Ind. J. Pharm. Edu. Res., 2026; 60(4s):s1563-s1572.
https://www.ijper.org

Original Article

Phytochemical Profiling, Cytotoxicity, and Molecular Docking of *Juglans regia* L. Leaf Extracts for Potential Breast Cancer Therapy

Nael Abutaha*, Fahd Abdo Al-Mekhlafi, Mohammed A. Wadaan

Department of Zoology, College of Science, King Saud University, Riyadh, SAUDI ARABIA.

ABSTRACT

Background: Juglans regia leaves contain bioactive compounds with potential anticancer effects. This study evaluated their cytotoxic activity and interactions with cancer-related targets. **Materials and Methods:** Leaves of J. regia were subjected to Soxhlet and ultrasonic extraction using different solvents. Extract yields were calculated, and antioxidant activity was assessed using the DPPH• assay. Cytotoxicity was evaluated against MCF-7, HepG2, and Huvec cell lines. Apoptotic changes were visualized using Hoechst 33342 staining. Ethyl acetate extract was analyzed by GC-MS. SwissTargetPrediction and gene enrichment analyses were used to identify cancer-related targets. Molecular docking was performed using AutoDock Vina. Results: Soxhlet extraction with ethyl acetate yielded 1.64 g of extract compared to 0.262 g by ultrasonic extraction. The isopropanol Soxhlet extract showed the highest antioxidant activity (IC_{50} = 0.68 µg/mL). The ethyl acetate extract exhibited cytotoxicity against MCF-7 (IC_{50} = 100.5 µg/mL), HepG2 (IC_{50} = 164.7 µg/mL), and Huvec cells (IC_{50} = 244.0 µg/mL). Nuclear condensation and fragmentation were observed in Hoechst-stained cells. GC-MS analysis identified 30 compounds, including γ-tocopherol (17.43%), 1-heptacosanol (14.06%), and β-sitosterol (13.76%). SwissTargetPrediction identified 752 targets for eight major compounds; 434 were drug-related and 117 overlapped with breast cancer-associated genes. Ten hub targets were identified, including ERBB2, HIF1A, and MTOR. β-sitosterol showed strong docking scores with 4YAY (−9.9 kcal/mol) and 4JSV (−9.0 kcal/mol). **Conclusion:** The observed cytotoxic and pro-apoptotic effects of the J. regia ethyl acetate extract correlate with network pharmacology and docking results, where β-sitosterol and γ-tocopherol showed strong affinities for MTOR, ERBB2, HSP90AB1, and AGTR1. These interactions suggest modulation of key oncogenic pathways such as PI3K-Akt, MAPK, and ErbB, supporting the extract's potential as a natural multi-target anticancer agent.

Keywords: Antioxidant, Cytotoxicity, Molecular Docking, Cancer Pathways, MCF7 Cells.

Correspondence:
Prof. Nael Abutaha
Department of Zoology, College of Science, King Saud University, Riyadh, SAUDI ARABIA.
Email: nabutaha@ksu.edu.sa

Received: 15-05-2026;
Revised: 13-07-2026;
Accepted: 26-08-2026.

INTRODUCTION

Juglans regia, commonly known as the walnut tree, is widely cultivated in temperate regions worldwide. The leaves are rich in various phytocopounds with significant biological potentials. The primary bioactive compounds in walnut leaves include phenolics, flavonoids, terpenoids, and tannins.[1,2] The leaves of *J. regia* contains various bioactive compounds. They exhibit potent antimicrobial and allelopathic properties, along with anti-inflammatory and bronchodilator effects. Additionally, these oils provide antioxidant, analgesic, and cholesterol-lowering benefits, while also possessing significant anticancer properties.[2,3]

Various methods, such as pressing, solvent extraction, supercritical fluid extraction, enzymatic processes, microwave-assisted extraction, and Ultrasound-Assisted Extraction (UAE), are employed for extracting bioactive compounds.[4,5] In this study, we focus on the extraction of phytochemicals from *J. regia* leaves using UAE and Conventional Soxhlet Extraction (CSE), comparing their efficiencies. CSE remains a widely adopted technique for phytochemical extraction due to its simplicity, scalability, and efficiency in extracting compounds such as phenolics and flavonoids. However, its drawbacks, including high solvent consumption, extended extraction times, and significant environmental impact, underscore the need for more sustainable alternatives.[6,7] On the other hand, UAE has gained popularity as an effective method for extracting bioactive phytocompounds from *J. regia*. By utilizing ultrasonic waves, UAE enhances solvent penetration and disrupts plant cell walls, leading to higher yields in shorter times. This technique offers advantages over traditional methods, including reduced solvent usage, preserved bioactivity of heat-sensitive

DOI: 10.5530/ijper.20263063

compounds, and environmentally friendly processing.[8-10] Each extraction method has its distinct characteristics in terms of the reagents or equipment required, extraction efficiency, process complexity, cost, and environmental impact. Therefore, selecting the most suitable extraction technique is crucial for obtaining high-value phytochemicals from *J. regia*, ensuring an optimal balance between yield, bioactivity, and environmental sustainability.

Cancer represents critical health challenges that demand innovative and effective therapeutic strategies. Network Pharmacology (NP) has developed as a powerful tool to elucidate multitarget and multipathway interactions associated with drugs and diseases.[11,12] Combined with molecular docking techniques, NP provides a comprehensive framework to investigate the therapeutic potential of bioactive compounds. This study explored the anticancer potential of J. regia phytochemicals against breast cancer by integrating diverse databases. This approach identified key bioactive compounds, molecular targets, and pathways involved in J. regia's anticancer effects. The findings highlight the activity of J. regia phytocompounds for plant-based cancer therapies, emphasizing sustainable extraction methods and advanced computational techniques to optimize their clinical application.

MATERIALS AND METHODS

Chemicals and Reagents

All solvents and reagents used in this study were of analytical or HPLC grade. n-Hexane (n-Hex, ≥95%), ethyl acetate (EtOAc, ≥99.8%), ethanol (EtOH, ≥99.8%), and 2-propanol (IPA, ≥99.5%) were purchased from Sigma-Aldrich (Germany). The free radical reagent 2,2-diphenyl-1-picrylhydrazyl and dimethyl sulfoxide were obtained from Sigma-Aldrich (Germany). Phosphate-buffered saline (PBS, pH 7.4). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, ≥98%) was supplied by Thermo Fisher Scientific (USA).

Plant material

Leaves of *Juglans regia* L. were collected manually in July 2024 in Tabarbour, Amman, Jordan. Post-harvest, the leaves underwent thorough rinsing with tap water and then distilled water. Subsequently, the harvested leaves were air-dried and stored in paper bags in a dry location until further processing. Before extraction, the leaves were crushed with an electrical blender (LC grinder, China). The ground leaves were used for extraction using HPLC-grade solvents (Sigma, Germany), namely n-hexane (n-Hex), absolute ethanol (EtOH), 2-propanol (IPA) and Ethyl Acetate (EtOAc).

