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A Study of Juglone in Georgian Walnuts (Juglon regia) by Chromato-Mass Spectrometry

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Abstract: The study concerns the identification and quantification of the biologically active naphthoquinone, juglone (5-hydroxy-1,4-naphthoquinone), in the walnut (*Juglans regia*), a species widespread in Georgia, using an ultra-high-performance liquid chromatograph. The relevance of this research lies in the need to valorize agri-food waste and adopt zero-waste principles, which constitute an important aspect of the circular economy. Walnut peel obtained during processing is considered a potentially rich source of juglone, which is used for its antimicrobial, anti-inflammatory, antifungal, and other effects. Mass spectrometric analysis identified juglone with a characteristic molecular mass (m/z 173 [M-H]). The effects of different extraction methods (ultrasound, classical, high pressure/temperature, hot extraction with reflux, lyophilization) and solvent on the yield of the target compound were compared. Quantitative data showed that the maximum juglone content was observed in the ethyl acetate extract of lyophilized walnut skin, exceeding that of the ethyl acetate extract of the sample dried at 40°C and of the raw skin. Relatively low concentrations were detected in the ultrasonic and classical extraction using 50% ethanol, while the minimum amount was found in the condensate formed during lyophilization. The results obtained indicate that walnut peel represents a promising raw material for juglone, and that lyophilization combined with ethyl acetate extraction can be considered the optimal technological approach for maximizing the extraction of the target compound. This study provides a basis for the targeted utilization of walnut agro-waste and for the optimization of technological processes aimed at obtaining bioactive compounds.

Keywords: Juglone, Mass-spectrometry, Zero-waste.

Introduction

In recent decades, environmental pollution has emerged as one of the most pressing challenges worldwide. The growing number of landfills and the accumulation of untreated waste pose significant threats to the environment and to all living organisms, contributing to climate change and the contamination of water, soil, and air. For instance, approximately 146 million tons of waste are disposed of in landfills annually in the United States. The food industry represents a particularly serious concern, as food waste constitutes one of the largest sources of

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pollution, accounting for about 24% of the total (World Economic Forum, 2023). Accordingly, increasing attention has been directed toward the zero-waste concept, first introduced in 1970 by chemist Paul Palmer, who demonstrated the potential for reusing chemicals discarded by emerging technology companies in Silicon Valley. The zero-waste approach has continued to evolve and has gained greater relevance in recent years, as it represents one of the key principles of the circular economy. In line with United Nations General Assembly Resolution 77/161, the Zero Waste Initiative has been endorsed by more than one hundred countries, including Georgia (Ministry of Environmental Protection and Agriculture of Georgia [MEPA], 2023). Thus, Georgia is actively aligning with global efforts to address the so-called "triple crisis." Nevertheless, waste recycling remains a complex challenge, particularly in our country. This is largely due to the novelty of the field, the limited availability of literature on specific facilities, recycling technologies, and methodologies, as well as a shortage of qualified professionals, all of which contribute to the overall complexity of the issue. In this context, our study focuses on plant-based biomass, specifically walnut (*Juglans regia*), which is often considered "waste" after food processing. Typically, these residues are discarded or incinerated, yet they hold considerable potential for various applications.

Walnut fruits, especially at the milk-ripe stage, are widely used for the production of preserves and other food products, generating substantial by-products and industrial residues. Plant-based biomass and the bioactive compounds derived from it are valuable resources for pharmaceuticals, sorbents, and other applications. Notably, naphthoquinones, such as juglone (5-hydroxy-1,4-naphthoquinone), are present in walnuts, leaves, roots, and bark (Kavtaradze, 2005). Walnut bark extracts are utilized as pigments in cosmetics, textiles, and the food industry due to their yellow coloration, which turns reddish-brown upon oxidation. Additionally, these extracts exhibit therapeutic properties, including antimicrobial, antifungal, and anticancer effects, and are applied in the treatment of skin disorders and infections. Therefore, the potential pharmaceutical application of walnut biomass is of considerable scientific and economic interest.

