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RESEARCH ARTICLE

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# Investigation of apoptotic, cytotoxic, and antioxidant effects of *Juglans regia* against MDA-MB-231 and A549 cell lines

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ABSTRACT

Cancer is one of the most common pathologies in the world, which for centuries has led to a decline in the standard of living and even death of many people. In short, cancer is a disease that occurs when mutations in genes that limit and regulate cell proliferation, survival, and movement are reflected in somatic cells. In recent years, there have been many new and promising developments in the world of science for cancer treatment. However, due to the side effects of some treatments, the cost of others, and the fact that some drugs are still in the trial phase, it has not yet reached the expected level of treatment and success. This has encouraged the scientific community to investigate natural agents due to their lower cost and limited side effects. *Juglans regia* L., popularly known as walnut, is a plant containing many natural compounds that have been used both as a foodstuff and for various medicinal purposes from past to present. As a result of the literature review, it is known that walnut has many medicinal and biological properties, especially anti-inflammatory, antifungal and antiallergic properties. In this study, the outer shells of *J. regia* were used and the components of these shells were extracted with three different solvents: methanol, ethanol, and hexane. These extracts were used in cytotoxic activity and antioxidant activity assays. The antioxidant activity of the extract was determined using a DPPH assay. Cytotoxic activity was determined by MTT assay using a breast cancer cell line (MDA-MB-231). *J. regia* extracts were found to have significant cytotoxic activity on the MDA-MB-231 cell line. It has been observed that the outer bark of *J. regia* showed more potent anticancer effects than antioxidant activity. According to the results obtained from the study, the antioxidant and cytotoxic effect of the outer bark of *J. regia* is thought to contribute significantly to the identification of new active substances for cancer treatment. Therefore, further studies are needed to fully determine the effects of the outer bark of *J. regia*.

## 1. Introduction

Cancer is one of the 20th century's most dreaded diseases, cancer is still occurring with persistence and increasing incidence. The situation is so alarming that one in four people is at a lifetime risk of cancer (Roy & Saikia, 2016). With more than 10 million deaths per year, cancer is one of the most important health problems on earth, and both its diagnosis and treatment have become a major challenge for the scientific community (Siddiqui et al., 2022). Breast cancer is the most common type of cancer and one of the most common causes of cancer death in women (Khan et al., 2022). Although treatments for breast cancer have made significant progress in recent years, many patients continue to experience metastasis and tumor growth due to chemoresistance as well as many disadvantages associated with chemotherapy and radiotherapy. For this reason, researchers are exploring new methods to better understand cancer cells' behavior and develop more effective therapies against them. Due to the serious side effects of synthetic drugs used for treatment and the medical and economic problems they cause, treatment with herbs has become popular (Akkol et al., 2020).

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Herbal medicines have become a very safe, non-toxic, and readily available source for compounds that treat cancer. Plants are believed to neutralize the effects of diseases in the body due to their various properties (Khan et al., 2019). Oilseeds and nuts play an important role in the nutrition of cancer patients because they are rich in biocomponents (Deniz Güneş & Acar Tek, 2021).

*Juglans regia* L., popularly known as walnut, is one of 64 species belonging to the Juglandaceae family. It is a plant thought to be native to the Middle East, Anatolia, northern parts of Iran, and the Himalayas. Since ancient times, *J. regia* has been used not only as a food but also in fields such as pharmacology and cosmetics with its different parts. It has also proved to be effective against coronary heart disease, diabetes, obesity, and rheumatism with regular use. Considering the results of the previous studies, it is seen that the regular consumption of walnuts causes a decrease in the incidence of cancer (Catanzaro et al., 2015; Schmiech et al., 2019).

## 2. Materials and methods

### *2.1. Extract preparation*

*J. regia* was collected from the Cip district of Elazığ in Turkey. The outer reddish shells were separated, dried, and ground to give one gram of powder. Then, 1 g was taken and kept for 72 hours with 10 ml of methanol, ethanol, and hexane solvents in a shaker incubator. All extracts were filtered with Whatman filter paper to remove extra particles of outer shells. The final stock was mixed with 10 ml of DMEM medium to gain the desired concentration, as shown in (Figure 1).

