Phytochemical Profile and Antioxidant Activity of *Juglans regia* L. Green Husk Extracts from Tuyserkan Region, Iran

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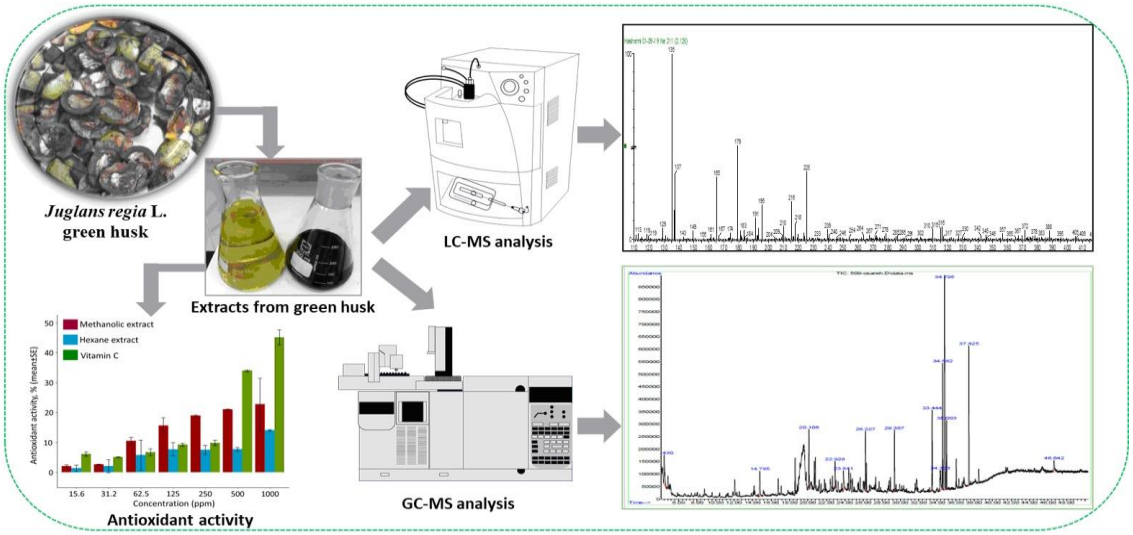
GRAPHICAL ABSTRACT



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Green husk is a byproduct of the walnut (*Juglans regia* L.) and could be a potential source of phytochemicals with important bioactivities. The extracts of *J. regia* L. green husk collected from the Tuyserkan region of Hamedan province were evaluated for their phytochemical profile and antioxidant activities. The chemical composition of crude extracts was analyzed by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). LC-MS analysis of green husk aqueous methanolic extract detected several compounds including phenolic acids, flavonoids, and hydrolyzable tannins. GC-MS analysis of the methanolic extracts revealed the presence of 1,2-dimethylbenzene (3.4%), methyl 14-methylpentadecanoate (2.82%), and methyl stearate (2.7%) as major compounds. The major components identified in the GC-MS analysis of non-polar hexane extract were (23*S*)-ethylcholest-5-en-3-*β*-ol (32.2%), *δ*-tocopherol (16.8%), lupeol (11.8%), and octadecane (5.7%). The antioxidant activity of the crude extracts was evaluated by DPPH assay, which showed aqueous methanol extract to be a more effective antioxidant agent (22.7%) compared to the hexane extract (14%) at the concentration of 1000 ppm. The findings suggest that methanolic extracts of walnut green husks from the Tuyserkan region are rich in bioactive compounds and exhibit more potent antioxidant activity than hexane extracts, demonstrating their potential use in pharmaceutical and food industries.

