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LC-MS Analysis, Total Phenolics Content, and DPPH Radical Scavenging Activity of Juvenile *Juglans regia* L. Fruit (walnut) Acetonitrile Extract

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ABSTRACT

Juglans regia L. is a fragrant transitory tree. Numerous health advantages are linked to the different components of the tree's phytochemistry, which has been the subject of substantial research. The current study evaluated the chemical composition of Jordanian *Juglans regia Linn.* acetonitrile extracts. The extract from the *Juglans regia L.* juvenile fruit, which grows in Amman, Jordan, was examined to determine its photochemical components. The extract was subjected to high-performance liquid chromatography-tandem mass spectrometry analysis. The fruit was harvested in May 2023. The juvenile fruit was extracted using Soxhlet, yielding 5.5 per cent. The semi-quantitative analysis identified secondary metabolites, including tannins. The total phenol content reached 9.83 gallic acid equivalents. Spectrophotometric analysis assessed antiradical activity after 2,2-diphenyl-1-picrylhydrazyl reduction, with an IC_{50} value of 0.75 mg/mL. The study revealed 79 chemical compositions, including gingerol, hyperoside flavonoids, prostaglandin derivatives, quercetin, naringenin, and ginkgolic acid II. These compounds have previously shown antibacterial, anti-inflammatory, and ulcer-prevention properties. Overall, this work shows that several phenolic acids, flavonoids, and fatty acids were identified that correspond to the pharmacological action of *Juglans regia L.* immature fruit extract previously described. The structural elucidation of unknown peaks requires more research.

***Keywords*:** *Juglans regia*; Juvenile fruit; High-performance liquid chromatography-tandem mass spectrometry.

Introduction

Plant medicine is used due to its potential to provide complex natural products with minimal side effects and adverse reactions.[1] Fruits, vegetables, and nuts are rich in active compounds with antioxidant, anti-inflammatory, antimicrobial, and antimutagenic properties, making them ideal for health due to their high concentration of vitamins, minerals, phytochemicals, and dietary fibre.[2-3] Unfortunately, these products are not entirely safe. Therefore, herbal remedies must be based on scientific evidence, including recent biological screening methods and analytical approaches.[4] Walnuts (*Juglans regia L.),* a valuable tree, are rich in minerals, proteins, phytosterols, and antioxidants. They have therapeutic benefits in preventing and treating diabetes, obesity, and cardiovascular illnesses.[1] Walnut leaves, husks, and shells contain essential phenolic chemicals used in traditional medicine. *Juglans regia L.* husk protects the seed from oxidation and microbial infection.[5-7] The husk of *Juglans regia L.*, which is typically discarded as debris during processing, has a phytochemical profile that determines its medicinal potential.[8]

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Recently, immature fruit and bioengineered silver nanoparticles have been studied for their wound-healing capability, anti-inflammatory activity, and anti-ulcer activity.[5;7-8] This green, juvenile fruit that contains the premature husk was studied for its phytochemical components.[1] *Juglans regia L.* is grown in Jordan and the Middle East.[5] The tree features fifteen species, two genera, and unisexual blooms. Its leaves are longitudinally fissured and have grey bark. The leaves are about 35 cm long and uniformly arranged.[1] Traditional medicine has utilized *Juglans regia L.* to treat various disorders, such as sinusitis, diarrhoea, helminthiasis, endocrine abnormalities, stomachaches, arthritis, asthma, scrofula, skin conditions, and eczema. When the autumn fruit ripens, the husk falls from the tree.[1-2] The ample seed tastes deep with a thin, edible shell. The tree's phytochemistry is thoroughly investigated, and several significant processes have been taken advantage of. However, the quantity of components may change according to variables, including time, place, temperature, genetic composition, etc. Research has indicated that the chemical composition of walnuts varies with climate. The fruits are valued, and the oil is rich in phytosterols, tocopherols, and polyunsaturated fatty acids. Aesculin, Epicatechin, Kaempferol-rhamnoside, Quercetin-glucuronide, Syringetin-O-Hexoside, Taxifolin-Pantocid, Juglone, Myricetin-3-*O*-Glucoside, and Myricetin-3-*O*-Pantocid are among the many compounds found in *Juglans regia L* leaves.[9-11]

