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# INVESTIGATION ON THE VOLATILE CONSTITUENTS OF *JUGLANS REGIA* AND THEIR *IN VITRO* ANTIOXIDANT POTENTIAL

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**Abstract:** Here, we report the chemical composition of the volatile constituents of Pakistani cultivar of walnut using Gas Chromatography-Mass Spectrometery (GC-MS), and its antioxidant potential using 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging and phosphomolybdenum complex (PC) method. This study revealed that Pakistani cultivar exhibited an adequate antioxidant potential having $IC_{50}$ of 51.25 ± 0.74 µL/mL.

***Keywords*:** *Juglans regia* L., common walnut, GC-MS analysis, steam distillation

## Introduction

The high protein and oil content of the kernels of *Juglans regia* L. (Juglandacea) makes this fruit indispensable for human nutrition. Therefore, the walnut is classified as a strategic species for human nutrition and is included in the FAO list of priority plants [1]. Walnut has been widely used as herbal medicine in the treatment of diabetes [2] and in folk medicine to treat prostate and vascular disturbance [3]. It also has high anti-atherogenic potential and a remarkable osteoblastic activity that adds to the beneficial effect of a walnut-enriched diet on cardioprotection and bone loss [4]. The cardiovascular protective effect of a walnut diet has been related to antioxidant and hypocholesterolaemic effects as well as via modulation of endothelial function [5]. Walnut extracts have been exhibited antioxidant capacity in a concentration-dependent manner in different performed assays. Their antimicrobial capacity was also checked against different gram positive and gram negative bacteria, and fungi, revealing activity against the different tested microorganisms [6].

Currently, the possible toxicity of synthetic antioxidants has been criticized. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea, and herbs may contribute to shift the balance toward an adequate antioxidant status [7]. Therefore, natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer. The defensive effects of natural antioxidants in fruits and vegetables are related to three major groups; vitamins, phenolics and carotenoides. Ascorbic acid and phenolics are known as hydrophilic antioxidants, while carotenoides are known as lipophilic antioxidants [8-11]. The present study was under taken to investigate the chemical composition of the volatile constituents of Pakistani cultivar of Walnut, and their antioxidant potential using 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging and phosphomolybdenum complex (PC) method.

## Materials and Methods

### *Plant Material*

The nuts of *Juglans regia* L. (common

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walnut) were purchased from a local dry-fruit market on Mall road, Lahore, in March 2009, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore.

*Extraction of Antioxidants*

The ground nuts (50 g) of *Juglans regia* L. were placed in a 2L round-bottom flask of steam-distillation assembly containing 150 mL of diethyl ether. The steam was thrashed on it for two hours and in the receiving flask two layers, an aqueous and an organic, were obtained. The organic layer was separated, dried over anhydrous sodium sulfate, filtered and the solvent was evaporated to obtain concentrated oil. This volatile oil was then analyzed by means of gas chromatography-mass spectrometry (GC-MS) and was also used to evaluate the antioxidant potential.

*Chemicals and standards*

DPPH· (1,1-Diphenyl-2-picrylhydrazyl radical) and BHT (Butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA) and diethyl ether solvent, sulphuric acid, sodium phosphate and ammonium molybdate from Merck (Pvt.) Ltd. (Germany). Individual standards for GC-MS were purchased from Aldrich (Milwaukee, WI).

*GC-MS Analysis*

A Shimadzu 2010 (Shimadzu, Japan) gas chromatograph equipped with a split-splitless auto-injector model AOCi, an auto sampler model AOC-20s and a MS-QP 2010 (Shimadzu, Japan) series mass selective detector was used for the analysis of the essential oil studied. A fused silica capillary column (J&W DB 5MS®), 5% phenyl polysiloxane as non-polar stationary phase (30 m × 0.25 mm i.d.) and 0.25 μm film thickness, supplied by Agilent (Palo Alto, CA, USA) was used for the GC separation, with helium as carrier gas at a constant flow at 1.27 mL/min. The temperature program used was as follows: initial temperature, 40°C held for 5 min, then at the rate of 5°C /min to 180°C, and 3°C /min to 240°C and then maintaining this temperature for 5 min. The temperature of the injection port was 200°C and a 1 μL volume was injected in split mode with 90 % split ratio. Mass selective detector was operated in electron impact (EI) ionization mode with an ionizing energy of 70 eV, scanning from *m/z* 40 to 950 at 0.5 s per scan. The ion source temperature was 250°C and the MS transfer line temperature was 205°C. The electron multiplier voltage (EM voltage) was maintained at 1000 V, and the solvent delay of 3.0 min was employed [12,13]. All the compounds were identified by comparison of their GC retention data and mass spectra with those of pure and authentic samples, and also by comparing fragmentation patterns of mass spectra with those stored in the spectrometer data base (NIST 147) and bibliography [14,15].

