract was added to the experimental group cells, which were incubated overnight, at a final concentration of 100 µg/µL, the next day. Experimental and control group cells that completed 24, 48, and 72 hours of incubation were detached from the surface with trypsin, and RNA was isolated from the cell pellets using the GeneJET RNA Purification Kit (Thermo Scientific, USA). Purified RNA samples were then used for cDNA synthesis. PCR reaction with human β -microglobulin-specific primers was performed to determine the quality of the obtained cDNAs. qRT-PCR was performed to determine the effective expression level of *Bax*, *Bcl-2*, and *p27* genes in JR using the determined cDNAs. The primer sequences used are listed in Table 1. The obtained data were calculated using the $2^{-\Delta\Delta C_t}$ method described by Livak and Schmittgen (2001).

Table 1. Primer sequence list used in qPCR.

Primer Name	Sequence
<i>Hβ-microglobulin</i>	5'-TTTCTGGCCTGGAGGCTATC- 3' 3'-CATGTCTCGATCCCCTTAAC- 5'
<i>Bax</i>	5'-TTTGCTTCAGGGTTTCATCC- 3' 3'-TGGTCACGGTCTGCCACGTG- 5'
<i>Bcl-2</i>	5'-GAGATAGTGATGAAGTACAT- 3' 3'-CAAAGAAGGCCACAATCCTC- 5'
<i>P27</i>	5'-TGAAGTCGACGACTTCTCCT- 3' 3'-GCCACACAGCTGGATGCTAGT- 5'

Statistical Analysis of Data

Real-Time PCR and MTT experiments were performed in triplicate. The results were statistically evaluated using One Way Anova in the GraphPad Prism 8 program ($p < 0.05^*$ was considered significant).

RESULTS

The effects of ethanol extract obtained from JR leaves on the viability of human breast carcinoma MDA-MB-231, human colon carcinoma HT-29, and human keratinocyte HaCaT cells were evaluated using dose- and time-dependent MTT assays. The obtained ethanol extract was dissolved in DMSO to prepare the main stock. The desired dose calculations of the extract studied in a dose-dependent manner were prepared by diluting the main stock. One day later, 1000, 800, 600, 400, 200, 100, and 50 µg/µL doses were applied to the experimental groups. The absorbance values obtained were compared with the control group to determine the dose- and time-dependent cytotoxic effect of JR on MDA-MB-231, HT-29, and HaCaT cells. According to the results of the MTT test performed on HaCaT cells to determine the cytotoxic

effect of JR leaf extract on healthy cells, it decreased cell proliferation at the two highest doses in all time periods and increased cell proliferation at the other doses. (Figure 1A). To determine the anticancer effect of JR leaf extract, its cytotoxic activity on MDA-MB-231 and HT-29 cells was evaluated in a dose and time-dependent manner.