Members of the walnut family comprise evergreen, semi-evergreen, and deciduous plants, encompassing approximately 60 species that are predominantly distributed in tropical and subtropical regions (Bai et al., 2024). Archaeological evidence suggests that the collection and use of walnuts originated in the Mediterranean region more than 7,000 years ago. The most widely distributed species include *Juglans regia* (English or Persian walnut), *Juglans nigra* (black walnut), *Juglans sieboldiana* (Japanese walnut), and *Juglans cinerea* (white walnut, also known as butternut) (Suzuki & Taseer, 2019). A walnut consists of several structural components. The fruit is initially enclosed in a green outer layer (husk or hull), which degrades and separates from the nut during the ripening process. Beneath this layer lies a hard shell, which is mechanically removed to obtain the edible portion. The seed itself is further covered by a thin skin. Walnut shells have traditionally been utilized for various purposes, including as a fuel source for heating, as an abrasive material for cleaning, and as a biofilter in the treatment of wastewater and oilfield effluents. In addition, they exhibit antioxidant and antimicrobial properties (Chamorro et al., 2022).

In recent years, the beneficial properties of walnuts have been widely discussed, and scientific interest in this raw material has increased due to its recognized value. The positive effects of walnuts are largely determined by their chemical composition. They are characterized by a low glycemic index and represent an important source of polyphenols, polyunsaturated fatty acids, and essential biogenic amino acids. In addition, walnuts possess high nutritional value (Sánchez-Morgado et al., 2013).

Figure 1. *Juglans regia*

Figure 2. *Juglans regia*, green husk

Leaves of walnut (*Juglans regia*) exhibit protective properties against various diseases, primarily due to their antioxidant activity. For example, compounds such as ascorbic acid, as well as organic acids including citric and malic acids, demonstrate antioxidant capacity through their ability to form chelate complexes with metal ions. Essential fatty acids, tocopherols, phytosterols, proteins, minerals, vitamins, and phenolic compounds collectively contribute to the health-promoting properties of walnuts. Recent studies indicate that walnuts serve as a source of natural antioxidants and may help prevent diseases associated with oxidative stress (Santos et al., 2013).

Juglone

Juglone has been shown to suppress the growth of various cancer cells, including those of the stomach, pancreas, and lungs, as well as certain human papillomavirus (HPV)-infected cells, such as HeLa and MIA PaCa-2, at concentrations ranging from 10 to 100 µM. The presence of juglone also gives walnuts allelopathic properties, making this compound a promising natural herbicide, particularly for weed control (Kavtaradze, 2005). In addition to plants, juglone exerts toxic effects on fungi, protists, and certain animals. Its antifungal activity has been compared with that of synthetic fungicides, such as clotrimazole and selenium sulfide, demonstrating moderate efficacy (Mominul Islam & Widhalm, 2020). Juglone is a natural toxin with multiple bioactivities, including the capacity to induce apoptosis, which varies depending on the cell type. Despite extensive research, the precise mechanisms underlying these effects remain unclear. Its cytotoxic and genotoxic actions include induction of oxidative stress, membrane damage, apoptosis, and necrotic cell death (Suzuki & Taseer, 2019). Cytotoxicity is primarily mediated through two mechanisms: redox cycling and interaction with glutathione. Recent studies have reported that juglone induces cell death in human lung carcinoma (A549), colorectal carcinoma (HT-29), and human keratinocyte (HaCaT) cell lines (Xu et al., 2010).

Walnuts contain various phenolic compounds, including flavonoids, terpenoids, and naphthoquinones. Juglone belongs to the naphthoquinone group, a class of aromatic organic compounds characterized by a phenyl moiety. Other widely distributed naphthoquinones include lawsone, plumbagin, and lapachol. Quinones are cyclic diketones, and naphthoquinones exist as three isomers: naphthoquinone, 0-amphi-naphthoquinone (Strugstad & Despotovski, 2012). Juglone typically appears as orange, needle-like crystals that are slightly soluble in water but readily soluble in organic solvents. It is primarily derived from naphthalene derivatives and is more effective when extracted from compounds containing functional groups in the 1,5-position (Kavtaradze, 2005). Juglone is particularly abundant in walnut husks and roots, with its concentration varying seasonally. In 1943, it was observed that juglone exists in the inner bark of walnut roots in a non-toxic form, α- hydrojuglone, which oxidizes to juglone upon exposure to air. Later studies identified that in walnut wood, juglone is stored as 1,4,5-trihydroxynaphthalene-5-glucoside, which, due to its lability, can easily degrade into glucose and juglone (Strugstad & Despotovski, 2012).