An essential analytical tool is liquid chromatography linked to mass spectrometry (LC/MS), which is used in metabolomics research, among other applications.[12] LC-MS-based methods are expected to be particularly significant in plants due to the incredibly rich biochemistry of plants, which comprises a large spectrum of semi-polar molecules, including significant secondary metabolite groups that are best separated and detected by LC-MS techniques.[11] Elution order on a liquid chromatography column affects the identification of structural isomers, such as differentiating between hexoses. LC-MS/MS is a thorough approach.

The literature has not adequately addressed data regarding the phytochemical composition of the juvenile fruit of *Juglans regia* L. As a result, a study on the phenolic profile is necessary since it can aid in

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analyzing the connection between nutrition and illness. Therefore, this work aimed to examine the phytochemical component profile, focusing on the acetonitrile fraction for the juvenile fruit, which was previously shown to have wound-healing properties. Thus, the fruit was extracted, and the phytochemical constituents were annotated and quantified using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemicals and Plant Material

All the chemicals and reagents used were of analytical reagent quality, and they were sourced from the Sigma-Aldrich Company.

A *Juglans regia* L. plant was collected in May 2023 from a Jordanian private garden. Its identity was confirmed through the websites "theplantlist.org" and "World Flora Online" and by taxonomist Dr. Shatat F. from the University of Jordan's Faculty of Agriculture.

The fresh, immature fruits are cleaned, sliced, and stored at −80°C. Acetonitrile was used as the solvent, and the Soxhlet equipment was used to extract 30 g of plant material. The extraction processes lasted for five hours, using a 1:9 solid-to-solvent ratio and accounting for the number of cycles of the Soxhlet apparatus following the thin layer chromatography technique's component separation.[13]

Phytochemical Analysis

The extracts' qualitative phytochemical analysis was put to the following tests: The ferric chloride test is used to detect tannins; the Kumar test looks for flavonoids; the creation of persistent foam is used to detect saponins; and the Wagner test looks for alkaloids.[13]

Total Phenolic Assay

The total phenolic content of walnut extracts was determined using the Folin-Ciocalteu technique, which involves reducing phenolic compounds in sodium carbonate.[5;13] Samples that have been diluted are pipetted into test tubes, the reagent is added, and the mixture is incubated for five minutes. After adding the sodium carbonate and stirring once more, the liquid is left for an hour. The lambda of 760 nm is the absorbance measured with a UV/VIS spectrophotometer (Biotech Engineering Management Co. Ltd., U.K.). The findings are given in milligrams of gallic acid equivalent for each gram of dry material.

DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used to measure the antioxidant activity of walnut extracts by assessing how well they could scavenge radicals. This procedure lowers DPPH radicals, which causes a change in hue. 25 mg of DPPH reagent are dissolved in one litre of methanol. Then, 100 mL of each sample diluted in a pure extraction solvent at different percentages is mixed with around 3900 mL of this solution. After the mixtures are incubated for 90 minutes, the absorbance of each sample is measured at 517–520 nm using a UV/VIS spectrophotometer. IC_{50} (µg/mL) is a measure of antiradical activity. The DPPH-scavenging effect was calculated using the formula:

$$DPPH\ scavenging\ effect\ (\%) = (A0 - A1)/A0) * 100 \quad \ldots\ldots Equation\ [1].$$

Every sample was examined three times.