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Keywords: Walnut green husk; Extracts; Chemical composition; Antioxidant activity; LC-MS; GC-MS

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INTRODUCTION

The green husk of the walnut fruit is an agricultural residue, which can be considered a source of natural compounds with diverse biological properties due to the presence of phenolic compounds. Walnut, with the scientific name *Juglans regia* L. belongs to the Juglandaceae family. It has been of interest as a medicinal plant and a source of antifungal substances by the natives of the villages (Salamat *et al*. 2006). Walnut seeds have many economic benefits in the food industry due to their nutritional and health-giving

properties and are famous worldwide (Lachman *et al*. 2010). The phenolic compounds from the green husk of the walnut fruit are rich in antioxidant properties and have several beneficial properties such as antiradicals, the ability to prevent low-density lipoprotein (LDL) oxidation and hardening of arteries, and anticancer properties that can be beneficial for human health (Pereira *et al*. 2007; Oliveira *et al*. 2008; Pereira *et al*. 2008). The green walnut husk is used in cosmetics and health industries and is a raw material for traditional walnut drinks (Stampar *et al*. 2006). Several studies have highlighted the valorization of green husk extracts for antioxidant and antimicrobial uses. Walnut green husk extract has been shown to have antibacterial properties against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* (Wang *et al.* 2023). The aqueous extract of green husks from different cultivars of Portugal showed antioxidant and antimicrobial activities (Oliveira *et al.* 2008). However, many of these studies used limited extraction techniques or lacked compound-level resolution (*e.g*., Li *et al.* 2006; Stampar *et al.* 2006; Oliveira *et al.* 2008), which this study addresses through comprehensive LC-MS and GC-MS profiling.

Walnut green peel extracts are rich in phenolic compounds, including hydroxycinnamic acids (chlorogenic acid, caffeic acid, ferulic acid, and sinapic acid), hydroxybenzoic acids (gallic acid, ellagic acid, protocatechuic acid, syringic acid, and vanillic acid), flavonoids (catechin, epicatechin, myricetin, and juglone) (Stampar *et al*. 2006). Four varieties of ripe walnut fruit were investigated to determine the phenolic constituents present in their green peel extracts, and juglone was found to be the major compound with the highest amount (Cosmulescu *et al*. 2010). As the green husk of walnuts contains many phenolic compounds, it can be considered an abundant source of bioactive compounds; therefore, it is necessary to identify the compounds of these extracts to determine their bioactivities.

In Iran, this plant grows from an altitude of 26 m below sea level in Mazandaran and up to an altitude of more than 2500 m above sea level in Chaharmahal and Bakhtiari and other provinces except for the coastal regions of the Persian Gulf and the Sea of Oman (Tabatabaei and Ahmadi 2008). Considering the cultivation of walnuts in many parts of Iran and the high percentage of green husk, the material has been practically considered waste upon harvest. Its use as a source of antioxidant and antimicrobial compounds can have significant economic benefits. Additionally, it can introduce new compounds with potent antimicrobial properties that could be effective in various industries, including pharmaceutical and healthcare. This study aimed to extract, isolate, and identify the chemical compounds present in the methanolic and hexane extracts of the walnut green husks and investigate their antioxidant activities.

Previous studies have used different methods to extract phenolic compounds from the green husk of walnuts, such as soaking (immersion), Soxhlet, ultrasound, and microwave-assisted extraction (Li *et al*. 2006). However, this study used the soaking method by mixing solvents, assisted by a decanter, to extract and isolate compounds from the green husk. The extracted compounds were identified by analyzing their mass using liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). The DPPH method was used to evaluate the antioxidant activity of different extracts. This study reports the bioactive compounds from the green husk of walnuts collected from the Tuyserkan region of Hamedan province that were not previously identified.

EXPERIMENTAL

Plant Materials

The fresh fruits of *J. regia* were obtained from the Tuyserkan plain (34°32'53″ N 48°26'49″ E), Hamedan province, Iran. The samples were collected in September 2022 (*i.e.*, the end of summer) when green husks are fully mature and phytochemically enriched. They were stored at room temperature (~25 °C) with estimated ambient humidity (~50%) until processing. The initial moisture content was approximately over 154%. Plant identification was performed at the Department of Horticulture, Faculty of Agriculture and Natural Resources at Ka.C., Islamic Azad University, Karaj, Iran (voucher specimen number 5239).