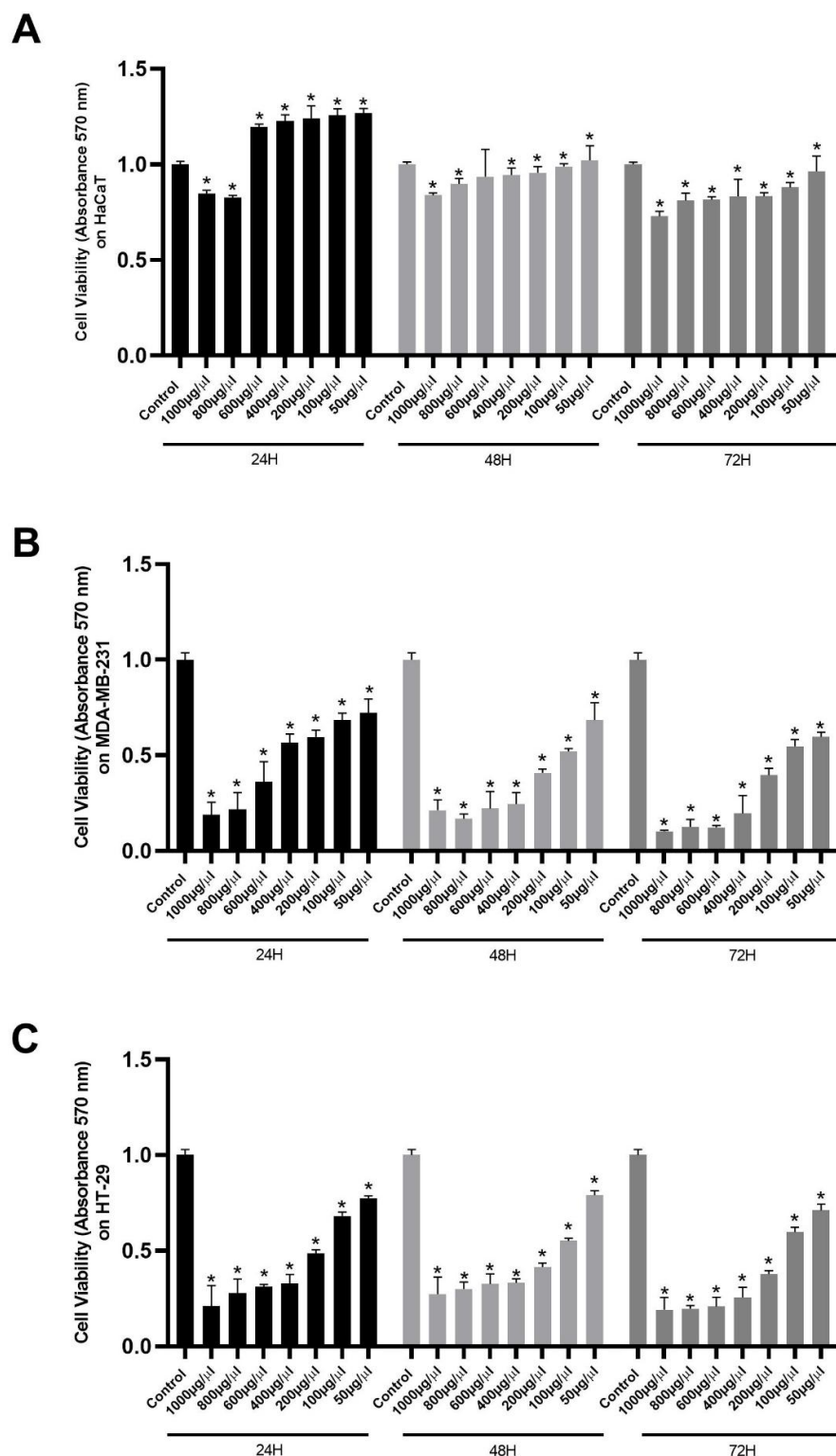


Figure 1. Cytotoxic effect of JR leaf ethanol extract. A: Cytotoxic effects on the HaCaT cell line are depending on dosage and timing. B: Cytotoxic effects on the MDA-MB-231 cell line are depending on dosage and timing. C: Cytotoxic effects on the HT-29 cell line are depending on dosage and timing. (* $p \leq 0.05$).

According to the results of the 24, 48, and 72 hours of application, the two highest doses in the 24-hour period maximally inhibited MDA-MB-231 cells, while the reducing effect diminished

as the dose decreased. At the 48 and 72-hour periods, cell viability decreased significantly at the higher doses, while a 50% cell death rate was determined at the 100 $\mu\text{g}/\mu\text{L}$ dose (Figure 1B). In HT-29 cells, doses of 1000, 800, 600, and 400 $\mu\text{g}/\mu\text{L}$ significantly reduced cell viability at all time points. The 100 $\mu\text{g}/\mu\text{L}$ dose demonstrated the same effect on MDA-MB-231 cells (Figure 1C).

