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The Extraction of Naphthoquinone and Tetralones from Walnut Green Husks Using Subcritical Water and Supercritical Carbon Dioxide Methods: Comparison of the Antioxidant Activities of the Extracts

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Abstract

The green husks of the walnut (*Juglans regia L.*), which is of industrial importance and cultivated in our country, were collected from the Hakkari province and extracted using subcritical water and supercritical carbon dioxide methods. The effects of different extraction methods under various temperature and pressure conditions on the chemical composition of the husks was examined using the obtained GC-MS (Gas Chromatography-Mass Spectrometry) analysis results.

The content of the walnut husks extracts was classified into tetralones, hydrocarbons, fatty acid esters and naphthoquinones. Furthermore, the antioxidant activities of the extracts were analyzed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The amount of tetralones in the extracts directly affected the DPPH antioxidant activity results.

Keywords: Naphthoquinone, Tocopherols, Antioxidant activity, Modern extraction techniques, *Juglan regia L.*

1. Introduction

Walnut, or *Juglans regia L.* (*J. regia L.*), belongs to the Juglandaceae family, traditionally cultivated for its valuable wood and fruit. All parts of the walnut tree, including the green outer husk, husk, green walnut, inner heart, root, and leaves, are widely used in the pharmaceutical chemistry and cosmetics industries [1]. The green husk is the characteristic by product of walnut cultivion areas. The use of natural waste materials is important to keep either effects on the environment via reduce waste generation or produce valuable molecules from a waste and natural materials. Additionally, these parts can serve as an excellent source of various compounds that exhibit a wide range of properties, such as antioxidant and antimicrobial potential, as well as analgesic, antiulcer, antidiabetic, antihistaminic, antiasthmatic, immunomodulatory, hepatoprotective, antiinflammatory, central nervous system stimulant, wound healing, lipolytic, insecticidal, and many other beneficial effects on human health [2].

According to the Food and Agriculture Organization (FAO), walnut is classified as a strategic species for human nutrition [3] and is also widely used in traditional folk medicine in some countries. Since the green outer husk of fresh walnuts contains a high amount of phenolic compounds and vitamins, healthy alcoholic beverage called walnut liquor is made from these husks in China [4]. Since, walnut is rich in phenolic compounds, it has excellent antioxidant properties, has been gaining increasing attention and is produced in large quantities in agricultural industries for use in various fields [5].

Naphthoquinones are biologically active compounds naturally found in plants, which can also be synthetically produced and contain a naphthalene ring (Figure 1a). Due to their strong cytotoxic effects, they exhibit antibacterial, antituberculosis, anticancer, and antifungal properties [6–14]. Plants containing naphthoquinones are also used in traditional medicine. In particular, they are widely used in China to reduce inflammation, lower fever, and treat parasitic diseases [11]. The naphthoquinone structure is also central to the vitamin K molecule.

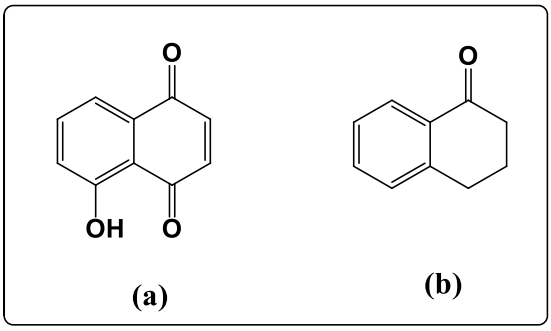


Figure 1. The structure of juglone (a) and α-tetralone (b)

Tetralones are suitable starting materials for the synthesis of biologically active pharmaceuticals and derivatives of natural products, and are important precursors and powerful reactive compounds in organic synthesis. Especially, α-tetralone is building block used in the synthesis of important functional compounds such as antidepressants, certain antibiotics, alkaloids with antitumor activity, and acetylcholinesterase inhibitors [15] (Figure 1b). In addition to acyl-CoA inhibitory activity [16], tetralones posses significant biological activities such as antimicrobial, antifungal and cytotoxic activity [17,18].