LC-MS/MS quadrupole

A quadrupole mass spectrometer was connected to an L.C. system to quantify the phytochemical components. Device model: SCIEX, USA, X500R LC-QTO. TOF for a mass range of up to 40 kDa. Ion selection for precursors: 5-2250 m/z. Accuracy in Mass Over Time: 12 hours of L.C. with less than two ppm root mean square (RMS)-Ionization of M.S. Sources: Twin Sprayer Atmospheric Pressure Chemical Ionization (APCI) Probe TOF-MS and Twin Sprayer Electrospray Ionization (ESI) Probe TOF-MS ≥ 42,000 in both resolution and speed. The full width of the peak at half the maximum height (FWHM) for bovine insulin at m/z 956 was recorded on the (M+6H)6+ charge isotope cluster. Injection of 6 µL of solvent, standards, and sample solution using the following chromatographic conditions: Column: Inertsustan C18 (25 cm x 4.6 mm x 5 µm) Flow rate: 1.0 mL\min Column Oven temperature: 40 °C Sample cooler temperature: 20°C Rinsing solution: 80:20 (methanol: water) with 0.1 formic acid Run time: 30 minutes. Mode: Gradient. Eluents A and B, or 0.1% formic acid in water and acetonitrile, respectively, were used as the mobile phase at a 1.0 mL/min flow rate. The following were the elution conditions: 0–5 minutes: 97% A; 5–18 minutes: 97–10% A; 18–23 minutes: 10% A; 23–27 minutes: 10–97% A; 27–30 minutes: 99% A. Both positive and negative ion modes were used to operate the ESI source.[4;8;11]

The primary chemicals were determined by contrasting the retention time (t_R) and fragmentation patterns of the *Juglans regia L*. acetonitrile extraction fraction with those of standards and an internal library. The two most abundant products and precursor ions [M+H] were chosen for the analysis. The mass-to-charge ratio (m/z) of the precursor and product ions is displayed in Table 1. Specific chemicals were identified and quantified by building calibration curves with the peak regions from the first transition. The findings were presented as µg/g E.W. (or micrograms per gram of extract weight).

Table 1: An overview of the retention times found in the positive ion mode for the *Juglans regia* L. acetonitrile juvenile fruit extract LC/MS chromatogram

Index component	Retention Time (min)	Library Hit	Percent (%)
C1	2.8	6-Gingerol	0.029
C2	4.6	D-Pyroglutamic acid	0.005
C3	10.9	S-Carboxymethyl-L-cysteine	0.185
C4	11.44	Ala-Gly	0.890
C5	11.45	N-Ethylamphetamine	0.121
C6	11.8	Ethylene glycol aminosalicylate	0.002
C7	12.04	13,14-Dihydro-15-ketoprostaglandin F2.alpha. isopropyl ester	0.012
C8	12.62	Syringic acid	0.086
C9	12.73	13,14-Epoxyfluprostenol isopropyl ester	0.059
C10	12.79	Hyperoside	0.005
C11	12.8	Dibenzylamine	0.345
C12	13.19	1,2,3,4-Tetra-O-acetyl-.beta.-D-glucopyranose	0.009

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C13	13.59	trans-3-Coumaric acid	0.129
C14	13.71	trans-Ferulic acid	0.002
C15	15.43	2-Oxoticlopidine	0.437
C16	15.64	N-Acetyl-D-galactosamine	0.073
C17	15.81	Mordant orange 1	4.790
C18	15.91	Tetradonium cation	0.468
C19	16.82	Benzyl nicotinate	0.033
C20	17.87	Heptaethylene glycol	0.211
C21	18.06	S-Benzyl-L-cysteine methyl ester	0.638
C22	18.06	Cyclohexanamine, N- 3-methoxy propyl)-1-phenyl-	0.099
C23	18.33	Methanone, 2-chloro-1-naphthalenyl) 1-pentyl-1H-indol-3-yl)-	0.049
C24	19.22	Dibenzylamine	0.091
C25	19.32	1-Stearoyl-2-hydroxy-sn-glycerol-3-phosphate	0.061
C26	19.38	Cyclohexanecarboxylic acid, 1-phenyl-, 2- 4-morpholinyl)ethyl ester	0.046
C27	19.57	Carbaprostacyclin methyl ester	0.060
C28	19.72	Gamma Muricholic acid	0.137
C29	19.91	Carbaprostacyclin methyl ester	0.360
C30	20.26	Fusidic acid	0.262
C31	20.4	Hexadecyltrimethylammonium cation	11.22
C32	20.73	N-Butylscopolaminium cation	0.136
C33	20.89	(+)-Pinoresinol	0.080
C34	21.12	Telmisartan	0.013
C35	22.58	N-2-4- Aminosulfonyl)phenylethylcarbamic acid tert-butyl ester	0.106
C36	22.75	(R)-Butaprost free acid)	0.475
C37	22.77	5-trans-Prostaglandin F2.alpha.	0.069
C38	23.28	16-Phenoxytetranorprostaglandin A2	0.172
C39	24.13	Prostaglandin F1.alpha. alcohol	0.218
C40	25.13	4-Diphenylmethoxymethylpiperidine	0.082
C41	25.3	16-Phenoxytetranorprostaglandin A2	0.242
C42	25.99	1,2-Dilinoleoyl-sn-glycero-3-phosphocholine	1.030

