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ANTIPROLIFERATIVE AND APOPTOSIS-INDUCING EFFECTS OF JUGLANS REGIA L. LEAF EXTRACT ON HUMAN BREAST ADENOCARCINOMA (MCF-7) CELLS

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Abstract
Background (Relevance): Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer-related death in women worldwide. The limitations of conventional therapies, including severe side effects and drug resistance, necessitate the exploration of novel therapeutic agents, particularly from natural sources. Juglans regia (walnut) leaves are known in traditional medicine for their astringent, antioxidant, and anti-inflammatory properties, attributed to a high content of phenolic compounds, flavonoids, and naphthoquinones like juglone. However, their specific cytotoxic mechanisms against breast cancer cells are not comprehensively understood. Objective: This study aimed to investigate the in vitro antiproliferative activity and the apoptosis-inducing potential of an ethanolic extract of Juglans regia leaves (JRLE) on the human breast cancer cell line MCF-7. Methods: Juglans regia leaves were collected from the Andijan region, Uzbekistan, identified, and subjected to ethanolic extraction. MCF-7 cells were cultured and treated with various concentrations of JRLE (0-600 µg/mL) for 24, 48, and 72 hours. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The induction of apoptosis was analyzed by Annexin V-FITC/Propidium Iodide (PI) double staining using flow cytometry. Results: JRLE demonstrated a significant dose- and time-dependent cytotoxic effect on MCF-7 cells. The half-maximal inhibitory concentration (IC_{50}) value was found to be 210.8 ± 14.5 µg/mL after 48 hours of exposure. Flow cytometry analysis confirmed that JRLE induced apoptosis. Treatment with JRLE (at 210 µg/mL and 420 µg/mL) for 48 hours led to a significant increase in the percentage of cells in the early and late apoptotic stages, totaling 31.4% and 58.2% respectively, compared to 5.1% in the untreated control group. Conclusion: The findings suggest that the ethanolic extract of Juglans regia leaves possesses potent anticancer properties by inhibiting MCF-7 cell proliferation and inducing programmed cell death via apoptosis. JRLE warrants further investigation as a potential source for the development of novel phytotherapeutic agents for breast cancer.
Keywords: Juglans regia, Walnut, Anticancer, Apoptosis, MCF-7 cells, Breast Cancer, Juglone, Cytotoxicity

INTRODUCTION

Breast cancer (BC) represents a major global health burden, with an estimated 2.3 million new cases and 685,000 deaths worldwide in 2020, making it the most prevalent cancer globally (Sung et al., 2021). Despite progress in diagnostic and therapeutic strategies, including surgery, chemotherapy, and targeted therapies, challenges such as acquired resistance, tumor recurrence, and significant systemic toxicity persist (Harbeck et al., 2019). This highlights the urgent need for the discovery and development of more effective, safer, and economically viable anticancer drugs.

Natural products, particularly those derived from medicinal plants, have always been a cornerstone of drug discovery, providing a vast array of structurally diverse and biologically active compounds (Newman & Cragg, 2020). Phytochemicals are known to interfere with various stages of carcinogenesis, including initiation, promotion, and progression, often by modulating multiple cellular signaling pathways, such as those controlling cell proliferation and apoptosis (Kopustinskiene et al., 2020).

Juglans regia L. (Persian walnut), belonging to the Juglandaceae family, is a tree valued not only for its nuts but also for its leaves, which are used extensively in traditional medicine systems for treating various ailments, including skin diseases, inflammation, and diabetes (Ahmad et al., 2018). The biological activities of walnut leaves are linked to their rich and diverse phytochemical profile, which includes high concentrations of phenolic acids (e.g., ellagic acid, chlorogenic acid), flavonoids (e.g., quercetin, kaempferol), and characteristic naphthoquinones, most notably juglone (5-hydroxy-1,4-naphthoquinone) (Hajji et al., 2019).

Juglone, the principal naphthoquinone in Juglans regia, has been reported to possess significant anticancer properties, including the ability to induce apoptosis and cell cycle arrest in various cancer cell lines (Zhang et al., 2021). While several studies have highlighted the antioxidant and general cytotoxic potential of walnut leaf extracts, detailed investigations into their specific pro-apoptotic mechanisms against human breast adenocarcinoma (MCF-7) cells are limited. This study was therefore designed to evaluate the in vitro cytotoxic and apoptosis-inducing effects of an ethanolic extract of Juglans regia leaves (JRLE) on the MCF-7 cell line.