Valuable natural compounds with biological activity can be extracted from plants. Traditional extraction methods such as maceration, infusion, shaking, vortexing, and Soxhlet have recently been replaced by modern extraction techniques. The use of auxiliary technologies like pressure, microwave, and ultrasonic waves has improved extraction performance, shortened process time, reduced energy and solvent usage, altered extraction mechanisms, and allowed for the extraction of various compound classes with high yields. Modern extraction techniques such as ultrasound-assisted extraction, microwave-assisted extraction, subcritical solvent extraction, supercritical fluid extraction, and pressurized liquid extraction offer opportunities for process optimization to extract desired compound classes or enrich an extract with biologically active compounds. Thus, extracts that can be used in more valuable and diverse applications can be obtained by avoiding unwanted compounds or contaminants [19].

In this study, the variation in the components of the green husks of walnuts grown in a high-altitude region such as Hakkâri was examined according to the type of extraction method used, and the antioxidant activities of the obtained extracts were compared. Walnut green husks were extracted using subcritical water and supercritical carbon dioxide extraction methods (Figure 2). The chemical compositions of the obtained extracts were determined using GC-MS. In addition, the antioxidant activities of these extracts were evaluated using the DPPH method, and inhibition rates were compared based on their contents. To the best of our knowledge, no comprehensive study has yet been conducted on the subcritical and supercritical solvent extraction of green walnut

husks grown in the Hakkâri region, as well as on the characterization of the obtained extracts and their antioxidant activities.

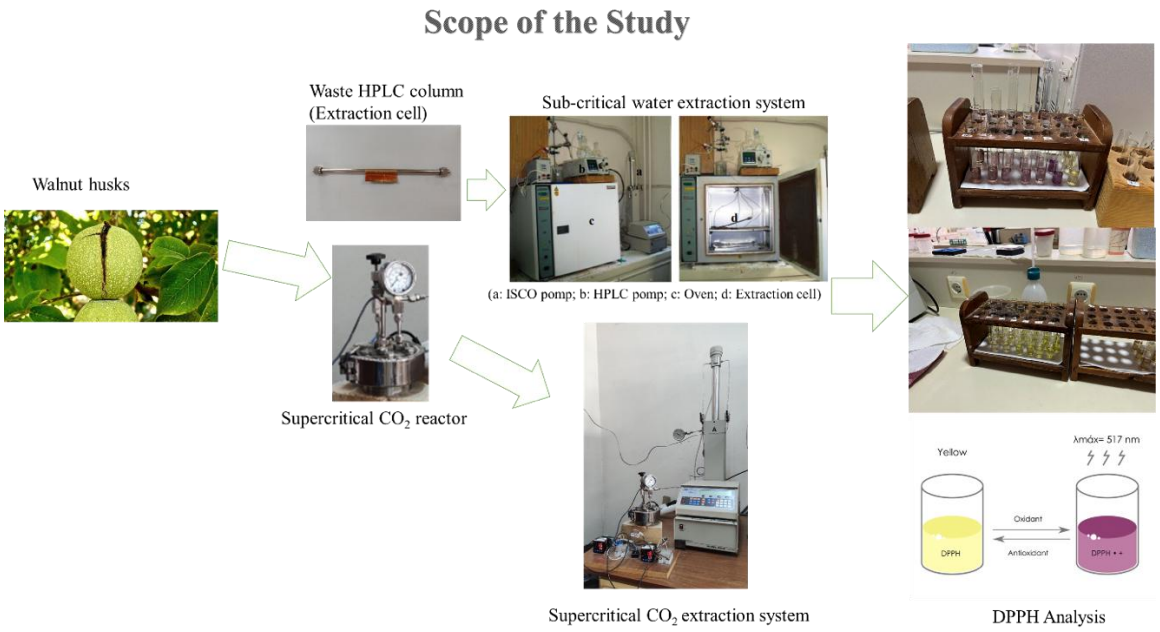


Figure 2. The scope of the study

2. Material and Method

2.1. Materials

2.1.1. Plant Source

The walnut green husks were collected in September 2022 from the Hakkâri province and were dried in the dark under room conditions for 2 weeks.

The dried samples were ground in a grinder to pass through a funnel with an inner diameter of 5 mm. They were stored in amber bottles in the refrigerator at 4^{o}C before extraction studies.

2.1.2. Chemicals

Unless noted otherwise, all of compounds were used as provided without further purification. All chemicals were procured from Merck, and carbon dioxide was obtained from Linde. Dichloromethane (analytical purity), methanol (analytical purity), ethanol (absolute purity), carbon dioxide (CO_2) (g) (99.9999% purity), 2,2-diphenyl-1-picrylhydrazyl (analytical purity), butylated hydroxytoluene (analytical purity).