Results and Discussion

The juvenile fruit of *Juglans regia* L. was extracted in fresh form using the Soxhlet method using acetonitrile as a solvent. The yield was about 5.5%, comparably higher to previously reported values: 4.4% for the pellicle and 3.71% for the kernel.[13] The semi-quantitative analysis was performed on the extract to identify secondary metabolites, including phenols, flavonoids, tannins, alkaloids, and saponins. It revealed the presence of tannins with high potential (score of 2) and the absence of alkaloids and saponins (score of -ve). The outcome is similar to earlier reports and roughly the same.[6,13]

The total content of phenol culminated in a value of 9.83 mg GAE/g. This activity reaches the activity of the recent data, that is, 8.5 and 10.8 mg GAE/g for pellicle and kernel parts, respectively.[13] Spectrophotometric analysis was used to assess the antiradical activity after the reduction of DPPH, which was accompanied by a change in colour from violet to yellow, detectable at 519 nm. This activity was assessed by determining the concentration resembling 50% inhibition (IC_{50}). Figure 1 displays the concentration versus percentages of the inhibition curve. The IC_{50} value is 0.75 mg/mL.

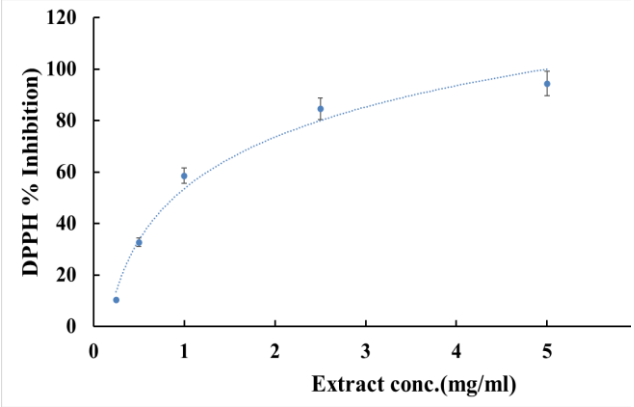

Figure 1: The DPPH radical scavenging activity is represented by the percentage of inhibition of Acetonitrile Extract versus concentration in mg/mL (IC_{50} is 0.75 mg/mL).

Figure 2 shows the chromatogram of an acetonitrile extract from *Juglans regia L*., grown in Jordan. Numerous chemicals were discovered because of this investigation. For most of them, the chemical structure is poorly resolved. The primary peaks with high intensity are resolved in Figure 2 and can be identified at retention periods of 10–10:15, 11:30, 12–12:10, 13–14, 15–17, 18–19, 20–21, and 24–25 minutes. Electrospray ionization was used to perform analysis at a relatively low fragmentation energy. Analysis of both positive and negative ions was done. The identification was completed by comparing their exact weight and fragmentation patterns to data kept in the internal database laboratory.