Recent innovative extraction methods have improved the stability and applicability of juglone for industrial and medical purposes. A study conducted in 2025 demonstrated high efficiency using a "green" approach that combines mechanochemistry with aqueous two-phase extraction to obtain high-purity juglone from *Juglans mandshurica* (Manchurian walnut) (Wei et al., 2025). Nano-encapsulation has been successfully employed to incorporate juglone into PLGA nanoparticles, achieving up to 90% efficacy. This method enhances water solubility and increases apoptotic activity in cancer cells (Yilmaz et al., 2025). Furthermore, cyclodextrin inclusion complexes have been shown to improve the thermal and pH stability of juglone, effectively doubling its antimicrobial potential against *Escherichia coli* and *Staphylococcus aureus* (Arayıcı et al., 2024).

Aim and Methods

Materials and Methods: Sample Preparation and Extraction

The aim of our study was to investigate the content of juglone in Georgian walnuts at the milk ripening stage using ultra-high-performance liquid chromatography coupled with a mass spectrometric detector (UPLC-MS-PDA), performing a comparative analysis based on solvents and extraction conditions. Initially, samples were prepared from lyophilized and fresh walnut skins, as well as skins dried at 40°C. Solvents included water, ethyl acetate, and ethanol at concentrations of 50% and 96%. Both ultrasonic probe-assisted and conventional extraction methods were employed, including ultrasonic bath extraction with vacuum filtration, hot extraction under reflux, and high-pressure, high-temperature extraction.

For the extraction procedures, the green husks were removed from fresh walnuts, and 50 g of skins were chopped. In one set of experiments, 200 mL of 50% ethanol was added to each portion, followed by homogenization for 1 min. The samples were then incubated in an ultrasonic bath for 15 min and filtered under vacuum. In the second set, an ultrasonic probe was used in place of the homogenizer. For the third set, hot extraction under reflux was applied, while the fourth set utilized high-pressure, high-temperature extraction. For classical extraction, dried walnut skins at 40°C were treated with 96% ethanol for 18 h, maintaining a solid-to-liquid ratio of 1:6. At the first stage, 7 g of fresh skins were mixed with 42 mL of 96% ethanol. In the subsequent stages, 3.64 g of skins were mixed with 21.84 mL of ethanol, with each extraction step lasting 1 min at 70°C. All extracts were filtered through paper filters. For ethyl acetate extraction, 1 g of dried, fresh, or lyophilized skins was mixed with 10 mL of ethyl acetate, homogenized, incubated in an ultrasonic bath, and filtered under vacuum. Prior to chromatography, all extracts were filtered through "Waters Acrodisc LC PVDF Filter 13 mm, 0.45 µm."

Results and Discussion

Identification of Compounds by Ultra-High-Performance Liquid Chromatography with a Mass Spectrometric Detector (UPLC-MS-PDA)

Analysis was performed using a Waters ACQUITY UPLC system coupled with a QDa detector (ESI). The column was ACQUITY BEH C18 (2.1 × 100 mm, 1.7 µm). Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B was acetonitrile. Gradient elution was applied as follows: 0–2.0 min, 5% B; 2.0–8.0 min, 20% B; 8.0–10.0 min, 35% B; 10.0–11.0 min, 95% B; 12.0–15.0 min, 5% B. The flow rate was 0.3 mL/min, injection volume 2 µL, and column temperature 40°C. MS conditions were as follows: Ionization, ESI+; Capillary voltage, 0.8 kV; Cone voltage, 15 V; Source temperature, 120°C; Desolvation temperature, 500°C; Mode, SIR; Scan range, m/z 100–1000. Calibration was performed with 5–7 points (0.1–100 µg/mL), $R^2 \geq 0.995$. **Statistical Analysis**: Results are expressed as mean ± SEM. Student's t-test was used to assess statistical significance between groups. A p-value < 0.05 was considered statistically significant (Putkaradze, et al., 2026).

Table 1. Tentative UPLC–MS profiling of walnut husks

No	RT(min)	m/z	Fragment ions (MS/MS)	Tentative identification	Class
1	0.75	133		Malic acid	Organic acid
2	6.648	173.03	145	Juglone	Naphthoquinone
3	8.372	446.91	301	Quercetin-3-O-glucoside	Flavanoid
4	8.795	447.17		Quercetin-galactoside	Flavanoid

Table 2. Juglone extraction efficiency across solvents and methods

Sample Name	Extraction Method	Solvent	Result mg/g
Green walnut husk	Ultrasound-assisted extraction (probe)	50% Ethanol	6.96
Green walnut husk	US extraction	50% Ethanol	20.81
Green walnut husk	High-pressure and high-temperature extraction	50% Ethanol	18.15
Green walnut husk	Hot extraction with reflux	50% Ethanol	17.60
Green walnut husk dried dried at 40 °C	classical extraction	96% Ethanol and water	9.89
Green walnut husk	Classical extraction	Ethyl acetate	32.81
Lyophilized Green walnut husk dried	Classical extraction	Ethyl acetate	38.80
Green walnut husk dried dried at 40 °C	Classical extraction	Ethyl acetate	35.27
Condensate formed during lyophilization			2.68