2.2. Methods

2.2.1. Preparation of Plant Extracts Using Supercritical CO_2 ($scCO_2$-E) Method



Figure 3. Supercritical and subcritical fluid extraction system A: ISCO pump; B: Oven; C: Extraction cell

The parameters used in the study and their ranges were determined through preliminary trials. Briefly, 2 grams of plant material are placed into a 100 mL volume reactor, which is wrapped with a heating jacket and connected to an ISCO tower pump (Figure 3). At the desired pressure, CO_2 is sent into the reactor by tower pump. Once the heater reaches the desired temperature, the system kept for one hour extraction time. After this period, the reactor is cooled down, and the reactor content is collected in an amber bottle containing dichloromethane (Figure 3). After evaporating dichloromethane, the extract is stored at +4^{o}C until further analysis.

2.2.2. Preparation of Plant Extracts Using Subcritical Water (sbcWE) Method

The parameters used in the study and their ranges were determined through preliminary trials [20]. Briefly, after filling the extraction cell with 2.0 g of the sample, it was placed in the oven to operate at the desired extraction temperature (Figure 3). Using an HPLC (High Performance Liquid Chromatography) pump, subcritical water was sent to the cell at the desired pressure, Extraction time was 30 min for static extraction with sbcWE and 20 min dinamic extraction with subcritical ethanol (sbcEtOH). Experimental conditions provided in Table 1.

Table 1. Experimental Conditions for sbcWE and $scCO_2E$ Methods

sbcWE		**$scCO_2E$**	
Temperature (^{o}C)	Pressure (atm)	Temperature (^{o}C)	Pressure (atm)
100	60 and 80	70	90
125	60 and 80		

140	60 and 80	70	300
160	60 and 80		

2.2.3. Chromatographic Separation and Identification

All extracts were analyzed by using a Thermo-Finnigan Trace GC-MS with electron impact (70 eV) assisted by an autosampler, and 1 µL of the sample was injected into the column for analysis using a 50 split ratio. Helium was used as the carrier gas, and the flow rate was set to 1.0 mL/min. A temperature programme was used between 50-240^{o}C at a rate of 3^{o}C/min. The injection port temperature was set to 250°C. A TR-MS-5 column (60 m x 0.25 mm x 0.25 µm, 5% phenyl polysiloxane) were used. Each component was identified by comparing mass spectra with the Wiley07 and NIST (National Institute of Standards and Technology) (2005) libraries.

2.2.4. DPPH Antioxidant Activity Test

The antioxidant activity of the husk extracts was tested according to the Blois method [21]. Briefly, 50 µL of extract was mixed in a 10 mL test tubes and 3 mL of a 0.001% 1,1-diphenyl-2-picrylhydrazyl ethanol solution. The mixture was allowed to stand in the dark for 15 minutes. After a time period, the absorbance value of the solution at 517 nm against ethanol was measured using a UV (Ultraviolet-Visible) spectrophotometer. The radical scavenging percentage was calculated using the following formula:

$$\text{Inhibition Percentage: } [(Ao - A1)/Ao]x100$$

Where, Ao: Absorbance value of the control solution, A1: Absorbance value of the sample or standard solution

A calibration curve of percentage inhibition of buthylatedhydroxytoluen (BHT) was drawn for 20, 40, 60, 80 and 100 µg/mL concentration to determine the percentage inhibition rate. All experiments were performed in triplicate. The curve equation used for calculating the percent inhibition values of the extracts and the standard calibration curve of BHT are shown in Figure 4.