For the molecular characterization of the anticancer effect of JR leaf extract, apoptosis-related *Bax*, *Bcl-2*, and *p27* gene expressions were analyzed (Figure 2). For this purpose, 100 $\mu\text{g}/\mu\text{L}$ JR leaf extract was applied to HaCaT, MDA-MB-231, and HT-29 cells. Following the application, RNA pellets were collected from the cells at 24, 48, and 72 hours, and then cDNA was synthesized. qRT-PCR was performed using the obtained cDNAs. When the mRNA level of the *Bax* gene was analyzed, the 24-fold increase in mRNA in HaCaT cells did not show a high increase compared to the control group (Figure 2A).

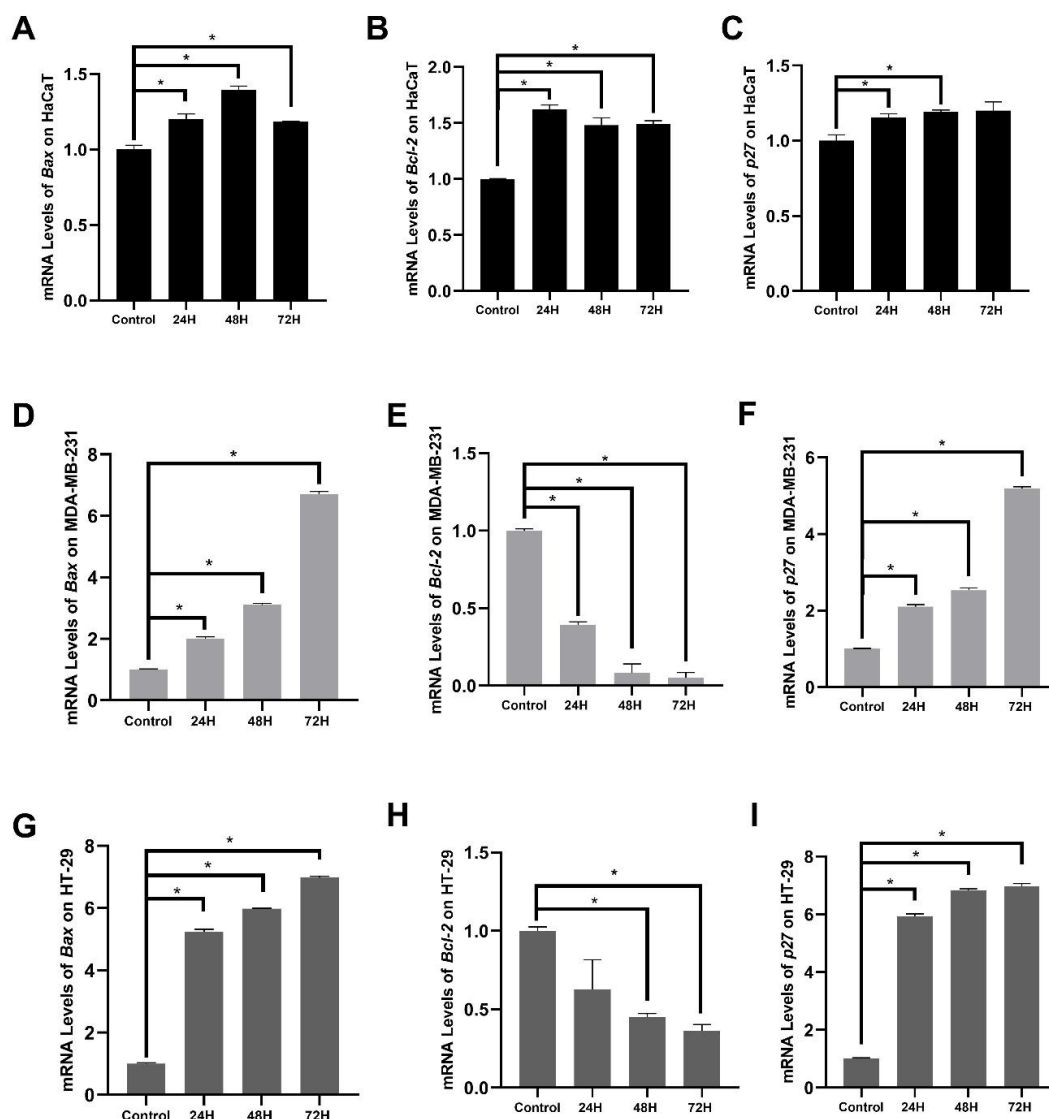


Figure 2. Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *Bax*, *Bcl-2* and *p27* genes. A: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *Bax* gene in HaCaT cell line. B: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *Bax* gene in MDA-MB-231 cell line. C: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *Bax* gene in HT-29 cell line. D: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *Bcl-2* gene in HaCaT cell line. E: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *Bcl-2* gene in MDA-MB-231 cell line. F: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *Bcl-2* gene in HT-29 cell line. G: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *p27* gene in HaCaT cell line. H: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *p27* gene in MDA-MB-231 cell line. I: Effect of 100 $\mu\text{g}/\mu\text{L}$ JR leaf ethanol extract on the expression level of *p27* gene in HT-29 cell line. (* $p \leq 0.05$).

However, the 24-fold increase in *Bax* mRNA level in MDA-MB-231 and HT-29 cells was time-dependent. Especially at 72 hours, it increased approximately 8-fold in both cell lines compared to the control group (Figure 2 B and C). When the mRNA level of the *Bcl-2* gene was analyzed, the expression of the *Bcl-2* gene, which increased in the early hours in HaCaT cells, was at similar levels at 48 and 72 hours (Figure 2D). In MDA-MB-231 and HT-29 cell lines, the expression level of the *Bcl-2* gene showed a time-dependent decrease (Figure 2E and F). The mRNA level of the *p27* gene did not show a significant increase in HaCaT cells at 24, 48, and 72 hours (Figure 2G). It showed a time-dependent increase in MDA-MB-231 and HT-29 cell lines. The increase in HT-29 cells was particularly notable (Figure 2H and I).