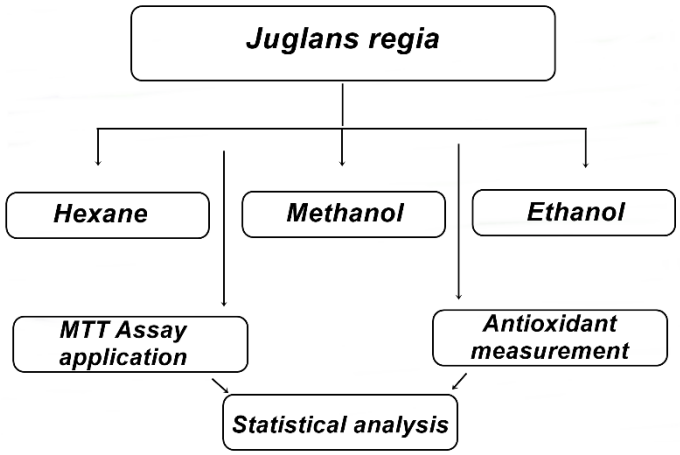


**Figure 1.** Experiment flowchart
The general design of the experiment has shown applied methods on the outer shells of *J. regia*.
Three different solvents; hexane, methanol, and ethanol were used to evaluate cytotoxic and antioxidant activities.

### *2.2. Supply of cell line*

MDA-MB-231 and A549 cell lines were obtained from Fırat University. Both cell lines were grown in DMEM [1% L-Glutamine, 1% Penicillin-Streptomycin, and 10% FBS (Fetal Bovine Serum)] in 25cm$^2$ flasks at 37 °C and 5% $CO_2$ atmosphere conditions.

#### *2.2.1. Cell culture and cell line*

When both cell lines grown in 25cm$^2$ flasks became 90% confluent, the medium in the flask was removed and washed with 5ml sterile PBS solution. 1ml Trypsin-EDTA was added to the flasks and incubated at 37 °C for 2 minutes in an incubator with 5% $CO_2$ and Trypsin-EDTA was inactivated with 5ml medium. Cells were removed from the flask and centrifuged at 1200 rpm for 5 minutes, then the supernatant was removed, the cell pellet was diluted with DMEM and 100 µl was seeded into 96-well plates according to the calculation after cell counting. Only medium was added to the first row to be used as blank and incubated for 24 hours at 37 °C in an incubator containing 5% $CO_2$. After incubation, *J. regia* outer shell extract diluted with the medium was added in three different concentrations, 12 replicates of each concentration, and incubated for 72 hours at 37 °C in an incubator containing 5% $CO_2$. 2.5 µg/ml doxorubicin was used as the positive control and DMEM was used as the negative control. MTT test was performed after incubation.

### *2.3. MTT assay application*

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a method used for measuring cell viability, proliferation, and cytotoxicity. The MTT method is based on the active mitochondria in living cells, reducing the MTT, a tetrazolium salt that can pass through the cell membrane, by taking electrons inside the cell and converting it into purple water-insoluble formazan crystals (Dalkilic et al., 2021; Riss & Moravec, 2004). The effect of plant extracts on cell viability at the end of incubation was determined by the MTT method. First, 20µl 5 mg/ml MTT solution was added to the wells containing cells from stock MTT (Thermo Fisher, USA) prepared in sterile phosphate buffer (pH: 7.2) and incubated for 4 hours at 37 °C in a dark environment containing 5% $CO_2$. After incubation, the medium was removed and formazan crystals were dissolved with 100µl DMSO (dimethylsulphoxide). The absorbance values were then determined using a plate reader (Synergy HT USA) device at a wavelength of 570 nm. By reading the control wells, the absorbance values obtained were averaged and this value was accepted as 100% live cell. The absorbance values obtained from the wells of plant extract were compared to the control absorbance value and percent viability values were calculated (Coskun et al., 2021; Dalkilic et al., 2021). The absorbance values measured with the plate reader device were recorded, then the measured absorbance values were compared with the control groups.

### 2.4. Antioxidant measurement

The antioxidant properties of *J. regia* were determined by DPPH free radical scavenging activity. DPPH (Sigma-Aldrich, USA) was prepared in methanol as a free radical. A stock solution of extracts was prepared and diluted with methanol as 2, 1, 0.5, 0.25, and 0.125 mg/ml. Methanol (2.5 ml) was used as a negative control. 0.3 mM DPPH methanol solution was added to the samples. The plate was left under incubation for 30 minutes in a dark environment, and the spectrophotometer absorbance values were measured at 517 nm. Due to the reduced absorbance, the remaining DPPH is detected as free radical removal activity (Flieger & Flieger, 2020; Huang et al., 2005).