Preparation of Green Husk Extracts

To prepare the green husks for the extraction process, mature walnut fruits were cut and sliced into approximately 0.5 to 1.0 cm pieces to enhance solvent penetration. The extraction was initiated by setting up a small piece of cotton that was compressed and placed at the bottom of a 500 mL separatory funnel at the opening of the outlet valve. Next, approximately 150 g of the tested plant sample was poured into a separatory funnel, followed by 300 mL of hexane:methanol (70:30, *v/v*), and the resulting mixture was macerated for 72 h. The outlet of the separatory funnel was opened, and the liquid was allowed to drop gradually, as described in the modified method by Rathi *et al*. (2006). The green husk sample was eluted again by adding 100 mL hexane:methanol (70:30, *v/v*) solution. The liquid was clarified by filtration twice and finally concentrated to dryness in several glass Petri plates. Then it was dried under a laminar hood at laboratory temperature (25 ± 5 °C). The residue extracts were accumulated and stored at 4 ºC until further separation (Hashemi *et al*. 2013). The green husk's hexane and methanolic solid extracts were obtained as 0.333 and 1.757 g, respectively, from 150 g of raw plant material. The preparation process of walnut green husk extracts is shown in Fig. 1.

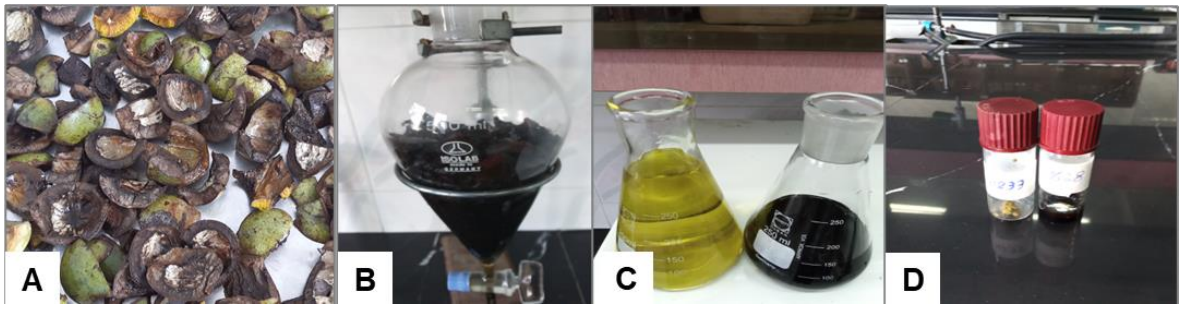

Fig. 1. Preparation process of extracts; A: Green husk; B: Separatory funnel containing green husks soaked in the solvent; C: Hexane (left) and methanolic extracts (right); D: Dried crude extracts.

Subsequently, 0.2 g of the dried methanolic extract was dissolved in a water:methanol (50:50, *v/v*) solution. Then, 50 mL of hexane solvent was added into the separatory funnel containing aqueous water:methanol solution to separate the mixture into two solvent layers: a lower layer of aqueous methanol-rich polar phase and an upper layer of hexane-rich non-polar phase. The mixture was shaken manually for 10 min to afford two phases (Hosseinihashemi *et al*. 2015a, b, 2017). The extracts obtained from each phase were used to identify the compounds and evaluate their antioxidant effects.

Phytochemical Analysis

LC-MS was selected for the analysis of green husk extracts because of its sensitivity to polar and phenolic compounds, whereas GC-MS is ideal for detecting non-polar and volatile substances.The chromatographic analysis of the green husk methanolic extract was conducted using an Alliance 2695 high-performance liquid chromatography (HPLC), and separation was carried out using an Atlantis T3-C18 column (3 µm, 2.1×150 mm). The column temperature was maintained at 40 °C. The mobile phase consisted of acetonitrile mixed with 0.1% formic acid (A) in water with 0.1% formic acid (B) at a flow rate of 0.2 mL/min. The gradient elution condition for separation was maintained as follows: 0-2 min 10% A, 2-10 min 10%-90% A, 10-16 min 90% A with a sample injection volume of 5 µL. Mass spectrometric detection of extracted compounds was performed using a Micromass Quattro micro-API mass spectrometer with an ESI source in positive and negative ion modes. The MS parameters were as follows: cone voltage, 25/20 V; capillary voltage, 3.5/4 kV; cone gas flow, 250 L/h; ion source temperature, 130 °C; desolvation gas temperature, 350 °C.