Ultrasonic extraction

Ultrasonic extraction was carried out using n-Hex, EtOAc, EtOH, IPA and adding 50 mL of solvent to 5.0 g of ground leaves in an ultrasonic bath device (WiseD, South Korea) at 175 W and a frequency of 40 kHz. The dried and grounded samples were immersed into a 100 mL glass beaker. The beaker was immersed in an ultrasonic bath and processed for 20 min while maintaining a constant temperature of 35°C. After extraction, the samples were filtered using filter paper and then through a 0.2 µm syringe filter, and the solvents were evaporated using a rotary evaporator at 40°C.

Soxhlet extraction

Soxhlet extraction was conducted using leaf powder (5 g) with four solvents: n-Hex, EtOH, IPA, and EtOAc. For each extraction, 450 mL of solvent was used, and the process was maintained at a constant temperature of 85°C to optimize extraction efficiency. After the extraction process, the mixture was filtered sequentially using standard filter paper and a 0.2 µm syringe filter. Solvent removal was performed using a rotary evaporator at 45°C under reduced pressure.

Assessment of free radical scavenging activity

The radical scavenging activity of extracts prepared with different solvents was determined using the DPPH• (1,1-diphenyl-2-picrylhydrazyl) free radical assay, with each extract solubilized in its corresponding solvent. Initially, dried solvent extracts were combined in equal amounts (5 mg each) and dissolved in HPLC-grade solvents, followed by filtration. The resulting filtrate was then used for antioxidant activity assessment. Similarly, a DPPH• solution was prepared in methanol at a concentration of 0.004%. Subsequently, 190 µL of this freshly prepared DPPH•solution was mixed with 10 µL of the extracted solution prepared in their respective solvent 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.07813, 0.03906, 0.01953, 0.00977, 0.00488 mg/mL). The mixture was thoroughly shaken for homogenization and incubated at 28°C for 30 min. After incubation, the absorbance of the resulting mixture was measured at 517 nm using a Multi Microplate Reader (ChromMate, USA). The percentage inhibition of the DPPH• solution was calculated based on the reduction in absorbance using Equation (Eq 1). Each treatment was replicated 3 times to ensure robustness and reliability of the data analysis.

$$\text{Percentage inhibition} = \frac{(\text{A blank} - \text{A sample})]}{\text{A blank}} \times 100$$

A_{blank}: represents the absorbance of the control (blank).

A_{sample}: represents the absorbance of the sample.

Assessment of cytotoxic activity

The cytotoxicity assay followed the methodology described by Abutaha *et al.*[13] utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay. The activities of the extracts were assessed within a concentration range of 500 to 15.6 µg/mL on HepG2 (Human Hepatocellular Carcinoma), MCF-7 (Human Breast Adenocarcinoma), and

HUVEC (human umbilical vein endothelial cells), as well as non-cancerous HUVEC cells. All cell lines were sourced from DSMZ. The compounds were stored at 4°C, and stock solutions were prepared in DMSO prior to analysis. Absorbance was measured at 570 nm using the Multi Microplate Reader (ChroMate, USA). Cell viability was expressed as a percentage relative to DMSO (0.1%) and used as a vehicle control. Each sample was assessed in triplicate. The linear regression method determined the half-maximal Inhibitory Concentration (IC_{50}).[14]

Observation of nuclear morphological changes

Cells were fixed and permeabilized using cold methanol for 20 min at 25°C, followed by three washes with phosphate-buffered saline. They were then stained with Hoechst dye (0.1 µg/mL) for 15 min in the dark. Afterwards, the plates were rinsed three times with PBS and examined under a fluorescence microscope with an excitation wavelength of 340-380 nm.[15]

Profiling of phyto-compounds by Gas Chromatography-Mass Spectroscopy (GC-MS)

A 1 µL aliquot of the sample was introduced using an autosampler in an Agilent Technologies 7890B GC-MS system (Santa Clara, CA, USA). The components were identified through the integrated NIST MS database software. Component analysis was conducted using gas chromatography coupled with a mass selective detector (GC-MS) as reported earlier.[13]

Identifying target genes for breast cancer treatment

To identify potential target genes associated with Breast Cancer (BC), we first collected BC-related genes from several comprehensive databases, including DisGeNET, GeneCards, Online Mendelian Inheritance in Man (OMIM), PharmGKB, and the Therapeutic Target Database (TTD) using the keyword *"breast cancer."* All retrieved genes were compiled, and duplicate entries were removed to generate a unified list of BC-associated genes. In parallel, we obtained the predicted target genes of the eight selected phytocompounds using the SwissTargetPrediction database. These targets were standardized and filtered to eliminate duplicates. To identify potential therapeutic targets, the two datasets the phytocompound target genes and the breast cancer-related genes were compared. The overlapping genes between these groups were considered candidate targets through which the phytocompounds may exert therapeutic effects against BC. This intersection was carried out using a Venn diagram, illustrating the shared genes between the compound targets and disease targets.[16]

Protein–Protein Interaction (PPI) analysis and identification of key target genes

The WNL target gene set was entered into the STRING database (https://string-db.org/) to retrieve PPI data. A PPI network was then visualized in Cytoscape v3.6.1. Using the cytoHubba plug-in, the top 10 genes, ranked by degree, were selected as key hub targets.[13]

GO and KEGG enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using the DAVID database to explore the potential functions of RD target genes. An FDR threshold of <0.05 was set to identify significant GO terms and KEGG pathways. The top 20 terms, ranked by the enriched genes, were visualized using a free online platform for visualization and data analysis at https://www.bioinformatics.com.cn.[13]

Molecular docking validation

Molecular docking was conducted to evaluate the binding affinities between selected hub targets and their respective phytocompounds. The PDB IDs of the target proteins included Mitogen-Activated Protein Kinase 3 (PDB ID: 4QTB), Protein Tyrosine Phosphatase Receptor Type C (PDB ID: 5FN7), Matrix Metalloproteinase 9 (PDB ID: 6ESM), Heat Shock Protein 90 Alpha, Cytosolic (PDB ID: 3NMQ), Angiotensin II Receptor Type 1 (PDB ID: 4YAY), Receptor Tyrosine-Protein Kinase erbB-2 (PDB ID: 3PP0), Mechanistic Target of Rapamycin (PDB ID: 4JSV), Hypoxia-Inducible Factor 1-alpha (PDB ID: 4Z1V), Enhancer of Zeste Homolog 2 (PDB ID: 5WG6), and Intercellular Adhesion Molecule 1 (PDB ID: 1IC1). Compound structures were sourced from PubChem in SDF format and converted to PDBQT format using the Open Babel GUI, while protein structures were obtained from the RCSB Protein Data Bank. Target protein preparation was carried out with AutoDock Tools version 1.5.6 (Morris *et al.*, 2009), where hydrogens were added, charges were assigned, and grid box centers were defined. Docking simulations were conducted using AutoDock Vina (Eberhardt *et al.*, 2021). The docking results were explored using PyMOL and Discovery Studio Visualizer to investigate ligand interactions and conformations within the active sites.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel software. Data are presented as mean ± Standard Deviation (SD) from at least three independent experiments, and a Student's t-test was used for comparison. A p-value of <0.05 was considered statistically significant. Additionally, the 50% Inhibitory Concentration (IC_{50}) was calculated using nonlinear regression (curve fit) in OriginPro software.