Results were calculated based on the formula given below:

$$Antioxidant\ activity\ (\%) = \frac{Control\ ABS - Sample\ ABS}{Control\ ABS} x\ 100$$

## 3. Results and discussion

The inhibition percentage of the DPPH radical in different concentrations of the methanol extract of the *J. regia* shell is mentioned in (Figure 2). According to the results, outer shell extracts of *J. regia* have shown antioxidant activities in a dose-dependent manner. The cytotoxic activity of the shell extract of *J. regia*, with ethanol, methanol, and hexane on the MDA-MB-231 (breast cancer) cell line at concentrations of 1/1000, 1/2000, and 1/4000 µg/ml were investigated. According to the results obtained from the study, methanol and hexane extracts exhibited significant differences compared to the control ($p$ <0.05) at 1/1000 µg/ml concentration.

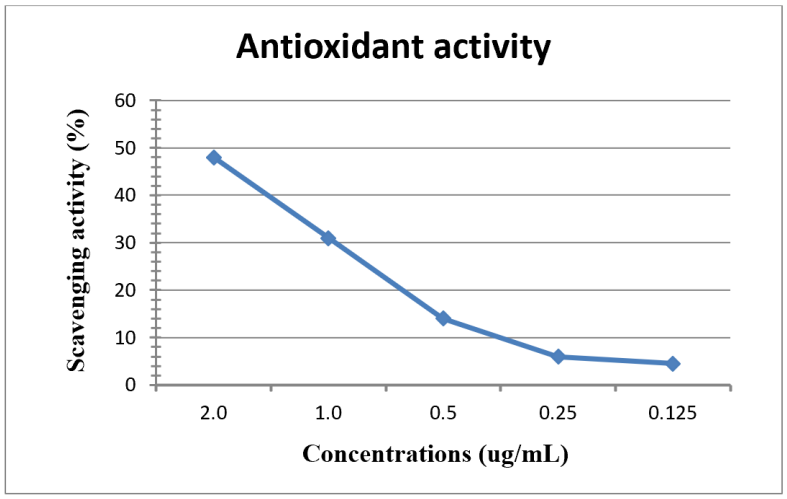


**Figure 2.** Percent inhibition of DPPH free radical scavenging activities
Percentage change of DPPH radical scavenging inhibition activity of methanol extract of *J. regia* according to concentrations.

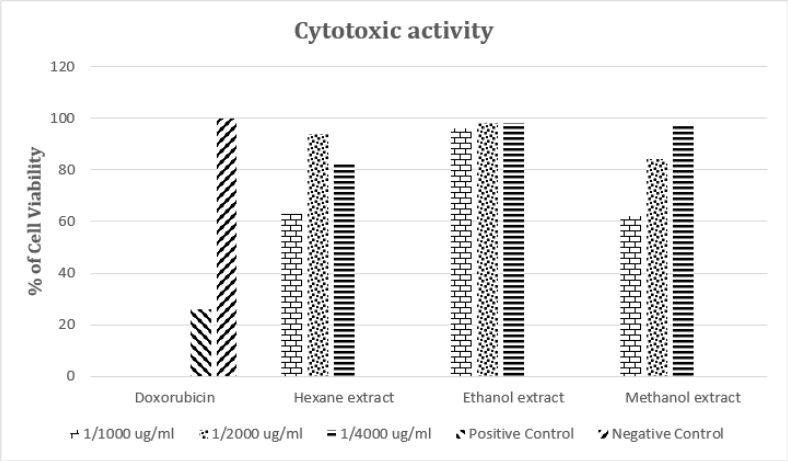


**Figure 3.** Results of cytotoxic activity in the MDA-MB-231 cell line
Represents the percentage of live cells, three extracts, three different concentrations, and analysis with MTT assay to observe effects of *J. regia* on MDA-MB 231 cell lines, DMEM: negative control (-), doxorubicin (2.5 µg/ml): positive control (+).

Cell viability was reduced and it was observed to have a cytotoxic effect at 1/1000 µg/ml concentration. While ethanol extract of *J. regia* showed non-significant differences compared to the control. Cell viability is not reduced and it was observed to have no cytotoxic effect at all concentrations. Also, similar results were detected in methanol and hexane extracts at (1/1000 µg/ml, 1/2000 µg/ml) concentration as shown in Figure 3. It was clear that the ethanol extract of *J. regia* did not show any cytotoxic activity at the concentrations of 1/1000 µg/ml, 1/2000 µg/ml, and 1/4000 µg/ml against the MDA-MB-231 cell line.