This study identified more chemicals by comparing their masses and fragmentation patterns to published research. Better identification of flavonoids, phenolic acids, and their glycosides results from this relatively low energy, which also positively affected the analysis. The investigation produced mass spectra that tentatively identified 79 substances (C1-C79) (Figure 2, Tables 1 and 2). Despite this, 3873 unidentified peaks in the positive mode and 2084 in the negative mode were found, indicating the extract's extreme complexity. The nature of these constituents ranges from a simple low molecular weight, such as 52.019 D, to a high molecular weight, such as 995.309 D. About 79 compounds were identified with a high confidence library score, as depicted in Tables 1 and 2. While the identified components with formulas can only reach 293 formulas,

Gingerol (C1 Table 1), an exciting phenol present in high proportion in ginger oil that exerts multiple pharmacological activities as anti-inflammatory through inhibiting the cyclooxygenase [14] and inhibiting metastasis of human breast cancer cells,[15] has been identified here for the first time with a low abundance of 0.029%. Even though it appears at a low percentage, it is recommended to analyze other extract portions with a lower polarity index (Table 1). Other phenolic components reported previously in walnuts include syringic acid (C8), which has here about 0.086%, commonly found as a plant phenolic metabolite, and trans-3-coumaric acid (C13), which was reported previously.[16] This extract portion has also identified hyperoside flavonoid (C10, 0.005%), the 3-O-galactoside of quercetin, an anti-prototype coronavirus.[17]

Prostaglandin derivatives are C7 (0.012%) (13,14-Dihydro-15-ketoprostaglandin F2- alpha.-isopropyl ester), C9 (0.059%) (13,14-Epoxyfluprostenol isopropyl ester); which is an F-series prostaglandin receptor agonist; and C37 (0.069%). 5-trans-Prostaglandin F2. alpha.; C38 (0.172%) 16-Phenoxytetranorprostaglandin A2; C39 (0.218%); Prostaglandin F1. Alpha; and C41 (0.242%) 16-Phenoxytetranorprostaglandin A2 can clarify previously reported anti-inflammatory action for walnut immature husk.[7] Mordant orange 1 (C17), a type of dye reported here in the positive mode separation, Neurodegenerative disorders are influenced by inflammation, and a walnut diet can effectively treat chronic inflammation and neurodegeneration by suppressing proinflammatory cytokines and reducing NF-κB, oxidative stress, and amyloid beta in mice.[7;18] Previous research suggests that walnuts contain anti-inflammatory compounds that may work alone or in combination to decrease inflammation. Previous research suggests that walnuts contain some anti-inflammatory compounds that may work alone or combined to decrease inflammation. These elements could include phenolic acids, peptides, flavonoids, linoleic acid, and ellagic acid.[18] According to Awdallah and Al-Nadaf's (2024) report,[6] investigations found prostaglandin-like components supporting this plant's anti-inflammatory activity.

The negative mode depicted in Table 2 clarifies previously reported components. Acids, as well as other chemical elements, were retrieved. Namely: threonic acid (0.024%), (-)-quanic acid (0.013%), maleic acid (0.03%), methylmalonic acid (0.01%), 1,2,3-benzene-triol (0.29%), neochlorogenic acid (0.617%), 2-(2-hydroxyethoxy)phenol (0.0023%), palatinose (0.014%), 5-Hydroxyisovanillic acid (0.034%), (3,4-Dichlorophenoxy)acetic acid (0.002%), 3-Hydroxybenzoic acid (0.039%), Mellein (0.014%), Caffeic acid (0.044%), Syringic acid (0.112%), Myricitrin (0.209%), Dicumarol (0.178%), and 17.beta - Estradiol 3-.beta. D-glucuronide (0.08%), 3-Hydroxybenzaldehyde (0.066%), 2,6-Dihydroxynaphthalene (0.0012%), Quercetin (0.161%), Naringenin (0.08%), Ginkgolic Acid II (0.183%), Jasmonic Acid (0.144%), ketoprofen (0.064%), 15-Hydroxy-11Z,13E-eicosadienoic acid (0.128%), 10E,12Z-octadecadienoic acid (0.752%), Benzenepropanoic acid, 4-(3-phenoxy phenyl)methylamino- (0.106%), Pinolenic acid (3.016%), (2,2`)-Methylene-bis(6-tert-butyl-4 methyl phenol) (34%), 16-Hydroxyhexadecanoic acid, (0.377%), Ethylene glycol tetradecyl ether sulfate (0.066%), 10E,12Z-octadecadienoic acid (8.6%), omega-3 Arachidonic acid (0.06%), and 9S,11R-Dihydroxy-15-oxoprostanoic acid (0.024%).