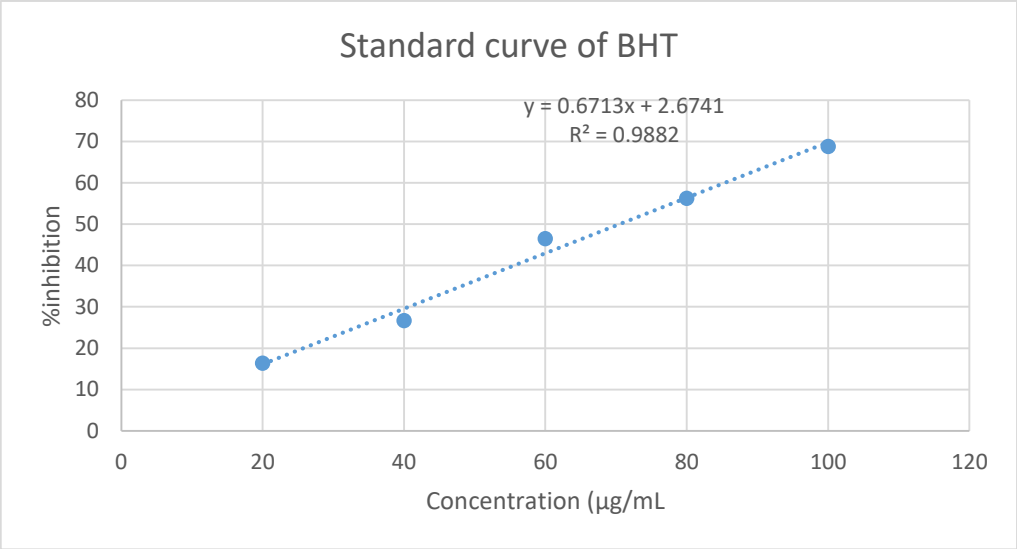


Figure 4. Standart curve of Buthylated hydroxytoluen (BHT)

3. Results

3.1. GC-MS analysis of sbcW and scCO₂ extracts

The GC-MS analysis of sbcW and $scCO_2$ extracts of walnut husks revealed that the main classes of molecules were naphthoquinones, tetralones, hydrocarbons (HC). The hydrocarbons (HC) included straight-chain and branched alkanes and alkenes, while fatty acid esters and fatty alcohols were also identified. The amount of components was calculated as relative abundance according to the peak area ratios from the GC chromatograms.

Table 2 presents the main phytochemicals in the extracts of husks obtained by two different methods. From Table 2, it can be seen that the most remarkable differencenses between $scCO_2E$ and sbcW extracts is efficiency of these two green methods on the extraction of naphtaquinones particullary in Juglone. Juglone is most abondant component of walnut organels [1–3,22,23].

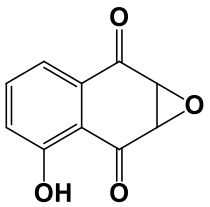
The maximum relative amount of juglone was determined at in the $scCO_2E$ extract at 70°C at 90 atm pressure. Under these conditions, the relative amount of juglone was 19.0, while it was 4.0 in the extract of 300 atm. On the other side, sbcW could only extract insignificant amounts of Juglone at 80 atm, 125 and 160ºC as 0.6 and 0.3, respectively. Similarly, for tetralone extraction, the $scCO_2E$ method was more effective than the sbcWE method. Tetralone derivatives were extracted at a maximum of 12.1 at 300 atm pressure, while the sbcW at 125°C and 60 atm extracted tetralone derivatives in maximum amount (9.5). The distribution of tetralones in the extract of $scCO_2E$ is given in Table 2. Accordingly, 2,5-dihydroxy-1,4-dihydronaphthoquinone (2.2), 2,6-dihydroxy-1,4-dihydronaphthoquinone (0.3), 7-hydroxy-3,4-dihydronaphthalenone (2.0), 7-methoxy-1-tetralone (1.4), isoscrelone (6.8) and sclerone (1.8) (Figure 5). At 300 atm extraction with $scCO_2$, same molecules, the same molecules were detected but in different relative abundances.

Table 2. The Relative Abundances of Chemical Composition of Husk Extracts

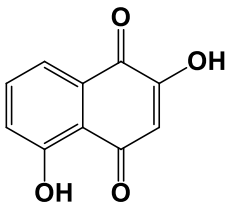
Extraction method	$scCO_2E$		sbcW E							
Temperature (°C)	70	70	100		125		140		160	
Pressure (atm)	90	300	60	80	60	80	60	80	60	80
Molecule classes	% Relative abundance									
*NQs & tetralones	24.8	16.7	1.4	1.9	9.5	7.6	1.5	3.5	6.8	6.9
Juglone	19.0	4.0	-	-	-	0.6	-	-	-	0.3
Tetralones	5.8	12.1	2.1	1.9	9.5	6.1	1.5	3.5	6.8	7.0
HCs	32.5	36.1	10.7	9.0	17.5	15.0	7.9	19.2	22.5	16.5
*FA esters	17.5	3.2	7.6	21.6	21.8	20.0	37.1	25.0	17.1	15.6
*F-alcohols										

*NQs: Naphthaquinones, FA: Fatty acids; F-Fatty.