The nuclear morphology images in the A549 cell line treated with the methanol and ethanol extracts of *J. regia* bark at a dose of 2.0 µg/ml are shown in Figure 4. Images were taken with a fluorescence-inverted microscope at 10X magnification. Cells were stained with Hoechst 33258 dye to visualize cell death [Positive control (doxorubicin 2.5 µg/ml), negative control (untreated cells)]. Hoechst stain was used to visualize the nuclear morphology of the cells.

Methanol and ethanol extract applied in the A549 cell line induced apoptosis in cells. However, when the two extracts were compared, it was observed that the methanol extract showed higher apoptotic activity than the ethanol extract.

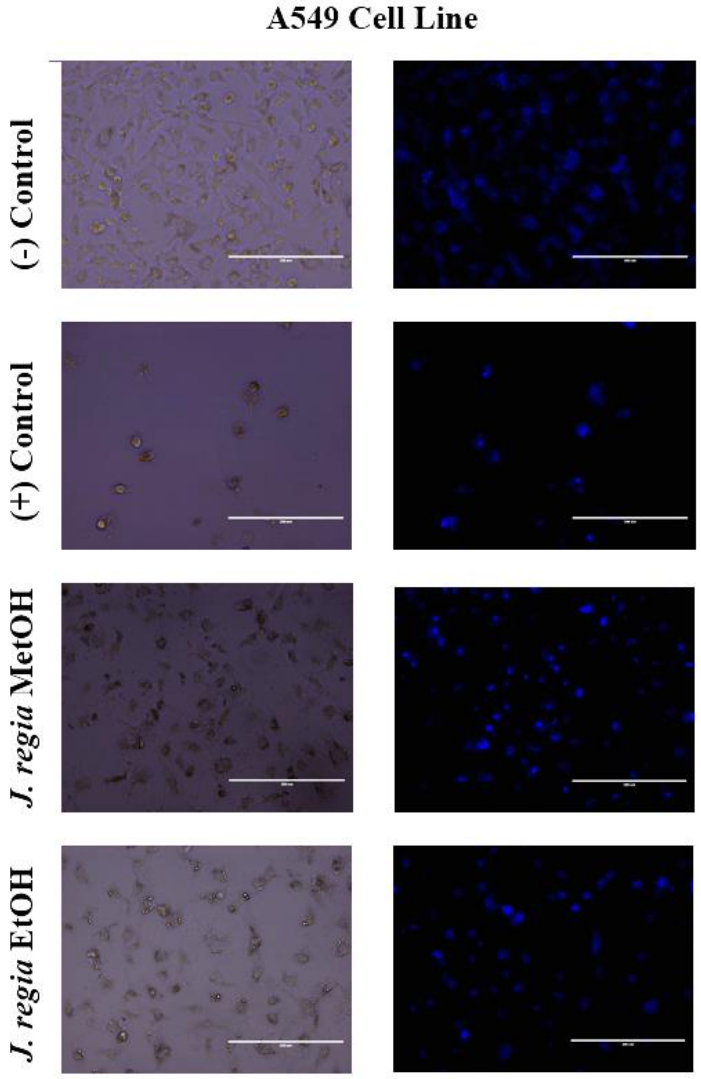


**Figure 4.** Results of the double staining method
Blue stained areas represent propidium iodide stain, (-) control represents untreated cells, and (+) control represents doxorubicin-treated cells.

*J. regia* flowers are known to contain multiple polyphenolic compounds that exhibit antioxidant effects through various mechanisms (Żurek et al., 2022). In addition, it has been reported by many authors that *J. regia* is one of the nuts with the highest antioxidant activity (Halvorsen et al., 2002; Kornsteiner et al., 2006; Mishra et al., 2010). Among various dried fruits, the methanolic extract of *J. regia* has been found to have very high antioxidant activity. Studies have also suggested that the shell or seed coat surrounding the kernel also has high antioxidant activity (Jahanban-Esfahlan et al., 2019). Some studies in the literature have shown that *J. regia* green bark has an antioxidant effect in the presence of polyphenolic compounds (Oliveira et al., 2008; Zhou et al., 2015). In a study, *J. regia* seed, green bark, and leaf parts were extracted with methanol and petroleum ether, and their antioxidant effect was investigated. As a result, it was observed that the radical effect increased with the increase in the concentrations of all methanolic extracts. At the same time, it was determined that the extracts of walnut leaf and green shell prepared with petroleum ether had the lowest radical effect (Carvalho et al., 2010). In another study, the antioxidant activity of *J. regia* green peel was determined by comparing the maturity stages. The results showed that the antioxidant capacity of ripe green peel was higher (Soto-Madrid et al., 2021). The flavonoids of *J. regia* leaves are known to have strong antioxidant properties (Zhao et al., 2014). In a previous study, *J. regia* leaves were powdered and their antioxidant effect was examined by dissolving with methanol. At the end of the study, it was seen that it had a higher value than ascorbic acid used as a reference, and the results obtained were determined to be significant (Shah et al., 2018). In another study, the antioxidant activity of the extract of powdered *J. regia* leaves with methanol