MATERIALS AND METHODS

Plant material and extract preparation - Fresh leaves of Juglans regia L. were collected from the Andijan region, Uzbekistan, in June 2024. The plant was taxonomically identified at the Department of Biological Chemistry, Andijan State Medical Institute. A voucher specimen (No. UZB-JR-2406-ADMI) was deposited at the institute's Herbarium. The leaves were washed with distilled water, shade-dried at room temperature, and pulverized into a fine powder. The powder (100 g) was extracted by maceration with 1 L of 80% ethanol (v/v) for 72 hours at 25°C with continuous stirring. The extraction, filtration (Whatman No. 1 paper), solvent evaporation (using a rotary evaporator at 40°C), and lyophilization to obtain a dry powder (JRLE) were all conducted at the scientific laboratory and the Department of Biological Chemistry of Andijan State Medical Institute. The resulting extract was stored at -20°C.

Cell line and culture - The MCF-7 human breast adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 0.01 mg/mL human recombinant insulin, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Gibco, USA). Cells were maintained in a humidified atmosphere at 37°C with 5% CO_2.

Cell viability (MTT) assay - The cytotoxicity of JRLE was assessed using the MTT assay (Mosmann, 1983). MCF-7 cells were seeded in 96-well plates (5×10^3 cells/well) and allowed to attach for 24 hours. The medium was then replaced with fresh medium containing various concentrations of JRLE (0, 50, 100, 200, 300, 400, 500, and 600 µg/mL) dissolved in 0.1% DMSO (vehicle). Control wells received medium with 0.1% DMSO. After incubation for 24, 48, and 72 hours, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours. The supernatant was discarded, and 150 µL of DMSO was added to dissolve the formazan crystals. Absorbance was read at 570 nm. Cell viability was calculated as a percentage relative to the control. The IC_{50} (half-maximal inhibitory concentration) was determined.

Apoptosis analysis by flow cytometry Apoptosis was quantified using an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, USA). MCF-7 cells were seeded in 6-well plates (2 ×

10^5 cells/well) and treated with JRLE at its IC_{50} (210 µg/mL) and 2x IC_{50} (420 µg/mL) concentrations for 48 hours. Untreated cells served as the control. After treatment, cells were harvested, washed with cold PBS, and resuspended in 1X binding buffer. Cells were stained with 5 µL of Annexin V-FITC and 5 µL of Propidium Iodide (PI) for 15 minutes in the dark. The samples were immediately analyzed by a FACSCalibur flow cytometer (BD Biosciences, USA). Data were analyzed to quantify the percentage of cells in different populations: viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and necrotic (Annexin V-/PI+).

Statistical analysis - All experiments were conducted in triplicate (n=3), and data were expressed as the mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (GraphPad Prism 9.0). A p-value < 0.05 was considered statistically significant.

RESULTS

JRLE inhibits proliferation of MCF-7 Cells The effect of JRLE on the viability of MCF-7 cells was evaluated using the MTT assay. As shown in Table 1, JRLE inhibited cell proliferation in a clear dose-dependent and time-dependent manner. The inhibitory effect was more pronounced at longer incubation times (72h > 48h > 24h). The IC_{50} values were calculated as 345.2 ± 18.1 µg/mL at 24 hours, 210.8 ± 14.5 µg/mL at 48 hours, and 168.9 ± 11.3 µg/mL at 72 hours. The 48-hour time point ($IC_{50} \approx 210$ µg/mL) was chosen for the subsequent apoptosis assays.

Table 1. Dose- and time-dependent cytotoxic effect of juglans regia leaf extract (JRLE) on MCF-7 Cell viability.

Concentration (µg/mL)	Cell viability (%) (Mean ± SD)	
	24 hours	48 hours
0 (Control)	100.0 ± 0.0	100.0 ± 0.0
50	92.5 ± 4.1	86.3 ± 3.8*
100	81.3 ± 3.5*	70.1 ± 3.1*
200	66.8 ± 2.9*	51.5 ± 2.8*
300	54.0 ± 3.0*	39.2 ± 2.2*
400	45.1 ± 2.4*	30.8 ± 1.9*
500	38.6 ± 2.1*	25.1 ± 1.5*
600	33.2 ± 1.9*	20.4 ± 1.3*
Data are presented as mean ± SD (n=3). p < 0.05 compared to the untreated control group.		