RESULTS

Yield calculation

In comparing yields of Soxhlet and ultrasonic extraction methods, distinct differences are observed. Soxhlet extraction generally produced higher yields than ultrasonic extraction for most

solvents. For instance, n-Hexane yielded 0.668 g with Soxhlet but only 0.448 g with ultrasonic extraction. Similarly, EtOAc yielded 1.64 g in Soxhlet, compared to 0.262 g in ultrasonic extraction. Soxhlet extraction produced 2.085 g of EtOH higher than the 0.360 g obtained with ultrasonic extraction. IPA followed this trend, with Soxhlet extraction yielding 1.283 g, compared to 0.443 g in ultrasonic extraction.

DPPH•radical scavenging activity

In comparing radical scavenging activity using DPPH• (IC_{50}) between Soxhlet and ultrasonic extraction methods, IPA demonstrated the strongest antioxidant activity in both methods, with the lowest IC_{50} values of 0.68 µg/mL for Soxhlet and 0.87 µg/mL for ultrasonic extraction indicating high radical scavenging potential. However, for EtOAc and EtOH, Soxhlet extraction yielded better antioxidant activity (IC_{50} of 1.9 µg/mL) than ultrasonic extraction, which showed slightly higher IC_{50} values of 2.2 µg/mL for EtOAc and 2.01 µg/mL for EtOH. In both methods, n-Hexane displayed the weakest antioxidant activity, with an IC_{50} of >5 mg/mL, reflecting minimal radical scavenging capacity.

Cell viability and nuclear morphology

Initially, eight extracts were evaluated for cytotoxicity against HepG2 cells at 250 mg/mL (Table 1). Result revealed that the ethyl acetate extract from Soxhlet extraction demonstrated the highest activity and was subsequently tested on additional cell lines based on these promising results. The MTT assay results, ranked from highest to lowest cytotoxicity, revealed that MCF7 cells (IC_{50} of 100.5 µg/mL) were the most sensitive, followed by HepG2 cells (IC_{50} of 164.7 µg/mL), while Huvec cells showed the least sensitivity (IC_{50} of 244.0 µg/mL). These findings indicate that the ethyl acetate extract of walnut leaves has variable cytotoxic effects depending on the cell line, with the strongest activity observed against liver and breast cancer cells and the weakest against endothelial cells. These results highlight the extract's potential for further investigation in cancer research, particularly for its stronger effects on cancer cell lines (Figure 1A). The impact of the ethyl acetate extract on the MCF-7 cells nuclear morphology was analyzed using the DNA-specific fluorescent dye Hoechst 33342. As illustrated in Figure 1B, exposure to the ethyl acetate extract at a concentration of 100 µg/mL for 24 hr induced significant alterations in chromatin architecture, including fragmentation, consistent condensation, and clustering near the nuclear periphery, which exhibited enhanced fluorescence intensity. In contrast, untreated cells displayed uniform staining patterns (Figure 1B).

Screening of Bioactive Compounds

A total of 28 compounds (Table 1) were identified via GC-MS. The analysis of the ethyl acetate extract revealed a compound profile dominated by γ-Tocopherol emerged as the most abundant compound, contributing 17.43% to the total area. 1-Heptacosanol followed closely with 14.06%, followed by β-Sitosterol (13.76%). Other significant compounds included 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)- (4.41%), Squalene (5.24%), n-Hexadecanoic acid, Phytol, 9,12-Octadecadienoic acid (Z,Z)-, Hexadecanoic acid, methyl ester, n-Hexadecanoic acid, and Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester. These findings suggest a diverse composition, with a significant presence of fatty acids, alcohols, sterols, and other lipidic compounds.

Target Prediction for *Juglans regia*

The gene targets associated with 1-Heptacosanol (1), β-Sitosterol (2), γ-Tocopherol (3), Squalene (4), Phytol (5), 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester (Z,Z,Z) (6), Neophytadiene (7), Eugenol (8) were predicted using SwissTargetPrediction, resulting in the identification of 752 targets for the 8 phytochemicals. Following the elimination of duplicates, 434 distinct drug-related gene targets were identified.

Identification of Target Genes for Breast Cancer Treatment

A total of 983 breast cancer-related gene targets were retrieved from the databases. After removing duplicates, 428 unique targets were identified. A comparison between compound-associated targets and breast cancer-related targets revealed overlapping genes, resulting in the selection of 117 target genes based on their interactions (Figure 2).

Construction of the PPI Network and Screening of Hub Targets

To identify potential Protein-Protein Interactions (PPIs), we utilized STRING 12.0, a comprehensive online database. A total of 117 proteins were analyzed, focusing on high-confidence interactions with a score threshold of 0.700. The resulting PPI network, constructed using Homo sapiens as the reference organism, consisted of 117 nodes and 508 edges, with an average node degree of 8.68. In this network, nodes represent individual proteins, and edges indicate potential functional associations, which may or may not involve direct physical interactions. To further analyze the network properties, we employed the Network Analyzer tool in Cytoscape v3.6.1 software. The network exhibited a density of 0.788, a heterogeneity of 0.156, and a centralization of 0.278. These metrics provide insights into the overall structure and complexity of the protein interaction network. The cytoHubba tool generated a PPI subnetwork featuring the 10 genes with the top degree values. The top 10 hub targets identified were presented in Figure 3.

Functional Enrichment Analysis

The enrichment analysis revealed the top 20 Gene Ontology (GO), Biological Process (BP) terms and KEGG pathways associated with the gene set, underscoring significant roles in

Table 1: List of compounds detected by GC-MS in the ethyl acetate extract from *Juglans regia* leaves extracted using the soxhlet apparatus.