For the GC-MS analyses, 1.0 µL of hexane extract was dissolved with 100 µL of hexane and 1.0 µL of water:methanol extract was dissolved with 100 µL of methanol separately and run on a GC Agilent 7890A and MS Agilent 5975C mass spectrometer detector (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5MS cross-linked capillary column (30 m long and 0.25 mm internal diameter, 0.25 µm film thickness). The GC-MS operation was carried out by following the method previously reported by Barzegari *et al*. (2023). Briefly, the conditions used were an injector temperature of 260 °C and transfer line temperature of 270 °C. The oven temperature program was performed at initial temperature of 60 °C for 4 min, 3 °C/min to 100 °C for 2 min, then 4 °C/min to 250 °C for 5 min; carrier gas was He at a constant flow rate of 1.0 mL/min. The intrinsic energy of the MS system sample was 70 eV. The split ratio of the sample was 50:1 with a split flow rate of 1.0 mL/min. Individual components were identified using mass spectra with data from the literature, two mass spectrometric libraries (Wiley 275 L, 1998 and NIST-05), mass database matching, and by comparing the retention times and mass spectra of constituents with published data (Joulain and König 1998; Adams 2001). Retention indices (R_I) were determined as described by Sabzikar *et al*. (2020) concerning a homologous series of normal alkanes (C_{10} to C_{32}) using Eq. 1 mentioned by Kováts (1958),

$$R_I = 100\,[(n + (N–n) \times \log t_{1R}\,(x) – \log t_{1R}\,(C_n)/\log t_{1R}\,(C_N) – \log t_{1R}(C_n)] \qquad (1)$$

where R_I denotes the retention index of a compound, t_{1R} (min) is the net retention time (t_R-t_0), t_0 (min) is the retention time of the solvent (dead time), t_R (min) is the retention time of the compound, and C_n and C_N are the number of carbons in the *n*-alkanes eluting immediately before and after the compound, respectively. *N* and *n* are the carbon atoms in the *n*-alkanes eluting immediately before and after the compound.

DPPH Scavenging Activity

The free radical scavenging activities of the methanolic and hexane extracts of the walnut green husk samples were determined using the DPPH method (Karau *et al*. 2013). Serial dilutions were carried out from the stock solutions (1.0 mg/mL) of the tested extract to obtain concentrations of 15.6, 31.3, 62.5, 125, 250, 500, and 1000 ppm. A working solution was freshly prepared by dissolving 2.4 mg of DPPH powder in 100 mL of

methanol. Subsequently, 250 µL of the crude extracts (1000 ppm) was added to the 1250 µL of the working solution of DPPH powder. The reaction mixture was mixed for 10 s and kept at room temperature for 30 min in the dark condition. The experiment was performed in four replicates, and the average absorbance was recorded for each concentration. The absorbance was measured using a UV scanning spectrophotometer at 517 nm.

To prepare the stock solution of a positive control sample, ascorbic acid was dissolved in pure methanol at a concentration of 1.0 mg/mL. The control samples consisted of all reaction reagents except the green husk extract or a positive control substance. Pure methanol (Sigma-Aldrich, Germany) was used as the blank in this assay. The DPPH free radical scavenging activity (%) was calculated using the following Eq. 2:

$$\text{Inhibition (\%)} = 100(A_c - A_s)/A_c \quad (2)$$

The percentage inhibition value was calculated from the absorbance value of the control, depicted as A_c, and the sample, as A_s. The values displayed in Fig. 2 represent the means of four replicate analyses.

RESULTS AND DISCUSSION

Chemical Composition of Methanolic Extracts from Walnut Green Husk

The methanolic extract from the green husk of *J. regia* L. was found to be a good antioxidant in the preliminary screening and thus evaluated for its phytochemical composition. Eighteen compounds were tentatively identified by comparing their ESI-MS data with those reported in the literature. The molecular ion mass, ion mode, molecular formula, identification, and classification results are summarized in Table 1.