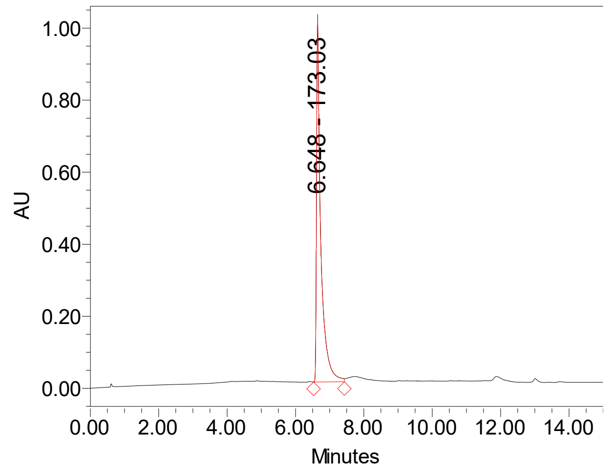

Figure 3. Juglone (5-hydroxy-1,4-naphthoquinone)

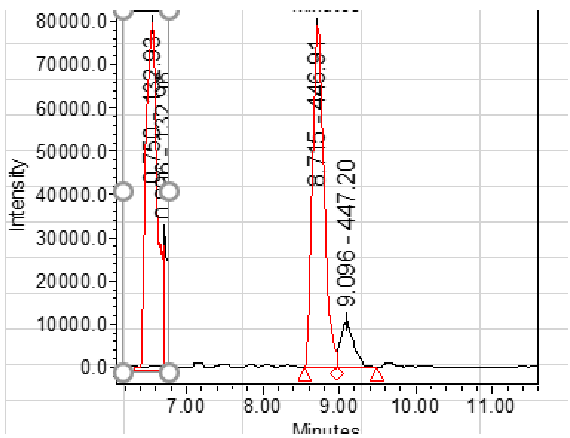

Figure 4. Phenolic compounds in walnut green husk

In addition to juglone (RT 6.648 min, m/z [M−H]⁻ 173.03), UPLC–ESI–MS analysis of the walnut extracts identified several other compounds, including malic acid (RT 0.75 min, m/z [M−H]⁻ 133), the flavonoid quercetin-3-O-glucoside (RT 8.372 min, m/z [M−H]⁻ 446.91), and quercetin-galactoside (RT 8.795 min, m/z [M−H]⁻ 447.17) (Table 1). Classical methods for isolating juglone from walnut husks involve grinding the husks, drying them at 30–35 °C, and extracting with 70% ethanol, which yields a solution containing approximately 1.45 mg/mL of juglone. Using ethyl acetate for extraction increases the concentration to 2.05 mg/mL. Furthermore, lyophilization of the starting material followed by ethyl acetate extraction enhances the yield to 3.25 mg/mL, representing an approximately twofold increase. Notably, during the drying process, juglone crystals also condense, resulting in about 2.0 g of juglone obtained from 1 kg of husks (Table 2).

Conclusion

Ultra-high-performance liquid chromatography coupled with mass spectrometric detection (UHPLC–MS) was used to identify the naphthoquinone compound juglone (5-hydroxy-1,4-naphthoquinone; m/z 173 $[M–H]^{-}$), as well as malic acid and several phenolic constituents, including quercetin glycosides such as quercetin-3-O-glucoside and quercetin-galactoside. A comparative analysis of the samples was conducted, taking into account the extraction conditions and the solvents used. The results showed that the highest yield of bioactive compounds was obtained with ethyl acetate, particularly when applied to lyophilized walnut husks. Lower, yet still substantial, extraction efficiency was observed for samples processed at 40 °C and for extracts prepared from raw material. When ethanol was employed as the solvent, the most efficient extraction was achieved under elevated temperature and pressure conditions, followed by ultrasonic probe-assisted extraction. These findings highlight the importance of optimizing the extraction method, temperature, and solvent type to maximize the recovery of bioactive compounds.

Scientific Ethics Declaration

* The authors declare that the scientific ethical and legal responsibility of this article published in EPSTEM journal belongs to the authors.

Conflict of Interest

* The authors declare that they have no conflicts of interest.

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