Peak	Retention time	area%	Name	Molecular formula	Molecular weight
1	7.17	0.669646	Hexanoic acid	$C_6H_{12}O_2$	116
2	16.125	1.1945	unknown	-	-
3	17.24	7.778186	Eugenol	$C_{10}H_{12}O_2$	164
4	18.04	0.351399	(-)-β-Bourbonene	$C_{15}H_{24}$	204
5	18.972	1.470083	Caryophyllene	$C_{15}H_{24}$	204
6	19.821	0.204935	cis-β-Farnesene	$C_{15}H_{24}$	204
7	20.529	2.155909	Germacrene D	$C_{15}H_{24}$	204
8	21.079	1.561164	Juglone	$C_{10}H_6O_3$	174
9	22.781	0.456355	Aromadendrene oxide-(2)	$C_{15}H_{24}O$	220
10	22.977	2.027536	Caryophyllene oxide	$C_{15}H_{24}O$	220
11	24.701	1.666106	3-Buten-2-one, 4-(6,6-dimethyl-1-cyc lohexen-1-yl)-	$C_{12}H_{18}O$	178
12	27.148	0.39612	4-(1,5-Dihydroxy-2,6,6-trimethylcyclohex-2-enyl)but-3-en-2-one	$C_{13}H_{20}O_3$	224
13	27.589	0.307868	Octahydrobenzo[b]pyran, 4a-acetoxy-5,5,8a-trimethyl-	$C_{14}H_{24}O_3$	240
14	28.649	6.771729	Neophytadiene	$C_{20}H_{38}$	278
15	29.146	0.575602	9-Eicosyne	$C_{20}H_{38}$	278
16	29.519	1.274771	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296
17	31.26	3.893371	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
18	33.96	4.725075	Phytol	$C_{20}H_{40}O$	296
19	34.534	4.701157	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278
20	34.957	0.660191	Octadecanoic acid	$C_{18}H_{36}O_2$	284
21	35.64	0.28046	1-Hydroxy-2-methylanthraquinone	$C_{15}H_{10}O_3$	238
22	36.032	0.339418	Ergolin-7-one, 6-methyl-8-methylene-	$C_{16}H_{16}N_2$	252
23	40.727	1.638484	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330
24	44.155	4.406172	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	$C_{21}H_{36}O_4$	352
25	47.04	5.237473	Squalene	$C_{30}H_{50}$	410
26	50.088	14.0638	1-Heptacosanol	$C_{27}H_{56}O$	396
27	59.816	17.43495	γ-Tocopherol	$C_{28}H_{48}O_2$	416
28	70.721	13.75754	β-Sitosterol	$C_{29}H_{50}O$	414

regulatory mechanisms and cancer-related pathways. GO BP enrichment highlighted processes predominantly involved in phosphorylation and metabolic regulation, including "Positive regulation of phosphorylation" and "Positive regulation of transferase activity." The high -log10 (FDR) values, with a range above 7, denote strong statistical significance, suggesting a substantial representation of these biological processes within the dataset. The KEGG pathway enrichment analysis indicated a pronounced association with cancer-related pathways, such as "Prostate cancer," "Pathways in cancer," and "MicroRNAs in cancer," which implies potential involvement of the gene set in oncogenic signalling and regulatory networks. The bar colour gradient in both panels represents the adjusted p-value, with red denoting the highest significance level, while dot size correlates with the number of genes contributing to each term or pathway. These findings suggest that the gene set is intricately involved in pathways critical to cancer progression, cellular metabolic regulation, and phosphorylation-mediated signalling, providing insights into its potential functional relevance in oncogenic and regulatory processes.

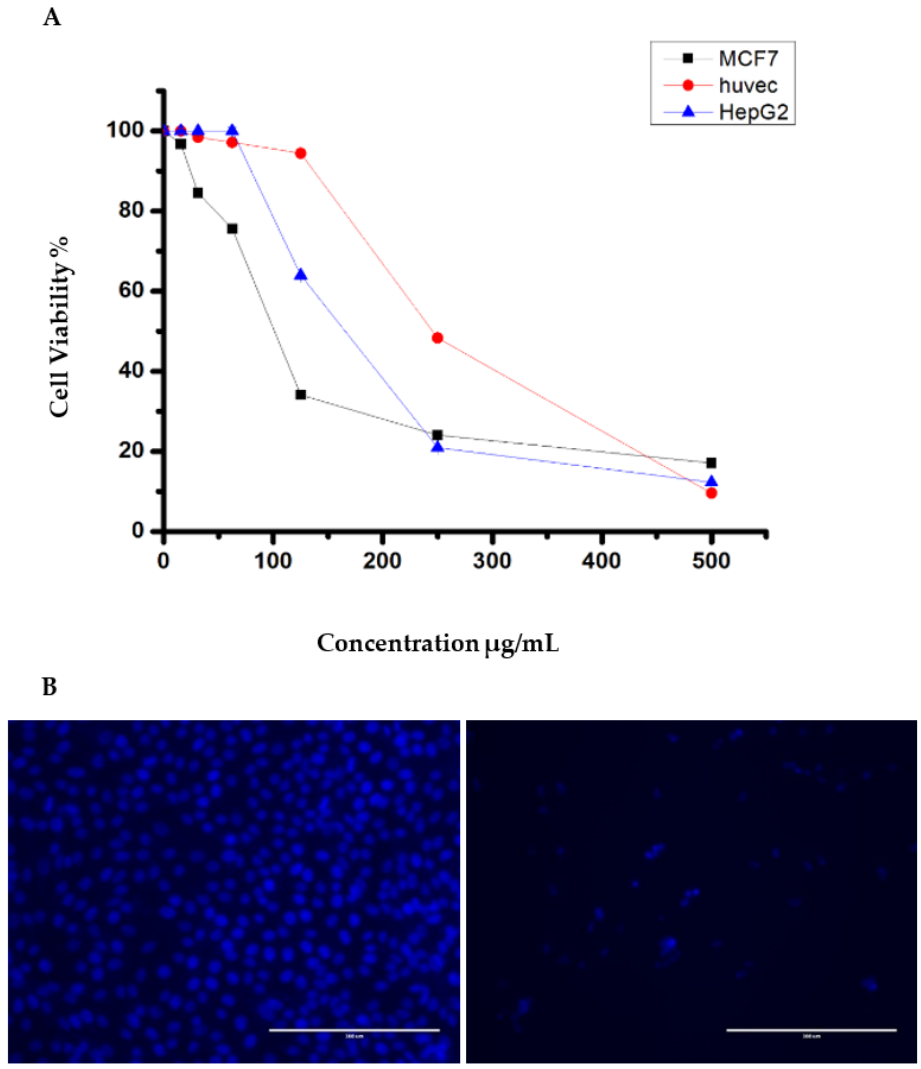


Figure 1: A: The *in vitro* cytotoxic activity (%) (mean ± SD) of the ethyl acetate extract of *Juglans regia* Leaves against various cancer cell lines, including HepG2, Huh7 and MCF-7, was assessed 24 hr post-treatment. The cytotoxicity data were compared to those of the normal Huvec cell line for reference. B: DNA fragmentation observed in cells treated with *J. regia* ethyl acetate extract, visualized using DAPI staining. Fragmentation is indicated by condensed and fragmented nuclei, characteristic of apoptotic cell death.