DISCUSSION and CONCLUSION

Cancer is one of the diseases with the highest mortality rate worldwide. Due to this high mortality rate, researchers are conducting numerous studies to discover new anticancer agents. More than 60% of the anticancer agents currently used are derived from plants (Cragg & Newman, 2005). Plants are used as chemotherapeutic agents due to the biological and pharmacological activities of their secondary metabolites (Cragg *et al.*, 1999; Verpoorte, 1998). Epidemiological studies have indicated that regular consumption of plants reduces the risk of certain cancers, such as stomach, colon, breast, and pharynx cancers (Jenab *et al.*, 2004; Mathew *et al.*, 2004; Reddy *et al.*, 2003).

Juglans regia is the best-known member of the Juglandaceae family. Due to the proven biological activities of its chemical constituents, many parts of the plant (root, bark, fruit, leaves, etc.) are used for therapeutic purposes. Various parts of the walnut have been used in medicine since ancient times. Later, its use in the pharmaceutical and cosmetic industries has attracted attention (Oliveira *et al.*, 2008). Walnut leaves contain a wide variety of substances, including tannins, naphthalene derivatives, flavonoids, phenolic acids, essential oils, ascorbic acid, cyclitols, and mucilage. The best-known naphthoquinone found in fresh leaves is juglone. The phenolic content in the leaves is highest between May and July (Cosmulescu *et al.*, 2011; Nour *et al.*, 2013). Therefore, fresh JR leaves were collected from the Susurluk district of Balıkesir Province in June and extracted with ethanol. After removing the solvent with an evaporator, the extract was dissolved in DMSO to prepare the master stock. MTT assay was performed to determine the cytotoxic effect of JR leaf extract. Three cell lines were used for this purpose: HaCaT, MDA-MB-231, and HT-29. HaCaT cells are human epidermal keratinocytes. They are a healthy cell line and do not form tumors. HaCaT cells were selected as the healthy cell line in our study.