JRLE induces apoptosis in MCF-7 Cells To confirm that JRLE-induced cytotoxicity was mediated by apoptosis, flow cytometry analysis with Annexin V-FITC and PI staining was performed. As detailed in Table 2, the untreated control cells showed a high viability (94.1%). However, after 48 hours of treatment with JRLE at its IC_{50} concentration (210 µg/mL), the percentage of viable cells decreased significantly to 65.9%. This was accompanied by a substantial increase in the early apoptotic population (18.6%) and the late apoptotic/secondary necrotic population (12.8%). The effect was more potent at the 2x IC_{50} concentration (420 µg/mL), where the viable cell population dropped to 39.1%, and the total apoptotic population (early + late) rose to 58.2% (30.5% early and 27.7% late). The percentage of primary necrotic cells remained low, suggesting that apoptosis is the dominant mode of cell death.

Table 2. Flow cytometry analysis of apoptosis induction by JRLE in MCF-7 Cells after 48-hour treatment.

Treatment Group	% Viable Cells (Q4)	% Early Apoptotic (Q3)	% Late Apoptotic (Q2)	% Necrotic (Q1)
Control (0	94.1 ± 1.8	3.0 ± 0.5	2.1 ± 0.3	0.8 ± 0.2

µg/mL)				
JRLE (210 µg/mL)	65.9 ± 3.1*	18.6 ± 1.9*	12.8 ± 1.4*	2.7 ± 0.6*
JRLE (420 µg/mL)	39.1 ± 2.7*	30.5 ± 2.4*	27.7 ± 2.0*	2.7 ± 0.4*
Data are presented as mean ± SD (n=3). $p < 0.05$ compared to the untreated control group.				

DISCUSSION

The search for novel, plant-derived anticancer compounds continues to be a high-priority area in cancer research. This study provides the first evidence, to our knowledge, of the specific pro-apoptotic effects of an ethanolic extract from Juglans regia leaves, collected in Uzbekistan, on the MCF-7 human breast cancer cell line. Our results clearly demonstrate that JRLE inhibits the proliferation of MCF-7 cells in a dose- and time-dependent manner. The IC_{50} value of 210.8 µg/mL at 48 hours indicates potent cytotoxic activity for a crude extract, falling within the criteria for promising anticancer activity (< 250 µg/mL) set by the US National Cancer Institute (NCI) (Syed et al., 2020).

Crucially, our study elucidates the mechanism underlying this cytotoxicity. The flow cytometry data (Table 2) confirm that JRLE induces programmed cell death, with a significant dose-dependent increase in both early and late apoptotic cell populations. The induction of apoptosis is a key therapeutic goal, as it allows for the elimination of cancer cells without triggering the inflammatory response associated with necrosis (Kerr et al., 1972).

The potent anticancer activity of JRLE is likely attributable to its complex mixture of bioactive phytochemicals. Walnut leaves are particularly rich in the naphthoquinone juglone, as well as various flavonoids (quercetin, kaempferol) and phenolic acids (ellagic acid, chlorogenic acid) (Hajji et al., 2019). Juglone, in particular, is a well-established cytotoxic agent that has been shown to induce apoptosis in breast cancer cells by generating reactive oxygen species (ROS), disrupting mitochondrial membrane potential, and inhibiting key signaling pathways like PI3K/Akt (Zhang et al., 2021; Ahmad et al., 2018). It is plausible that the observed effects of the crude extract result from a synergistic interaction between juglone and other phenolic compounds, which are also known to possess anticancer properties (Kopustinskiene et al., 2020).

This study's findings are in agreement with other research on different extracts of Juglans regia. For example, extracts have shown cytotoxicity against other cancer cell lines, such as colon (HCT-116) and prostate (PC-3) cancer (Carvalho et al., 2010). However, our work provides specific, mechanistic insight into the pro-apoptotic pathway in MCF-7 cells.

The limitations of this study include its in vitro nature, which may not fully represent the complex in vivo environment. Furthermore, the specific compound(s) responsible for the anticancer activity were not isolated. Future research should focus on bio-guided fractionation to identify the most potent active molecules and to further elucidate their precise molecular targets. In vivo studies using animal xenograft models are also required to confirm the extract's efficacy and safety profile.

CONCLUSION

In conclusion, this study demonstrates that an ethanolic extract of Juglans regia leaves exhibits significant anticancer activity against the MCF-7 human breast cancer cell line. This activity is mediated by the inhibition of cell proliferation and the induction of apoptosis. These results highlight Juglans regia leaves as a valuable natural resource for the discovery of novel phytotherapeutic compounds that could be developed for breast cancer treatment.

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