LC-MS analysis of phenolic compounds in the crude methanolic extract revealed the presence of phenolic acids, hydrolyzable tannins, flavonoids, and other compounds. Walnut green husks are an abundant source of phenolic and flavonoid compounds (Jahanban-Esfahlan *et al*. 2019). The presence of several phenolic acids such as caffeic acid (*m/z* 179), hydroxyphenyl propionic acid (*m/z* 165), hydroxymandelic acid (*m/z* 167), protocatechuic acid-O-hexoside (*m/z* 315), and 3-*p*-coumaroylquinic acid (*m/z* 337) was detected in the methanolic extract. These phenolic acids were also found in walnut green husks collected from Xinjiang Uygur Autonomous Region, China (Sheng *et al*. 2021). Phenolic acids are commonly found in higher plants, mostly involved in the defense against pathogens, and have antioxidant properties (Chrzanowski *et al.* 2011).

The presence of these phenolic acids in the methanol extract in this study might be related to their antioxidant activities. Flavan-3-ol type flavonoid (-)-epicatechin gallate (*m/z* 441) isomer was detected in the extract along with quercitrin (*m/z* 447). (-)-Epicatechin gallate was also found previously in black walnut kernels (Vu *et al.* 2018). The extract also contained hydrolyzable tannins, two tetralone derivatives, and a coumarin compound.

Tetralone derivatives are widely found in different parts of *Juglans* spp. including green husks (Vieira *et al*. 2020). The green husks of *J. regia* L. have been found to contain tetralone derivatives, specifically hydroxyl, hexosyl, and hydroxybenzoyl derivatives (Zhou *et al*. 2015). This work identified two tetralone derivatives, scytalone (*m/z* 193) and dihydroxytetralone galloyl-hexoside isomer (*m/z* 491), from walnut green husk. Only one naphthoquinone derivative, 5-hydroxy-2,3-dihydro-1,4-naphthalenedione, was detected in

the methanol extract, where the commonly identified juglone was not detected in the walnut green husks in this study.

Table 1. Tentative Identification of Compounds from Walnut Green Husk Methanolic Extracts by LC-MS Analysis

Molecular Ion Mass (*m/z*)	Ion Mode	Molecular Formula	Tentative Identification	Class
133	M-H	$C_4H_6O_5$	Malic acid	Dicarboxylic acid
137	M-H	$C_7H_6O_3$	Protocatechualdehyde	Phenolic aldehyde
191	M-H	$C_7H_{12}O_6$	Quinic acid	Hydrolysable tannin
179	M-H	$C_9H_8O_4$	Caffeic acid	Phenolic acid
165	M-H	$C_9H_{10}O_3$	Hydroxyphenyl propionic acid	Phenolic acid
167	M-H	$C_8H_8O_4$	Hydroxymandelic acid	Phenolic acid
183	M-H	$C_8H_8O_5$	Methyl gallate	Phenolic acid
193	M-H	$C_{10}H_{10}O_4$	Scytalone	Tetralone derivative
315	M-H	$C_{13}H_{16}O_9$	Protocatechuic acid-O-hexoside	Phenolic acid
281	M-H	$C_{15}H_{22}O_5$	Dihydrophaseic acid	Abscisic acid derivative
337	M-H	$C_{16}H_{18}O_8$	3-*p*-Coumaroylquinic acid	Phenolic acid
441	M-H	$C_{22}H_{18}O_{10}$	(-)-Epicatechin 3-O-gallate	Flavonoid
447	M-H	$C_{21}H_{20}O_{11}$	Quercitrin	Flavonoid
359	M-H	$C_{15}H_{20}O_{10}$	Dimethyl galloyl hexoside	Hydrolysable Tannin
491	M-H	$C_{23}H_{24}O_{12}$	Dihydroxytetralone galloyl-hexoside isomer	Tetralone derivative
481	M-H	$C_{21}H_{22}O_{13}$	Galloyl methylgalloyldeoxyhexoside isomer	Hydrolysable tannin
163	M+H	$C_9H_6O_3$	7-Hydroxycoumarin	Coumarin derivative
177	M+H	$C_{10}H_8O_3$	5-Hydroxy-2,3-dihydro-1,4-naphthalenedione	Naphthoquinone

The methanolic extract from walnut green husks contained the highest number of compounds by GC-MS analysis compared to the hexane extract. The chemical components identified by the GC-MS analysis in the methanolic extract are presented in Table 2.