The MDA-MB-231 cell line is highly invasive due to the lack of estrogen receptors (ER), progesterone receptors (PR), and E-cadherin, and is a model for late-stage breast carcinoma (Welsh, 2013). The HT-29 cell line is used for human colon carcinoma studies and to study the properties of mature intestinal cells. HT-29 cells carry a mutation in the p53 gene. Consequently, altered tumor suppressor functions, increased cellular proliferation, and impaired apoptosis mechanisms are observed (Verhoeckx *et al.*, 2015). Seven different doses of JR leaf ethanol extract (1000, 800, 600, 400, 200, 100, and 50 µg/µL) were analyzed. JR leaf extract, which did not show cytotoxic effects on HaCaT cells at different doses examined over time, showed cytotoxic effects on MDA-MB-231 and HT-29 cell lines. Especially at high doses, JR 100 µg/µL showed a potent anti-proliferative effect, significantly reducing cell viability.

Apoptosis is an evolutionarily conserved programmed cell death mechanism that plays an important role in physiological processes and is actively involved in tumor suppression mechanisms. The B-cell lymphoma/leukemia type 2 (*Bcl-2*) protein family is the most important family regulating apoptosis. *Bcl-2* and *Bax*, two important members of the apoptosis-associated family, act alone or by dimerizing. Inducing *Bax* expression drives cell apoptosis, while inducing *Bcl-2* inhibits apoptosis (Babacan, 2023). The study using methanol, chloroform, and hexane extracts from JR root bark demonstrated the anticancer activity of these extracts in the MDA-MB-231 cell line. All extracts studied increased the expression of *Bax*,

caspase 3-8, *TNF- α* , and *Tp53*, while decreasing the expression of *Mdm-2* and *Bcl-2* (Alshatwi, 2011). In a study on PC3 cells, a human prostate carcinoma cell line, it was determined that hexane extract of JR leaves exhibited dose- and time-dependent anti-proliferative effects (Li *et al.*, 2015). Methanol extracts of JR leaves showed dose-dependent growth inhibition against human kidney carcinoma (A-498 and 769-P) and human colorectal cancer cell line (Caco-2) cells (Carvalho *et al.*, 2010). In this study, the effect of ethanol extract of JR L. leaves on the regulation of apoptosis-related genes in MDA-MB-231, HT-29, and HaCaT cells was analyzed. After application of JR extract, the expression level of the apoptosis-associated gene *Bax* was induced in MDA-MB-231 and HT-29 cells, but did not show any significant change in HaCaT cells. The expression level of the anti-apoptotic gene *Bcl-2* did not show any significant change in HaCaT cells but showed a significant decrease in MDA-MB-231 and HT-29 cells, particularly over time. *p27*, a tumor suppressor, is a member of the CDK inhibitor proteins that bind and inhibit cyclin-CDK to arrest the cell cycle and has an important role in cell cycle regulation in response to antimitogenic signals. *p27*, a tumor suppressor, is a member of the CDK inhibitor proteins, which bind to and inhibit cyclin-CDK to arrest the cell cycle and play a crucial role in cell cycle regulation in response to antimitogenic signals. We analyzed *p27* expression levels in selected JR-treated cell lines. While *p27* expression levels were induced in a time-dependent manner in the MDA-MB-231 and HT-29 cell lines, no significant changes were observed in HaCaT cells.

This study determined the anticancer and antiproliferative effects of JR L leaf ethanol extract on HaCaT, MDA-MB-231, and HT-29 cells. The data obtained indicate that JR leaf ethanol extract has anticancer effects, exerting this effect by regulating the *Bax* and *Bcl-2* genes and also regulating the mitotic phase via the *p27* gene.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

Data Availability Statement

Research data is available and can be sent via email upon request.

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