*DPPH Radical Scavenging Activity*

The DPPH radical scavenging activity of volatile fraction of Pakistani walnut was examined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the method of Lee *et al.* [16]. Briefly, various amounts of the volatile extract (300 μL, 200 μL, 100 μL) were mixed with 3 mL of chloroform solution of DPPH (0.1 mM). The mixture was shaken vigorously and allowed to stand at room temperature for an hour. Then Absorbance was measured at 517 nm against chloroform as a blank in the spectrophotometer. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity.

The percent of DPPH decoloration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = A_{control} - A_{sample} / A_{control} \times 100$$

Each extract was assayed in triplicate and mean values were calculated.

*Total Antioxidant Activity by Phosphomolybdenum Method*

The total antioxidant activity of volatile fraction was evaluated by phosphomolybdenum complex formation method [17]. Briefly, various amounts of volatile fraction (300 µL, 200 µL, 100 µL) were mixed with 4ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution. The vials were capped and incubated in water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of butylated hydroxytoluene (BHT).

*Statistical Analysis*

All the measurements were done in triplicate and statistical analysis was performed by Microsoft excel 2003. Results are presented as average ± SEM.

**Results and Discussion**

The chemical composition of volatile extract of Pakistani cultivar of walnut was investigated using Gas Chromatography-Mass Spectrometery (GC-MS) analysis. When the GC profile [Fig. 1] of walnut was studied, it exhibited twenty one constituents, which were identified by comparison of their GC retention data and mass spectra with those of pure and authentic samples, and also by comparing fragmentation patterns of mass spectra with those stored in the spectrometer data base (NIST 147) and bibliography [9,10]. The names of identified compounds, their retention times and area percentages are shown in Table 1. Some volatiles e.g. α-thujene, Sabinene, *p*-cymene, 1,8-cineol, Linalool, Myretenal, Pinocarveol, Verbenol, Myrtenol, *p*-cymen-8-ol, Isobutyl cyanide and Benzyl alcohol have already been reported from walnut [18] while the remaining constituents namely α-cadinol, α-bisabolol [19], Isopulegol [20], Carvacrol, [21], Estragol [22], Globulol [23], Viridiflorol [22, 23], Nerolidol [24], and Neo-iso-3-thujanol [25] are reported for the first time from this species. In a search for potential bioactive substances from plant origin volatile oil of Pakistani walnut was studied, for free radical scavenging activity using 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) and its total antioxidant activity by Phosphomolybdenum method and the results are shown in Table 2 and Table 3, respectively. The results showed that greater the concentration of volatile extract, greater its antioxidant effect. Due to the presence of various oxygenated constituents [Table 1], it also exhibited an adequate *in vitro* 'total antioxidant activity' and 'DPPH scavenging activity' relative to butylated hydroxytoluene (standard). The $IC_{50}$ of the oil was calculated as 51.25 ± 0.74 µL/mL, relative to butylated hydroxytoluene (BHT), having $IC_{50}$ of 12.1 ± 0.92 µL/mL. Hydrogen and electron transfer from antioxidant analytes to DPPH radical and Mo(VI) complex occur in DPPH and phosphomolybdenum assay methods. The transfer occurs at different redox potentials in