Sixteen peaks belonging to a diverse group of compounds were detected in the methanolic extract. The main constituents of the extract were 1,2-dimethylbenzene (3.4%), methyl 14-methylpentadecanoate (2.82%), methyl stearate (2.7%), 2,6-dimethoxyphenol (1.81%), and myristic acid (1.74%). Several phenolic compounds have previously been identified in the walnut shell pyroligneous acid (Ma *et al*. 2011; Jahanban-Esfahlan and Amarowicz 2018; Arslan *et al*. 2023). This work found 2,6-dimethoxyphenol (syringol, 1.81%), 2,4-bis(1,1-dimethylethyl)phenol (1.72%), and 2-methoxy-4-vinylphenol (0.90%) in the extract.

Table 2. Tentative Identification of Compounds from Walnut Green Husk Methanolic Extracts by GC-MS Analysis

RT (min)	Name	Molecular formula	Class	Area (%)	KI (Exp.)
4.429	1,2-Dimethylbenzene	C_8H_{10}	AH	3.37	95
12.062	2,3-Dihydro-benzofuran	C_8H_8O	Coumaran	1.41	1085
14.168	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	Phenol	0.90	1133
14.786	2,6-Dimethoxyphenol	$C_8H_{10}O_3$	Phenol	1.81	1147
16.778	Tetradecane	$C_{14}H_{30}$	ALH	0.62	1189
17.105	*trans*-Caryophyllene	$C_{15}H_{24}$	SH	0.71	1195
18.610	2,4-Bis(1,1-dimethylethyl)phenol	$C_{14}H_{22}O$	Phenol	1.72	1227
20.794	Hexadecane	$C_{16}H_{34}$	ALH	1.30	1271
21.225	5-Phenylundecane	$C_{17}H_{28}$	ALB	0.73	1279
22.652	Heptadecane	$C_{17}H_{36}$	ALH	0.72	1307
23.254	4-Phenyldodecane	$C_{18}H_{30}$	ALB	0.64	1322
24.411	Octadecane	$C_{18}H_{38}$	ALH	0.69	1350
24.608	Myristic acid	$C_{14}H_{28}O_2$	FA	1.74	1355
25.651	7,9-Di-*tert*-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	Oxaspiro	0.74	1379
26.227	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	FAME	2.82	1392
29.366	Methyl stearate	$C_{19}H_{38}O_2$	FAME	2.70	1479

RT: Retention time; AH: Aromatic hydrocarbon; ALH: Alkane hydrocarbon; SH: Sesquiterpene hydrocarbon; ALB: Alkyl benzene; FA: Fatty acid; FAME; Fatty acid methyl ester; KI (Exp.): experimental Kovats indices on HP-5MS column in reference to *n*-alkanes

A long-chain saturated fatty acid, myristic acid (1.74%), was found along with fatty acid methyl ester, and methyl stearate (2.7%). Myristic acid has been shown to exhibit antifungal, antiviral, and anticancer activities (Javid *et al.*, 2020), and methyl stearate was reported for its antimicrobial activity (Suresh *et al.* 2014). Several short-chain alkanes such as hexadecane (1.30%), heptadecane (0.72%), octadecane (0.69%), and tetradecane (0.62%) were also detected by GC-MS analysis similar to previous research (Barekat *et al*. 2023). *trans*-Caryophyllene (0.71%) was identified in the extract as a minor component previously detected in the walnut leaves (Buttery *et al*. 1986). *trans*-Caryophyllene has several biological properties reported in the previous studies, including antimicrobial and anti-inflammatory activities (Astani *et al.* 2011; Fernandes *et al.* 2007).