Compared to simply using the husk, the existence of these components suggests a possible purpose for this immature fruit. Mellein is a significant fungal metabolite and a member of the isocoumarin family, with well-known antibacterial and phytotoxic qualities may appear here as a contaminant or from fruit that has been infected; further research is required to understand this phenomenon.[19] In addition, dicoumarol, a natural anticoagulant, is reported here (Table 2). The presence of benzentriol, which has antiseptic properties, dihydroxy naphthalene, hydroxybenzaldehyde, and ginkolic acid II, which has been proven for its activity against biofilms, antiseptic, and anti-proliferative activity, gives the possibility of evaluating this extract portion as an anticancer.[20-21] Hydroxyisovanillic acid has anti-inflammatory activity.[22] Caffeic acid has anti-inflammatory and antioxidant effects on the immune system effect.[23-25] Myricitrin, which is glycosyoxyflavone, exhibits antiallergic activity via nitric oxide synthase inhibition as well as protein kinase c.[26]

As a plant-derived fatty acid, pinolenic acid has gained popularity recently for its potential to reduce weight and suppress appetite. 26 Furthermore, it has been demonstrated that derivatives of eicosadienoic acid, which are pinolinc acid metabolites, have anti-inflammatory action. These components were reported in wide different ranges in other plants worldwide. [26] This study is the first to report on the presence of 6-gingerol, prostaglandin derivatives, and eicosadienoic acid derivatives in *Juglans regia L*. The substantial variety in the chemical composition of the component studied to the extraction process and solvent utilized can be observed when comparing the chemical constituents acquired here with those obtained from previous research. Furthermore, the vegetative phase strongly influences this variability, just as the plant's maturity stage significantly influences the kind and quantity of metabolites across existence. As was indicated, several significant factors could be responsible for this variance. These include the habitat from which the plant was gathered, the time of year it was collected, the extraction technique, and the plant's developmental stage. More research is required to investigate various solvent fractions and to extract and separate chemical ingredients.

Table 2: An overview of the retention times found in the negative ion mode for the *Juglans regia* L. acetonitrile juvenile fruit extract LC/MS chromatogram

Index component	Retention Time (min)	Library Hit	Percent
C43	2.69	D-Fructose	0.0037
C44	2.8	Threonic acid	0.024
C45	2.93	(-)-Quinic acid	0.013
C46	3.43	Maleic acid	0.030
C47	5.49	Methylmalonic acid	0.010

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C48	9.35	1,2,3-Benzenetriol	0.290
C49	10.87	Neochlorogenic acid	0.617
C50	11.08	2-(2-Hydroxyethoxy)phenol	0.0023
C51	11.15	Palatinose	0.014
C52	11.26	5-Hydroxyisovanillic acid	0.034
C53	11.32	(3,4-Dichlorophenoxy)acetic acid	0.002
C54	11.94	1,3,6-Tri-O-galloyl-.beta.-D-glucose	0.008
C55	12.31	3-Hydroxybenzoic acid	0.039
C56	12.38	Mellein	0.014
C57	12.44	Caffeic acid	0.044
C58	12.61	Syringic acid	0.112
C59	12.76	Myricitrin	0.209
C60	12.78	Dicumarol	0.178
C61	12.84	17.beta.-Estradiol 3-.beta.-D-glucuronide	0.080
C62	13.33	3-Hydroxybenzaldehyde	0.066
C63	13.71	2,6-Dihydroxynaphthalene	0.0012
C64	15.02	Quercetin	0.161
C65	15.78	Naringenin	0.080
C66	17.17	Ginkgolic acid II	0.183
C67	17.32	Jasmonic acid	0.144
C68	17.32	Ketoprofen	0.064
C69	18.55	15-Hydroxy-11Z,13E-eicosadienoic acid	0.128
C70	20.25	10E,12Z-octadecadienoic acid	0.752
C71	20.81	Benzenepropanoic acid, 4-(3-phenoxyphenyl)methylamino-	0.106
C72	22.01	2,2'-Methylene-bis(6-tert-butyl-4 methylphenol)	0.137
C73	22.57	Pinolenic acid	3.016
C74	23.12	2,2'-Methylene-bis(6-tert-butyl-4 methylphenol)	33.995
C75	23.4	16-Hydroxyhexadecanoic acid	0.377
C76	23.54	Ethylene glycol tetradecyl ether sulfate	0.066
C77	24.12	10E,12Z-octadecadienoic acid	8.600
C78	25.69	omega.-3 Arachidonic acid	0.060
C79	25.93	9S,11R-Dihydroxy-15-oxoprostanoic acid	0.024