Molecular Docking

The molecular docking analysis across 8 compounds and a negative control (glycerol) against ten target proteins (1IC1, 3NMQ, 3PP0, 4JSV, 4QTB, 4YAY, 4Z1V, 5FN7, 5WG6, and 6ESM) demonstrated binding affinities ranging from −3.3 to −9.9 kcal/mol. The targets 4YAY and 4JSV consistently exhibited the strongest binding interactions, with notable scores of −9.9 kcal/mol for β-sitosterol and −9 kcal/mol for γ-tocopherol, highlighting their potential as favorable binding environments due to compatible structural or electrostatic features at their active sites. In contrast, the negative control, glycerol, exhibited the weakest affinities across all targets, with scores between −3.3 and −4.1 kcal/mol, validating its role as a baseline. Compounds displayed varying affinities within similar protein classes, indicating differential selectivity, with β-sitosterol and γ-tocopherol emerging as the strongest binders overall. Targets such as 5WG6 and 3PP0 demonstrated moderate to weak binding scores, while 4YAY and 4JSV clustered in the heatmap with strong, negative binding scores across several compounds, especially for rows representing β-sitosterol and γ-tocopherol. These findings suggest that 4YAY and 4JSV may serve as promising targets for further exploration, particularly under docking conditions favoring ligands like β-sitosterol and γ-tocopherol. This study provides a foundation for subsequent structural and functional analyses to validate the therapeutic relevance and specificity of these interactions (Figure 3).

DISCUSSION

Currently, the primary clinical treatment options for Breast Cancer (BC) include targeted therapies, hormone therapy, chemotherapy, surgical resection, and radiotherapy. However, surgical intervention often fails to improve long-term prognosis significantly, and prolonged exposure to radiotherapy and chemoradiotherapy can lead to severe side effects.[17,18]

Plants synthesize phytochemicals as a defense mechanism during growth and development, providing a diverse array of protective and therapeutic benefits.[19,20] Over a thousand phytochemicals are known for their health benefits.[21] However, different extraction methods can affect the yield, types and activities of these phytochemicals.[22]

The cytotoxic effects of ethyl acetate extracts were particularly noteworthy, showing pronounced activity against HepG2 cells, as evidenced by low IC_{50} values. The observed cell line sensitivity ranking (HepG2 > MCF-7 > HUVEC) aligns with the hypothesis that certain bioactive compounds selectively target cancerous cells while sparing normal cells. In the present study. This is the first report presenting quantitative results for *J. juglans* extracts obtained through various extraction methods.

The observed higher cytotoxicity of the Ethyl Acetate (EtOAc) Soxhlet extract, despite its moderate antioxidant activity, can be attributed to several key factors related to the extraction process and solvent properties. Ethyl acetate, with its intermediate polarity, is highly effective at extracting a wide range of bioactive compounds, including polyphenols, flavonoids, tannins, and alkaloids, all of which can exhibit both antioxidant and cytotoxic properties.[23,24] This aligns with studies on *Citrullus colocynthis* (L.), where ethyl acetate demonstrated superior cytotoxic activity against MIAPaca-2 (human pancreatic cancer cell lines) compared to other solvents of different polarities. These findings emphasize that the chemical nature of the solvent plays a crucial role in influencing the biological activities of plant extracts.[25]

The Soxhlet extraction method, characterized by prolonged solvent exposure, increases the extraction efficiency and concentration of these bioactive compounds, leading to a more potent cytotoxic effect. Additionally, the solvent's polarity allows it to extract both polar and moderate polar compounds, some of which may have cytotoxic effects on cancer cells by inducing apoptosis. This contrasts with other solvents, such as n-hexane, which are less effective at extracting polar bioactive compounds. This finding is similar to study carried out by Hussain *et al.*, who reported the Cytotoxic effects of ethyl acetate extract from *Ifloga spicata* (forssk) against HepG-2 cancer cell line.[26]

Moreover, the higher yield of the EtOAc Soxhlet extract (1.64 g) compared to other extracts likely contributes to the increased cytotoxicity, as higher concentrations of bioactive molecules are available. In comparison, ultrasonic extraction, which uses shorter exposure times, may not extract the same diversity or concentration of cytotoxic compounds, resulting in lower

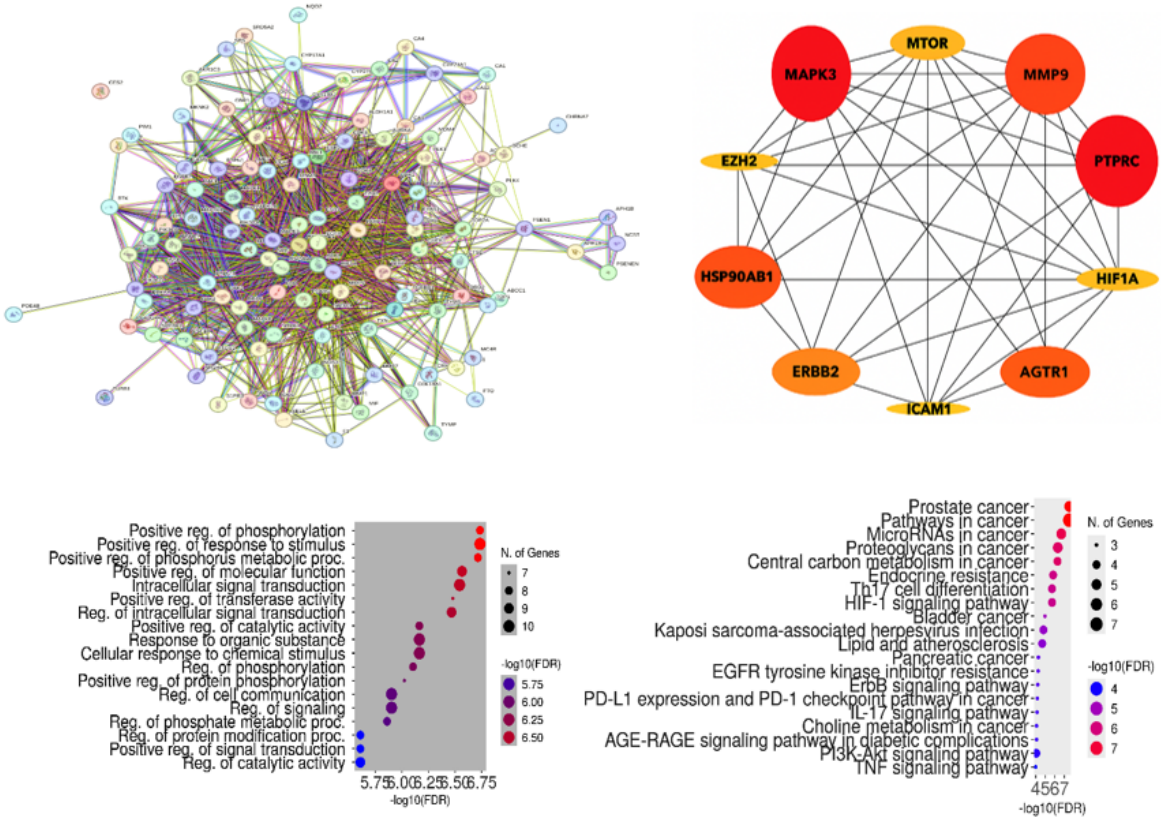
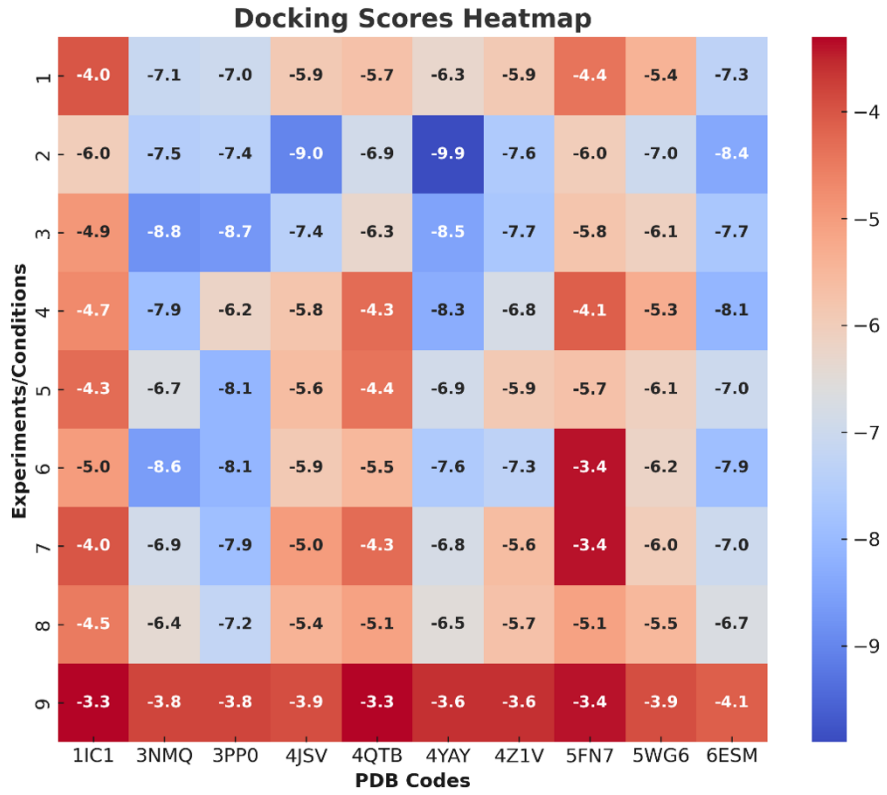