Chemical Composition of Walnut Green Husk Hexane Extracts

The hexane extract of *J. regia* L. green husks exhibited weak antioxidant activity compared to its polar methanolic extract. The chemical components identified in the hexane extract are listed in Table 3.

The non-polar hexane extract of green husk contained different groups of compounds, including alkanes, terpene, and methyl esters. Alkanes with varying chain lengths were found (n-C16-n-C23) in the extract. Walnut green husks produce long- and short-chain alkanes (Seabra *et al*. 2019; Barekat *et al*. 2023). Nevertheless, in the present work, long-chain alkanes were detected in the non-polar extract. Eicosane was previously reported for its antifungal activities against fungal pathogens (Bhat *et al*. 2024). The analysis of GC-MS concluded that (23*S*)-ethylcholest-5-en-3*β*-ol (32.23%) is the most lipophilic and the major component detected in the walnut green husk hexane extract. The antibacterial activities were reported in the crude extracts of *Capparis spinosa* L. containing (23*S*)-ethylcholest-5-en-3*β*-ol (Al-Khafagi and Mohammed 2023). *δ*-

Tocopherol and lupeol were also found to be the prevailing compounds in the hexane extract, with an area of 16.8% and 11.8%, respectively. However, *α*-tocopherol/vitamin E was present in lower amounts, with an area of 1.59%. Tocopherols are known as antioxidants and are widely found in nuts (Yıldız and Karaca 2021). Lupeol exhibits a wide range of bioactivities, including anticancer, antimicrobial, and anti-inflammatory activities (Gallo and Sarachine 2009).

Table 3. Tentative Identification of Compounds from Walnut Green Husk Hexane Extracts by GC-MS Analysis

RT (min)	Name	Molecular Formula	Class	Area (%)	KI (Exp.)
19.606	Hexadecane	$C_{16}H_{34}$	Alkane	2.35	1248
28.281	Phytol Isomer	$C_{20}H_{40}O$	Terpene	1.63	1449
29.480	Docosane	$C_{22}H_{46}$	Alkane	1.38	1482
30.896	Tricosane	$C_{23}H_{48}$	Alkane	1.69	1525
32.256	Eicosane	$C_{20}H_{42}$	Alkane	3.22	1568
36.033	Nonadecane	$C_{19}H_{40}$	Alkane	2.96	1694
37.683	Z-9-Tetradecenal	$C_{14}H_{26}O$	Fatty aldehyde	1.33	1755
38.347	Octadecane	$C_{18}H_{38}$	Alkane	5.73	1779
38.934	δ-Tocopherol	$C_{27}H_{46}O_2$	Terpene	16.80	1800
41.009	D-α-Tocopherol	$C_{29}H_{50}O_2$	Terpene	1.59	1882
43.422	(23S)-Ethylcholest-5-en-3*β*-ol	$C_{29}H_{50}O$	Stigmastane	32.23	1980
44.667	Lupeol	$C_{30}H_{50}O$	Terpene	11.76	2033

RT: Retention time; KI (Exp.): Experimental Kovats indices on HP-5MS column in reference to *n*-alkanes

Antioxidant Activity

Walnut green husk extracts with proven bioactive properties for commercial extraction are considered to have antioxidant activities (Soto-Maldonado *et al*. 2019; Arslan *et al*. 2023). Polar methanolic extracts of green husk exhibited relatively higher antioxidant activity than non-polar hexane extract in comparison with standard vitamin C (Fig. 2).

For both crude extracts, the scavenging activity increased with increasing concentration. The methanolic extract showed the lowest antioxidant activity (2.04%) at 15.6 ppm, which was lower than that of vitamin C (6.13%) at the same concentration. The highest activity was observed in the methanolic extract (22.7%) at 1000 ppm, which was also lower than that of vitamin C (45. 14%) at the same concentration. The hexane extracts exhibited weaker antioxidant activity for all working concentrations than methanolic extract and standard vitamin C. The lowest antioxidant activity (1.40%) was observed for hexane extract at 15.6 ppm, significantly lower than vitamin C (6.13%) at the same concentration. The highest activity was observed with the hexane extract (14.06%) at 1000 ppm, which was also lower than that of vitamin C (45.14%) at the same concentration.