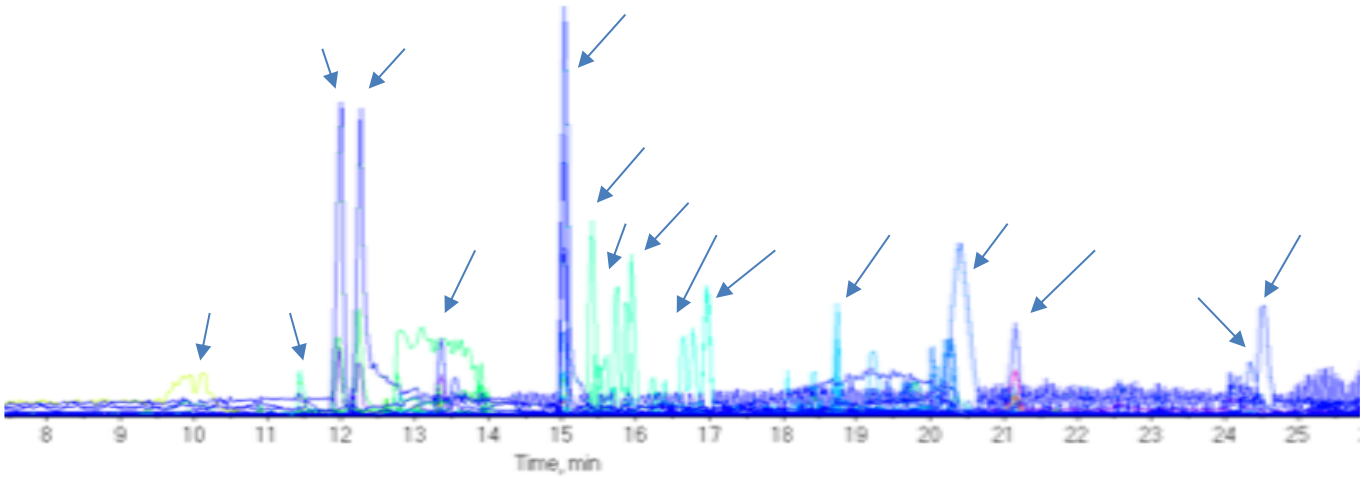

Figure 2: LC/MS chromatogram of *Juglans regia* L. acetonitrile extract retention time (X-axis) versus intensity (Y-axis). Orange arrows specify the peaks at retention times: 10-10:15, 11:30, 12-12:10, 13-14, 15-17, 18-19, 20-21, and 24-25 min

Conclusion

This investigation clarifies the chemical composition of the *Juglans regia* L acetonitrile juvenile fruit extract from the Jordanian Amman area. Using HPLC/MS analysis, the pellicle's 77 unique chemical compositions were identified. The main ones were ginkgolic acid II, quercetin, naringenin, prostaglandin derivatives, hyperoside flavonoids, and many more unidentified compounds. It was discovered that these unnamed peaks needed closer examination. This clarifies the previously documented antimicrobial, anti-inflammatory, and ulcer-prevention characteristics of the *Juglans regia* L. juvenile fruit pellicle extract extracted using acetonitrile. All known phytochemicals can be used to explore and support other medicinal applications. Furthermore, additional investigation is needed to determine the structure and distinguish between unidentified chemical components.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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