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ORIGINAL ARTICLE

Quality Profiling and Estimation of Total Phenols, Flavonoids, Flavonols and Antioxidative Potential of Walnut kernel (*Juglans regia*) from Kashmir valley

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A R T I C L E I N F O

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Secondary metabolites

A B S T R A C T

Present research study was carried out with four walnut (*Juglans regia* L.) genotypes viz. *KG* (Kulgam), *CS* (Char-e-Sharief), *KW* (Kupwara) and *TM* (Tangmarg) from major walnut producing areas of Kashmir valley. The aim of the study was the selection of superior genotypes having better quality nuts and kernels with higher antioxidant potential, for its exploitation at farm and consumer level. Nut and kernel external quality traits were recorded. The results revealed that the nut weight ranged between 11.70 to 14.51g; nut diameter between 9.60 to 14.27mm; nut length between 17.40 to 20.51mm. The maximum variability was observed in nut weight, kernel weight, kernel recovery, nut length and nut diameter. The fat content accounted for more than 60% of walnut kernel weight and ranged between 49.83 to 83.76%. The antioxidant potential, proximate and mineral composition, total phenolic content, flavonoids, as well as, flavonols, were all evaluated. Mineral content; zinc (Zn), iron (Fe), manganese (Mn), copper (Cu) magnesium (Mg) were determined. The Total Phenolic Content varied between 19.8 to 50.19mgGAE/g while the total flavonoid and flavonols ranged from 188.5 to 815.08mgQE/100mg and 3.46 to 7.77mgQE/g respectively. The walnut extracts (0.5mg/mL) showed 82.60 to 97.19% DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity. This study demonstrates that owing to maximum nut and kernel weight, as well as, nut recovery, better free radical scavenging properties and higher phenolic profile of *KW* (Kupwara) extract, this genotype seems to serve as a potential and promising one for production of walnut on large scale to avoid non uniformity.

Introduction

The Juglandaceae family includes walnuts (*Juglans regia* L.). Walnut production in the globe is estimated to be 1,500,000 metric tonnes. China, the United States, and Iran are the major walnut-producing countries in the world, accounting for around 25%, 20%, and 11% of global production, respectively (Gharebzahedi *et al*., 2014). Walnuts provide a lot of nutritional and medicinal benefits (Jahanbani *et al*., 2018). Walnut has a long history of therapeutic use in Silk Road countries to cure a range of ailments (Vahdati, 2014). Walnut (*Juglans regia* L.) is economically significant due to the nutritious content of the nut and its wood (Khodadadi *et al*., 2020). All plant organisms have abundant phenolic

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chemicals as secondary metabolites, with a role in fruit tree growth and development, as well as pre- and post-harvest fruit life (Cheniany *et al*., 2010). Walnuts are a healthy nut that include alpha-linolenic acid in their lipid fraction and poly phenolics in their skin, both of which have potential free radical scavenging qualities (Gao *et al*., 2019). Synthetic antioxidants such as Butylated Hydroxyl Anisole (BHA), Butylated Hydroxy Toulene (BHT), tert. Butyl Hydroquinone (TBHQ) and Propyl Gallate (PG) have been utilised widely in the past, owing to their chemical stabilities and significant antioxidant activities. However, due to the potential negative consequences of these synthetic oxidants, academics and the food industry have been looking for alternative potent natural molecules with outstanding antioxidant qualities over the past two decades (Gursul *et al*., 2019).

Natural antioxidants, such as phenolic compounds and flavonoids, are gaining popularity as a result of their health benefits, which include lowering the risk of degenerative diseases by reducing oxidative stress and inhibiting macromolecular oxidation (Oliviera *et al*., 2008 and Yang *et al*., 2014). Walnuts contain a variety of phenolic molecules, some of which play a function in pathogen defence (Khodadadi *et al*., 2016). Walnut proteins and protein hydrolysates have garnered a lot of attention for their anti-atherogenic, anti-mutagenic, and antioxidant properties (Jahanbani *et al*., 2016). Walnut kernels also have a high oil content, which is beneficial because the main fatty acids found in walnut kernel oil are polyunsaturated fatty acids (PUFA) (linoleic [18:2, omega-6] and linolenic [18:3, omega-3]) and monounsaturated fatty acids (oleic [18:1, omega-9]). Walnut kernels have a higher concentration of linoleic acid and a better linoleic acid/linolenic acid ratio, making them one of the greatest sources of these substances for humans (Pakrah *et al*., 2021). Walnuts have a somewhat astringent flavour, and phenolics together with tocopherols, serve an important function in shielding unsaturated fatty acids from oxidation (Trandafir *et al*., 2016). Ellagic acid, Gallic acid, Ellagitannins (tannins) and many other such compounds with excellent anti-oxidant potential, in the form of polyphenols are found in walnuts (Ahad *et al*., 2020). Antioxidant-rich diets, on the other hand, have been shown to lower the risk of acquiring a variety of diseases, including cancer, and to scavenge numerous free radicals (Jahanbani *et al*., 2016). The aim of this study was to determine the phytochemical efficacy of walnut kernels from four different genotypes grown under different environmental conditions, but gathered at the same time, by measuring TPC, total flavonoids, flavonols, and free radical scavenging capability. The scavenging action on DPPH was used to access antioxidant potential (2,2-diphenyl-1-picrylhydrazyl).