The methanolic extract exhibited the highest activity in the ranges of 62.5 ppm to 250 ppm, which was very muchhigher than that of vitamin C at the same concentration. These results were expected, since 200 ppm of juglone was shown to inhibit the white-rot fungus (*Pleurotus sajor-caju*) by 72.1% (Curreli *et al*. 2001). Similarly, the toxicity relative threshold was estimated to be around 0.15 mg/mL (150 ppm) for *Trametes versicolor* fungus with ethanol-toluene wood extractives (Hosseini Hashemi *et al*. 2008).

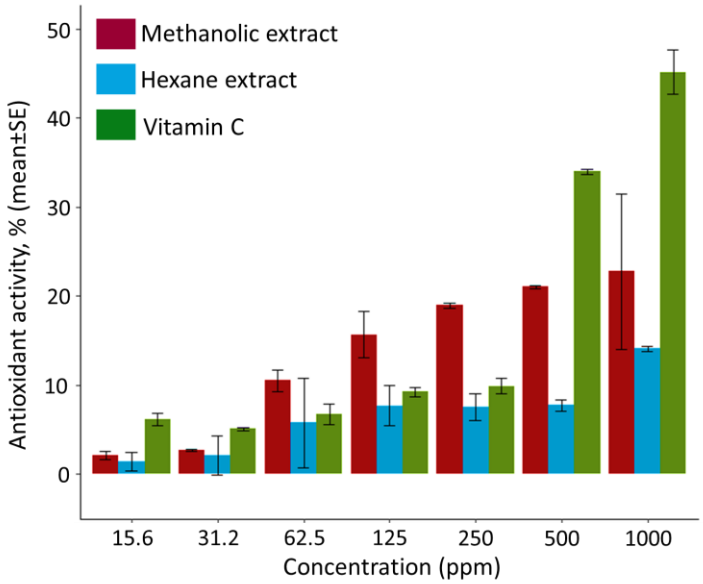

Fig. 2. A plot shows the antioxidant activity (%; mean ± SE) as affected by the concentration of walnut green husk compared with vitamin C

Both extracts exhibited concentration-dependent responses in the scavenging effect. These concentration-dependent patterns have been observed in most previous findings, with higher activity in methanol extracts. Agullo *et al.* (2013) demonstrated higher antioxidant activity in extracts obtained from methanol and ethanol solutions. Arslan *et al.* (2023) reported a similar increasing pattern to the current study, with a corresponding trend in concentrations. The increasing amount of phenol content with rising concentration can enhance antioxidant activity, as is also evident in our research. Consequently, it can prevent the detrimental effects of free radicals on specific diseases. Thus, it can be concluded that the methanolic extracts of walnut green husks may contain relatively higher amounts of antioxidant compounds than hexane extracts and that they can be effectively utilized in the formulation and advancement of future research, particularly in the pharmaceutical and cosmetics industries.

CONCLUSIONS

1. In summary, this study demonstrated the chemical profile of walnut green husks collected from the Tuyserkan region.
2. Liquid chromatography – mass spectrometry (LC-MS) analysis of polar methanolic extract showed that it was rich in phenolic compounds and more effective antioxidants than non-polar hexane extract.
3. The gas chromatography – mass spectrometry (GC-MS) analysis revealed the presence of bioactive metabolites including phenolics, fatty acid methyl esters, terpenes, stigmastane, and coumaran.
4. The antioxidant activities of both extracts were lower than that of the positive control, vitamin C; however, the presence of diverse compounds in walnut green husks demonstrates the potential of this agricultural byproduct as a natural source of bioactive compounds with essential health benefits that are yet to be discovered.

5. Further research on the antioxidant activity and other bioactivities of individual compounds present in the extracts could extend the prospects of walnut green husks as a significant source of natural compounds to the healthcare sector.

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