Figure 2: A: (a) Visualization of the Protein-Protein Interaction (PPI) network generated in Cytoscape. (b) Identification of the top ten key targets using CytoHubba analysis. Node size reflects target importance, with larger nodes indicating greater significance in the network. B: Top 20 GO BP terms and KEGG pathways. (a) BP enrichment. (b) KEGG pathway enrichment. The color of the dot chart represents the adjusted *p*-value.



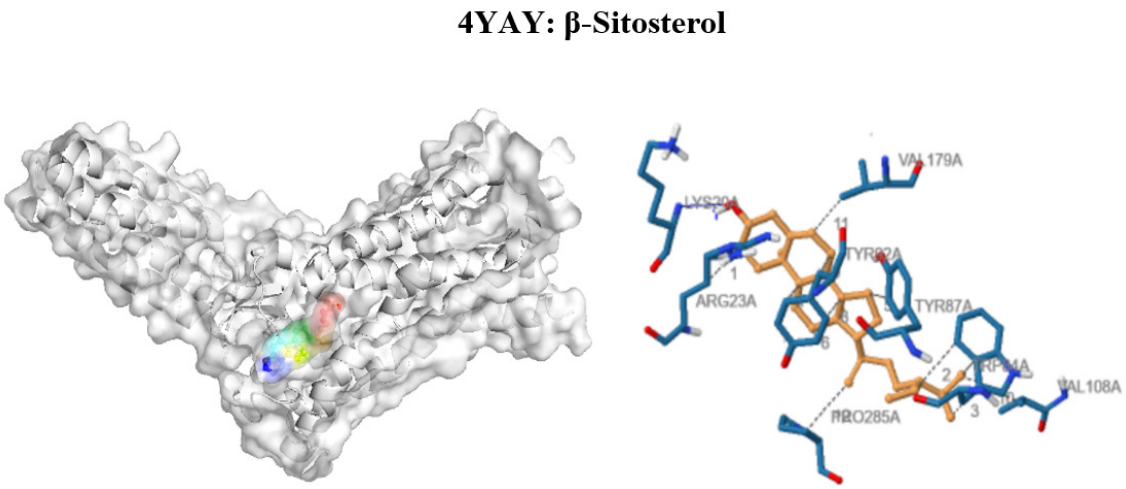


Figure 3: A: heatmap visualizes binding scores across various compounds (rows 1–9) and target protein (4QTB, 6SEM, 3NMQ, etc.), where each cell represents a specific score. 1-Hepta (1), β-Sitosterol (2), γ-Tocopherol (3), Squalene (4), Phytol (5), 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester (Z,Z,Z) (6), Neophytadiene (7), Eugenol (8), and negative control Glycerol (9). B: Molecular docking visualizations of β-Sitosterol with the 4JSV and 4YAY receptors. The left panels display the surface view of the receptor binding pocket with the ligands (β-Sitosterol) shown in stick and sphere representations, respectively, highlighting their binding positions within the receptor site. The right panels detail the specific interactions between the ligands and key amino acid residues within the binding pocket.

cytotoxicity. Overall, the combination of solvent polarity, prolonged extraction time, and higher yield of bioactive compounds likely explains why the EtOAc Soxhlet extract demonstrates significantly higher cytotoxicity, suggesting that ethyl acetate is a suitable solvent for extracting potent bioactive compounds with therapeutic potential.

This study also investigated the potential of *J. regia* ethyl acetate extract for treating cancer through network pharmacology, molecular docking. The study identifies several signaling pathways including Prostate Cancer, Pathways in Cancer, MicroRNAs in Cancer, Central Carbon Metabolism in Cancer, PD-L1 expression and PD-1 checkpoint pathway in cancer, ErbB signaling pathway, PI3K-Akt signaling pathway, TNF signaling pathway. These pathways are linked to several hub genes, such as AGTR1, ERBB2, EZH2, HIF1A, HSP90AB1, ICAM1, MAPK3, MMP9, MTOR, and PTPRC, which are involved in breast

cancer, though their roles vary between cancer types. MAPK3 (ERK1) in the MAPK pathway promotes cell proliferation and survival, often leading to poor prognosis and chemotherapy resistance.[27] PTPRC (CD45) affects immune cell interactions in the tumour microenvironment, influencing tumour progression and immunotherapy response.[28] MMP-9 aids invasion and metastasis,[29] while HSP90AB1 stabilizes oncogenic proteins, contributing to tumor growth and drug resistance.[30] AGTR1 promotes tumor angiogenesis and metastasis via angiotensin II signaling,[31] and ERBB2 (HER2) is critical in aggressive HER2-positive cancers targeted with therapies like trastuzumab.[32] MTOR regulates cell growth and is associated with chemotherapy resistance.[9] HIF1A supports angiogenesis and metastasis under hypoxic conditions, while EZH2 silences tumor suppressor genes, enhancing metastasis and cancer stem cell properties.[33] Finally, ICAM1 facilitates tumor cell invasion and metastasis by interacting with endothelial cells, correlating with poor prognosis in breast cancer.[34]