was determined at different concentrations, and in line with the results obtained, it was determined that it exhibited an effective scavenging capacity depending on the concentrations (Pereira et al., 2007).

Natural products are a good source of oncogene protein modulators and many of them or their derivatives have been developed as anticancer drugs such as doxorubicin and bleomycin (Yuan et al., 2020). In the current era, natural agents have a very important place in the treatment of cancer, either because of their various components or as a whole (Catanzaro et al., 2018). *J. regia* peel is part of the residue from the consumption of the fruit and its components are used as antimicrobial, anticancer, and antioxidant agents (Vieira et al., 2020; Yin et al., 2019; Zakavi et al., 2013). In a study, the cytotoxicity of the membrane surrounding the *J. regia* nucleus was determined in MCF-7, Caco-2, and a primary fibroblast cell line (HFF-1) with different concentrations of *J. regia* nucleus membrane. The results showed a significant decrease in the Caco-2 cell line at 24 and 48 hours. However, no cytotoxic effect was found on HFF-1 and MCF-7 cell lines (D'Angeli et al., 2021). In another study, the anticancer effect of the extract from *J. regia* seeds on breast cancer cell line MDA-MB231 and colon cancer cell line HT-29 was determined using MTT [3-(4, 5-dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide]. In line with the results obtained, it was determined that *J. regia* seeds showed inhibitory properties in HT-29 and MDA-MB231 cells and had the same antioxidant effect (Jahanbani et al., 2016). In another study, the polyphenol fraction isolated from the pollen of male flowers of the *J. regia* tree was investigated for its antioxidant and anticancer effects in cell lines such as breast cancer cell line (MCF-7), colorectal cancer cell lines (DLD-1, Caco-2) and glioblastoma cell line (U87MG). According to the results of this study, the strongest cytotoxic activity of *J. regia* pollen was observed in Caco-2 and MCF-7 cell lines, while the lowest cytotoxic activity was observed in melanoma (SK-Mel-29) and astrocytoma (U251MG) cell lines (Żurek et al., 2022). The anticancer effect of *J. regia* extracts on the human neuroblastoma cell line (IMR-32); human breast epithelial cell line (HBL-100); human osteosarcoma cell line (U2OS) was investigated. The results obtained from this study emphasized that the *J. regia* tree constitutes a very good source of effective natural antioxidants that can act as anti-cancer agents, considering that *J. regia* extracts exhibited greater cell viability in U2OS cells versus IMR-32 and HBL-100 cells (Shah et al., 2018).

## 4. Conclusions

When the apoptotic, cytotoxic, and antioxidant activities of *J. regia* against MDA-MB-231 and A549 cell lines were examined, the highest effect in the antioxidant study was reported at a concentration of 2.0 µg/ml of *J. regia* bark prepared in methanol solvent, and the lowest effect was reported at a concentration of 0.125 µg/ml. When the cytotoxic activity assay results were examined, the most effective result in the MDA-MB-231 cell line was seen in the highest concentrations of methanol and ethanol extracts of a walnut shell. When the results of apoptotic activity were examined, the shell extracts of *J. regia* showed evident apoptotic activity even if it was not as potent as doxorubicin. Our results indicate that *J. regia* bark extract may be an effective agent in the fight against cancer, and also support previous studies.

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## Conflict of interest

The authors confirm that there are no known conflicts of interest.

## Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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## CRediT authorship contribution statement

**Lütfiye Kadıoğlu Dalkılıç:** Project administration, Conceptualization
**Semih Dalkılıç:** Data curation, Supervision, Validation
**Lütfü Uygur:** Writing-reviewing & editing

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## Supplementary File

None.

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