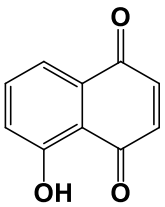
Naphthoquinones



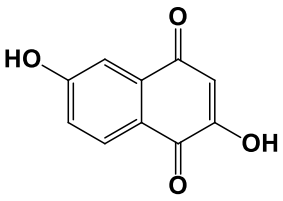
1,2,3,4-tetrahydro-2,3-epoxy-5-hydroxy-1,4-naphthaquinone



2,5-dihydroxy-1,4-naphthaquinone

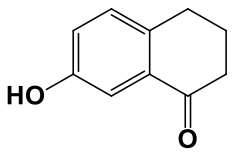


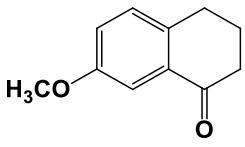
5-hydroxy-1,4-naphthaquinone (juglone)

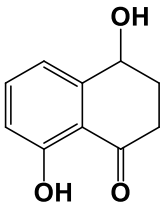


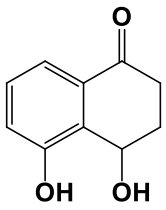
2,6-dihydroxynaphthalene-1,4-dione

Tetralones









1,5-dihydroxy-

7-hydroxy-3,4-dihydro-1-(2H)-naphthalenone	7-methoxy-3,4-dihydro-1-(2H)-naphthalenone	3,4-dihydro-1-(2H)-naphthalenone (isosclerone)	4,5-dihydroxy-3,4-dihydronaphthalen-1(2H)-one (sclerone)

Figure 5. Naphthoquinone and tetralone structures obtained in husk extracts

3.1.1. The effects of sbcW extraction method on the extraction of tetralones from walnut husks

The most effective method for the extraction of biologically active compounds, such as tetralones from green husk is the $scCO_2$ extraction at 300 atm (12.1) Table 2. Hovewer, in sbcWE, tetralones have been detected in extracts obtained at lower pressures and at all temperatures. Figure 6 show the effecet of temperature and pressure on tetralon extraction in sbcWE. According to Figure 6, the tetralone content reaches its maximum value at 125ºC and 60 atm, similarly the minimum content was obtained at 140ºC and 60 atm.

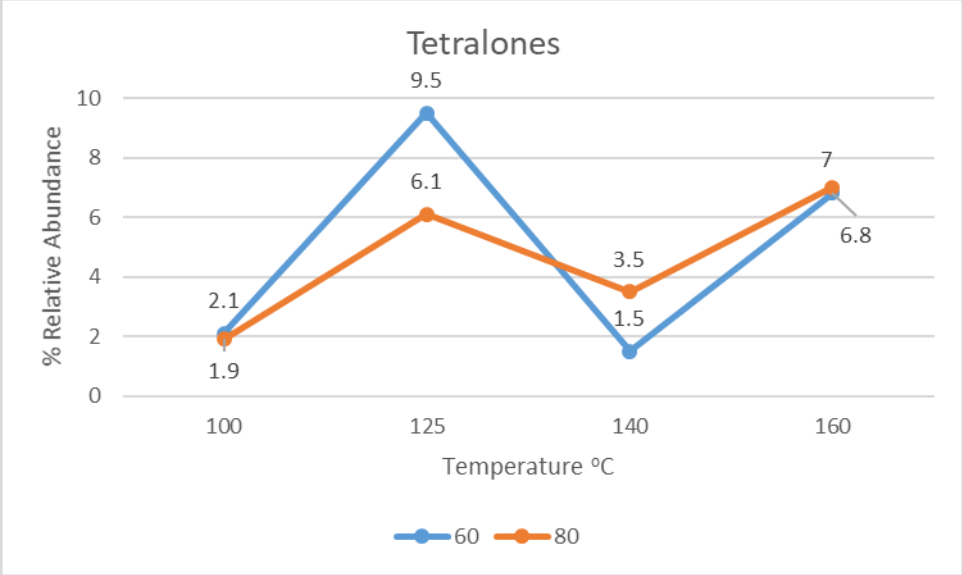


Figure 6. Effect of temperature and pressure on tetralone extraction in sbcW extraction

3.1.2. The effects of sbcW and $scCO_2$ extraction methods on the extraction of hydrocarbons (HCs) from walnut husks

Supercritical CO_2 is a much more nonpolar solvent than subcritical water. As expected, hydrocarbons most effectively extracted by $scCO_2$. The recovery of HCs of husk was 36.1% at 300 atm and 32.5% at 90 atm. This indicates that pressure has an effect on the extraction of HCs (Table 3).