The in silico docking analysis demonstrated significant interactions between eight compounds and ten key target proteins. Notably, β-sitosterol exhibited a high binding affinity towards AGTR1 (4YAY) with a binding energy of −9.9 kcal/mol. Additionally, β-sitosterol showed a strong interaction with MTOR (4JSV), with a binding energy of −9 kcal/mol. β-Sitosterol, a major phytosterol found in plants, shares chemical similarities with cholesterol and has significant pharmacological potential in cancer treatment, particularly against leukaemia, lung, breast, colon, ovarian, and prostate cancer. β-Sitosterol modulates pathways related to cell proliferation, apoptosis, metastasis, and inflammation and has shown chemosensitizing effects, enhancing conventional chemotherapy efficacy. However, it is less potent than traditional chemotherapeutics, and more research is needed to understand its detailed mechanisms, drug delivery methods, and potential as a non-toxic nutraceutical for cancer therapy.[17,35,36]

γ-tocopherol displayed strong affinities towards HSP90AB1 (3NMQ) with a binding energy of −8.8 kcal/mol**,** and ERBB2 (3PP0) with a binding energy of −8.7 kcal/mol highlighting their potential as promising ligands for these target proteins. Recent studies have indicated that γ-tocopherol (γT), δ-tocopherol (δT), γ-tocotrienol (γTE), and δ-tocotrienol (δTE), possess significantly stronger cancer-preventive effects. These forms inhibit cancer-promoting pathways such as COX, 5-LOX, NF-κB, and STAT3, inducing pro-death and antiproliferative effects in cancer cells by modulating sphingolipid metabolism. These forms are rapidly metabolized into more potent compounds, such as 13′-carboxychromanols, which exhibit stronger anti-inflammatory and anticancer properties. Preclinical studies suggest that these vitamin E forms effectively prevent cancer progression, positioning them as potential agents for cancer prevention and therapy.[37]

These findings highlight *J. regia* ethyl acetate extract and its components as promising natural agents against breast and liver cancer, with further research and clinical validation needed to fully understand their therapeutic potential and mechanisms. Future studies should (i) assess the pharmacological effects of *J. regia* extract across various cancer types, (ii) investigate the mechanisms behind its anticancer activity, including potential immunomodulatory effects, and (iii) evaluate the safety and efficacy of combining *J. regia* with other common therapeutic strategies, such as chemotherapy, endocrine therapy, and targeted molecular therapy. These efforts could facilitate the development of safer and more effective herbal medicine-based treatments for breast cancer, potentially improving patient outcomes.

CONCLUSION

This study highlights the anticancer potential of bioactive compounds from *Juglans regia* (walnut) leaves. Soxhlet extraction proved more efficient than ultrasonic methods, with ethyl acetate extracts showing the highest cytotoxicity and antioxidant activity. The extract was most effective against MCF7 cells and induced apoptosis. GC-MS analysis identified 28 compounds, with β-sitosterol and γ-tocopherol emerging as key bioactives. Molecular docking confirmed strong interactions, particularly for β-sitosterol with targets 4YAY and 4JSV, suggesting its therapeutic relevance. These findings establish *J. regia* leaves as a promising source of anticancer agents, necessitating further exploration in cancer therapy.

ACKNOWLEDGEMENT

The authors express their sincere appreciation to Ongoing Research Funding program (ORF-2026-757), King Saud University, Riyadh, Saudi Arabia.

ABBREVIATIONS

N-HEX: n-hexane; **ETOH:** Ethanol; **IPA:** 2-propanol; **ETOAC:** Ethyl acetate; **DPPH•:** 1,1-diphenyl-2-picrylhydrazyl; **EQ 1:** Equation 1; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **HEPG2:** Human hepatocellular carcinoma; **MCF-7:** Human breast adenocarcinoma; **HUVEC:** Human umbilical vein endothelial cells; **DSMZ:** Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures); **DMSO:** Dimethyl sulfoxide; **IC_{50}:** Half-maximal inhibitory concentration; **GC-MS:** Gas chromatography–mass spectroscopy; **NIST:** National Institute of Standards and Technology; **BC:** Breast cancer; **GO:** Gene ontology; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **DAVID:** Database for Annotation, Visualization and Integrated Discovery; **FDR:** False discovery rate; **PDB ID:** Protein data bank identifier.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

This project was funded by Ongoing Research Funding program (ORF-2026-757), King Saud University, Riyadh, Saudi Arabia.

AUTHORS' CONTRIBUTIONS

Conceptualization: NA, Data curation: NA and FAA, Formal analysis: FAA and NA, Funding acquisition: MAW. Investigation: MAW, Methodology: NA, Project administration: MAW. Resources: FAA. Supervision: MAW. Writing-original draft: NA and FAA. Writing-review and editing: NA, FAA

SUMMARY

This study investigated the cytotoxic and antioxidant activities of *Juglans regia* leaf extracts using Soxhlet and ultrasonic extraction methods. Ethyl acetate Soxhlet extract showed the highest yield and notable cytotoxicity against MCF-7 and HepG2 cancer cells, along with nuclear changes indicative of apoptosis. GC-MS analysis identified 30 compounds, including β-sitosterol, which demonstrated strong molecular docking interactions with cancer-related proteins such as 4YAY and 4JSV. The findings support the therapeutic potential of J. regia extracts, warranting further pharmacological and clinical investigations.