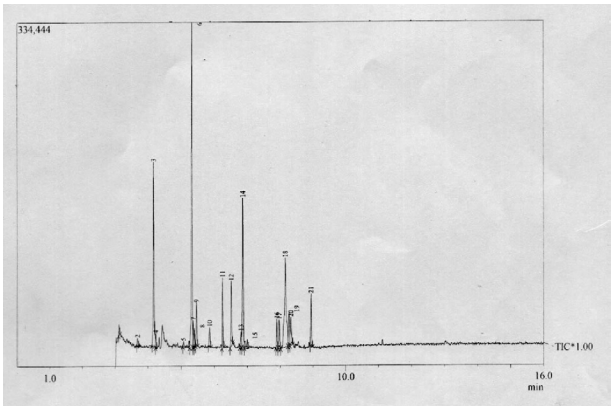


**Figure 1.** Peak profile for the volatile oil of *Juglans regia* L. (Retention time on X-axis; intensity on Y-axis).

**Table 1.** Volatile Constituents of *Juglans regia* L. with retention time and area%.

| Peak No. | Retention Time | Compound Name | Area% |
|---|---|---|---|
| 1 | 3.119 | α-Thujene | 0.50 |
| 2 | 3.682 | Sabinene | 0.60 |
| 3 | 4.168 | *p*-Cymene | 10.94 |
| 4 | 4.233 | 1,8-Cineol | 0.67 |
| 5 | 5.068 | α-Cadinol | 0.40 |
| 6 | 5.317 | Benzyl alcohol | 18.14 |
| 7 | 5.359 | α-Bisabolol | 1.36 |
| 8 | 5.400 | Linalool | 1.07 |
| 9 | 5.461 | Isopulegol | 2.63 |
| 10 | 5.857 | Carvacrol | 1.59 |
| 11 | 6.251 | Myretenal | 3.88 |
| 12 | 6.511 | Estragol | 4.19 |
| 13 | 6.810 | Pinocarveol | 1.21 |
| 14 | 6.863 | Globulol | 10.95 |
| 15 | 6.993 | Verbenol | 1.17 |
| 16 | 7.903 | Viridiflorol | 1.85 |
| 17 | 7.967 | Myrtenol | 2.03 |
| 18 | 8.156 | Nerolidol | 13.54 |
| 19 | 8.265 | *p*-Cymen-8-ol | 2.83 |
| 20 | 8.324 | Neo-iso-3-thujanol | 2.67 |
| 21 | 8.941 | Isobutyl cyanide | 3.30 |
| | | | 100.00 |

**Table 2.** Free radical scavenging activity of *Juglans regia* L. (common walnut) using 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH).

| S. No. | Sample | Concentration in Assay (µL/3mL) | %age Scavenging of DPPH· ± S.E.M.[a]) |
|---|---|---|---|
| 1 | *Juglans regia* L. | 300 | 89.09 ± 0.74 |
| | | 200 | 63.08 ± 0.56 |
| | | 100 | 49.51 ± 0.64 |
| 2 | BHT[b]) | 500 | 91.74 ± 0.62 |
| | | 100 | 85.11 ± 0.25 |
| | | 50 | 61.34 ± 0.06 |

[a] Standard error of means of three assays. [b] Standard antioxidant

the two assays and also depends on the structure of the antioxidant. Several hydroxylated compounds have been isolated from plant extracts with DPPH radical scavenging activities, whereas the phosphomolybdenum method usually detects

**Table 3.** Total antioxidant activity of *Juglans regia L.* (common walnut) by phosphomolybdenum method (absorbance at 695 nm).

| S. No. | Sample | Concentration in Assay (µL/4mL) | Total Antioxidant Activity ± S.E.M.[a]) |
|---|---|---|---|
| 1 | *Juglans regia* L. | 300 | 0.892 ± 0.72 |
| | | 200 | 0.585 ± 0.61 |
| | | 100 | 0.382 ± 0.59 |
| 2 | BHT[b]) | 500 | 1.893 ± 0.31 |
| | | 100 | 1.760 ± 0.56 |
| | | 50 | 1.452 ± 0.47 |
| 3 | Blank | 75 | 0.217 ± 0.75 |

[a] Standard error of means of three assays. [b] Standard antioxidant

antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoides. Ascorbic acid, glutathione, cysteine, tocopherols, polyphenols and aromatic amines have the ablity to donate hydrogen and electrons and can thus be detected by the two assay models [16,17].

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