Table 3. Comparison of the Relative Abundances of Certain Molecular Classes in Walnut Husk Extracts and the DPPH Percentage Inhibition Values of the Extracts

Extracts	Tetralones	HCs	FAEs-F-Alcohols	Juglone	DPPH activity %
sbcWE					
100°C-60 atm	2.1	10.7	7.6	-	84.68
100°C-80 atm	1.9	9.0	21.6	-	61.00
125°C-60 atm	9.5	17.5	21.8	-	83.84
125°C-80 atm	6.1	15.0	20.0	0.6	64.07
140°C-60 atm	1.5	7.9	37.1	-	58.50
140°C-80 atm	3.5	19.2	25.0	-	63.51
160°C-60 atm	6.8	22.5	17.1	-	80.78
160°C-80 atm	7.0	16.5	15.6	0.3	83.01
scCO$_2$E@70°C					
90 atm	5.8	32.5	17.5	19.0	55.99
300 atm	12.1	36.1	3.2	4.0	86.91

In sbcWE extraction, HC extraction efficiency reached its maximum at 160ºC and 60 atm (22.5%). As the temperature of sbcW increased, its polarity decreases [24]. Therefore, this result was not surprising. Figure 7 shows the effect of temperature and pressure on HC extraction in sbcW extraction. Upon examining the graph, it can be said that at 60 atm pressure, there is a fluctuation in HC extraction efficiency depending on temperature, while at 80 atm pressure, there is an increase up to 140°C, followed by a decrease at 160°C. Thus, it can be said that higher temperatures are effective in HC extraction in sbcW extraction.

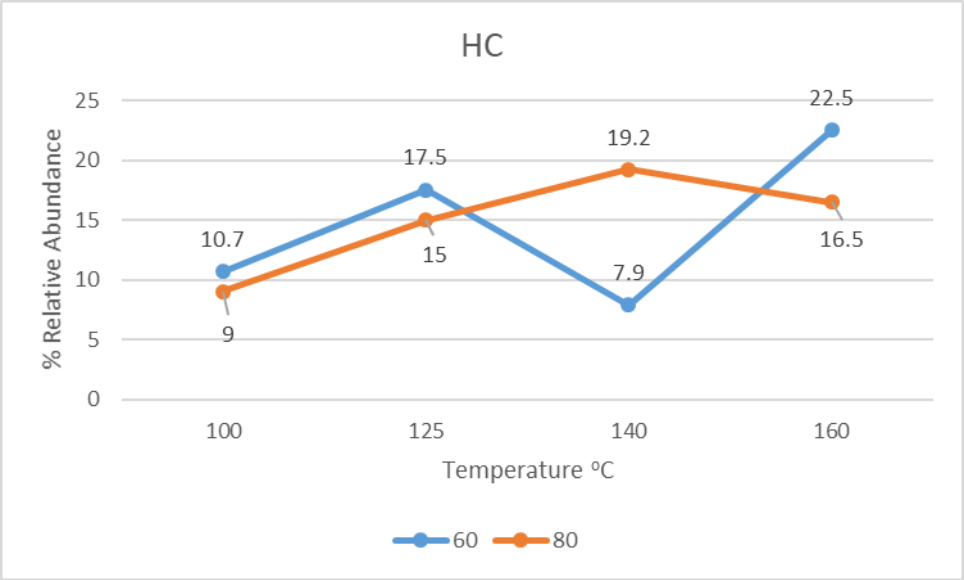


Figure 7. Effect of temperature and pressure on HC extraction in sbcW extraction

3.2. DPPH assay results

The DPPH method was chosen in this study because it is simple and rapid to apply, the color changes are visually observable, it offers advantages over other tests in determining the activities of fat-soluble antioxidants, it involves a true free radical, and it can directly indicate radical-scavenging capacity. Antioxidant activity of natural products depends on their chemical composition. Among them phenolic compounds suggested as potential sources of antioxidant compounds. Phenolic compounds act as reducing agent due to their redox properties. They are also hydrogen donators and singlet oxygen querchers. As mentioned above, sbcW and $scCO_2$ extracts of green husk include tetralones and juglone in remarkable amount (Table 2). As seen from Figure 8, tetralons and Juglone are phenolic compounds. DPPH antioxidant activity results of the extracts are presented in Table 3.