REFERENCES

1. Sharma M, Sharma M, Sharma M. A comprehensive review on ethnobotanical, medicinal and nutritional potential of walnut (*Juglans regia* L.). Proceedings of the Indian National Science Academy. 2022;88(4):601-16.
2. Taha NA, Al-wadaan MA. Utility and importance of walnut, *Juglans regia* Linn: A. Afr J Microbiol Res. 2011;5(32):5796-805.
3. Bhat AA, Shakeel A, Rafiq S, *et al. Juglans regia* Linn.: A natural repository of vital phytochemical and pharmacological compounds. Life. 2023;13(2):380.
4. Patil PD, Patil SP, Kelkar RK, *et al.* Enzyme-assisted supercritical fluid extraction: An integral approach to extract bioactive compounds. Trends in Food Science & Technology. 2021;116:357-69.
5. Quero-Jiménez P, Felipe L, López L. Oil extraction and derivatization method: A review. Open Access J Sci. 2020;4:110-120.
6. Bitwell C, Indra SS, Luke C, *et al.* A review of modern and conventional extraction techniques and their applications for extracting phytochemicals from plants. Scientific African. 2023;19:e01585.
7. Patel K, Panchal N, Ingle P. Review of extraction techniques. Int J Adv Res Chem Sci. 2019;6(3):6-21.
8. Wen C, Zhang J, Zhang H, *et al.* Advances in ultrasound assisted extraction of bioactive compounds from cash crops–A review. Ultrasonics sonochemistry. 2018;48:538-49.
9. Shrivastav G, Prava Jyoti T, Chandel S, *et al.* Eco-Friendly Extraction: Innovations, Principles, and Comparison with Traditional Methods. Separation & Purification Reviews. 2024: 1-17.
10. Bhadange YA, Carpenter J, Saharan VK. A Comprehensive Review on Advanced Extraction Techniques for Retrieving Bioactive Components from Natural Sources. ACS omega. 2024;9(29):31274-97.
11. Chandran U, Mehendale N, Patil S, *et al.* Network pharmacology. Innovative approaches in drug discovery. 2016: 127.
12. Jiashuo W, ZHANG F, Zhuangzhuang L, *et al.* Integration strategy of network pharmacology in Traditional Chinese Medicine: a narrative review. Journal of Traditional Chinese Medicine. 2022;42(3):479.
13. Abutaha N, Wadaan MA, Alghamdi RA, *et al.* Potential Anticancer Activity of Alcea rosea and Molecular Docking of Selected Target Proteins in Liver Cancer Cells. International Journal of Morphology. 2024;42(6).
14. Alghamdi R, Abutaha N, Al-Wadaan M. Cytotoxic potential of Commicarpus plumbagineus extracts against liver cancer cell lines through In-Vitro and In-Silico methods. Journal of King Saud University-Science. 2024;36(7):103253.
15. Abutaha N. In vitro antiproliferative activity of partially purified *Withania somnifera* fruit extract on different cancer cell lines. J BUON. 2015;20(2):625-30.
16. Dwivedi PS, Shastry C. System biology mediated assessment of molecular mechanism for sinapic acid against breast cancer: via network pharmacology and molecular dynamic simulation. Scientific Reports. 2023;13(1):21982.
17. Li N, Yang J, Wang C, *et al.* Screening bifunctional flavonoids of anti-cholinesterase and anti-glucosidase by *in vitro* and *in silico* studies: Quercetin, kaempferol and myricetin. Food Bioscience. 2023;51:102312.
18. Hong R, Xu B. Breast cancer: an up-to-date review and future perspectives. Cancer communications. 2022;42(10):913-36.
19. Dillard CJ, German JB. Phytochemicals: nutraceuticals and human health. Journal of the Science of Food and Agriculture. 2000;80(12):1744-56.
20. Kumar A, P N, Kumar M, *et al.* Major phytochemicals: recent advances in health benefits and extraction method. Molecules. 2023;28(2):887.
21. Rodríguez-Negrete EV, Morales-González Á, Madrigal-Santillán EO, *et al.* Phytochemicals and Their Usefulness in the Maintenance of Health. Plants. 2024;13(4):523.
22. Dhanani T, Shah S, Gajbhiye N, *et al.* Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera. Arabian journal of chemistry. 2017;10:S1193-9.
23. Lezoul NEH, Belkadi M, Habibi F, *et al.* Extraction processes with several solvents on total bioactive compounds in different organs of three medicinal plants. Molecules. 2020;25(20):4672.
24. Lefebvre T, Destandau E, Lesellier E. Selective extraction of bioactive compounds from plants using recent extraction techniques: A review. Journal of Chromatography A. 2021;1635:461770.
25. Mandour YM, Refaat E, Hassanein HD. Anticancer activity, phytochemical investigation and molecular docking insights of *Citrullus colocynthis* (L.) fruits. Scientific Reports. 2023;13(1):20038.
26. Hussain S, Liufang H, Shah SM, *et al.* Cytotoxic effects of extracts and isolated compounds from *Ifloga spicata* (forssk.) sch. bip against HepG-2 cancer cell line: Supported by ADMET analysis and molecular docking. Frontiers in Pharmacology. 2022;13:986456.
27. Yu T-T, Wang C-Y, Tong R. ERBB2 gene expression silencing involved in ovarian cancer cell migration and invasion through mediating MAPK1/MAPK3 signaling pathway. European Review for Medical & Pharmacological Sciences. 2020;24(10).
28. Noblejas-López MdM, Baliu-Piqué M, Nieto-Jiménez C, *et al.* Transcriptomic profiles of CD47 in breast tumors predict outcome and are associated with immune activation. International Journal of Molecular Sciences. 2021;22(8):3836.
29. Barillari G. The impact of matrix metalloproteinase-9 on the sequential steps of the metastatic process. International journal of molecular sciences. 2020;21(12):4526.
30. Liu H, Zhang Z, Huang Y, *et al.* Plasma HSP90AA1 predicts the risk of breast cancer onset and distant metastasis. Frontiers in Cell and Developmental Biology. 2021;9:639596.
31. Ma Y, Xia Z, Ye C, *et al.* AGTR1 promotes lymph node metastasis in breast cancer by upregulating CXCR4/SDF-1α and inducing cell migration and invasion. Aging (Albany NY). 2019;11(12):3969.
32. Ahuja S, Khan AA, Zaheer S. Understanding the spectrum of HER2 status in breast cancer: From HER2-positive to ultra-low HER2. Pathology-Research and Practice. 2024: 155550.
33. Corbin J, Yu X, Jin J, *et al.* EZH2 PROTACs target EZH2-and FOXM1-associated oncogenic nodes, suppressing breast cancer cell growth. Oncogene. 2024;43(36):2722-2736.
34. Zhang Y, Fan J, Wang X, *et al.* Role of ICAM-1 in triple-negative breast cancer. Open Medicine. 2024;19(1):20240969.
35. Nandi S, Nag A, Khatua S, *et al.* Anticancer activity and other biomedical properties of β-sitosterol: Bridging phytochemistry and current pharmacological evidence for future translational approaches. Phytotherapy Research. 2024;38(2):592-619.
36. Durrani AK, Khalid M, Raza A, *et al.* Clinical improvement, toxicity and future prospects of β-sitosterol: a review. CyTA-Journal of Food. 2024;22(1):2337886.
37. Jiang Q. Natural forms of Vitamin E as effective agents for cancer prevention and therapy. Advances in Nutrition. 2017;8(6):850-67.

Cite this article: Abutaha N, Al-Mekhlafi FA, Wadaan MA. Phytochemical Profiling, Cytotoxicity, and Molecular Docking of *Juglans regia* L. Leaf Extracts for Potential Breast Cancer Therapy. Indian J of Pharmaceutical Education and Research. 2026;60(4s):s1563-s1572.