As can be seen from Table 3, different sbcW and $scCO_2$ extracts different antioxidant properties depending on the used extraction fluid. $scCO_2$ at 70°C and 300 atm is the most efficient extraction fluid for the extraction of tetralones (12.1%) and further for the highest DPPH antioxidant activity (86.91%). In addition, the relative yield of Juglone is also found in the highest amount in extracts. We could say that tetralones and Juglone have a positive effect on the antioxidant activity of the extracts. As seen from Table 3, another molecule class as in the extracts didn't show an influence on the antioxidant activity.

In case of sbcW extracts of green husk, a very good correlation was observed between tetralones content and DPPH antioxidant activity (Figure 8). At 125°C and 60 atm, relative abundance of tetralones was 9.5 and this extract show highest DPPH antioxidant activity (83.84%). According to presented data, the second highest antioxidant property was obtained at 160°C and 80 atm, and the weakest for extracts of 140°C and 60 atm. Tetralones contents of these last two extracts is also gave similar relationship with antioxidant activty.

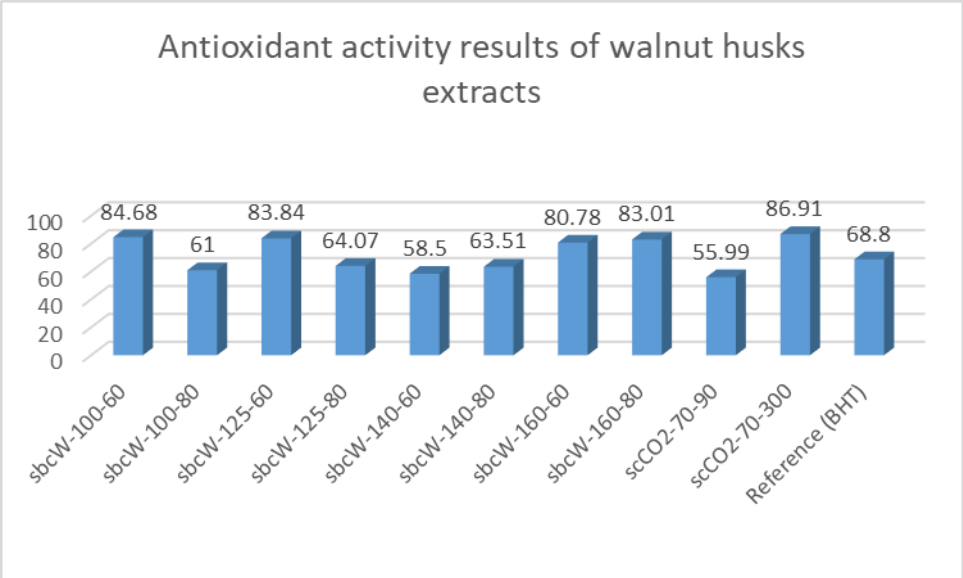


Figure 8. Antioxidant activity results of walnut husks extracts

4. Conclusion

During the valnut harvesting process considerable amounts of husks produced as a by-products rich in juglone and tetralones phenolic which are beneficial compounds. In this study, for the first time, walnut husks collected from a high-altitude region such as Hakkari were extracted using modern extraction techniques, their chemical compositions were determined, and their antioxidant contents were revealed in order to investigate the relationship between their antioxidant activities and chemical compositions. The green husk of walnut fruit were extracted by sbcw-E and $scCO_2$-E methods. The investigations of the extracts indicated that it contains different chemicals which mainly belongs to the classes of naphthoquinones, α-tetralones, hydrocarbons, fatty acid esters and fatty alcohols. The effects of extraction temperature and pressures during sbcW-E and $scCO_2$-E on the chemical composition of the extracts and their DPPH antioxidant activities were examined. It was found that the DPPH inhibition activities of the walnut husk extracts and their tetralones content were in very good correlation and they could be a good alternative to synthetic antioxidants. Investigation of the pharmacological activities of these extracts is among our ongoing studies. Based on the study results, the biochemical recycling of green walnut husk wastes, rich in valuable bioactive compounds and showing high antioxidant activity, offers potential benefits for environmental sustainability.

Authorship contribution statement

M.Erşatır: *Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resource, Data Curation, Original Draft Writing, Review and Editing,*

Declaration of competing interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Committee Approval and/or Informed Consent Information

As the author of this study, I declare that I do not have any ethics committee approval and/or informed consent statement.

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