Materials and Methods

Chemicals and reagents

Merck supplied the Folin-Ciocalteu reagent (2N), hydrochloric acid (37%) and potassium chloride, as well as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and methanol (Germany). Sigma (Germany) provided Gallic acid (99% purity), Anhydrous Sodium carbonate (99% purity), Anhydrous Sodium acetate (98%), Aluminium nitrate, Potassium acetate, and Quercetin (HPLC-grade); and Sigma-Aldrich Co., St Louis, USA provided 2, 2-diphenyl-1-picrylhydrazyl (DPPH, 90% purity). The tests were conducted with analytical-grade chemicals and reagents. The water used for analysis was purified using a Milli-Q water purification device (Millipore, Bedford, MA, USA).

Plant material

Samples of walnut (*Juglans regia* L.) were obtained from various eco geographical regions of Jammu and Kashmir, covering a wide range of altitude areas (Table1). All walnut samples were collected from mature trees grown in the designated zones in 2020. Nuts were gathered from three trees chosen as duplicates for each tree. Once harvested,

nuts were instantly taken to the laboratory within two hours. Peeling of the green husks was immediately carried out and the shelled walnuts were rinsed with water. For about four days, the cleaned walnuts were dried under sun and in the open air conditions. After drying, the hard shells of the nuts were manually cracked, and the samples were visually inspected, to detect them with defects, such as insect damage, fungus development, odd colour, or harmful conditions. Only healthy kernels, devoid of illnesses macroscopically, were chosen for analysis, and defective samples were rejected. For later use, the selected nuts were kept in polyethylene bags and stocked at 3-5 ^{0}C and 65-75% relative humidity.

Table1. Areas Specifications

Sampling areas	Latitude	Longitude	Altitude(m)
Kupwara (Sogam)	34 ^{0} 07 / 92.5 // N	74 ^{0} 72 / 86 // E	1615
Char-e-Sharief (Nagam)	33 ^{0} 55 / 29 // N	74 ^{0} 47 / 23 // E	1933
Tangmarg (Dhobiwan)	34 ^{0} 05 / 35.2 // N	74 ^{0} 32 / 41.6 // E	2080
Kulgam Munad)	33 ^{0} 44 / 21.08 // N	74 ^{0} 57 / 45.75 // E	1639

Morphological analysis

During the harvest season, nearly 20-30 nuts from mature trees were arbitrary collected and their traits like shell colour, shell texture, shell integrity shell thickness, shell strength, nut length, nut weight, nut diameter at suture, nut diameter at cheek, nut shape, kernel weight, kernel colour and kernel percentage were evaluated according to the international descriptor for walnuts (Eriksson, 1998).

Extraction preparation

Sun dried walnut kernels were milled to a fine powder with the help of a grinder. Briefly about 5 g of each sample was extracted with 100 mL of methanol, incubated in an ultrasonic bath for 40 min at 50°C, and then centrifuged at 8000 rpm for 10 min. For analysis, the supernatants were filtered through Whatman no. 4 filter paper and stored at 4^{0}C.

Physico-proximate composition

Colorimetry

For colorimetry, a Hunter colour lab spectro-colorimeter was employed. Colour indices, indicated by the CIE
(Commission International de I'Eclairage) are being measured by the instrument. For the determination of the kernel colour of the walnuts of each samples, the CIE, L^*, a^* and b^* colour indices were assessed. The colour index L^* (lightness component) extends from (0 to 100) and the other two colour indices viz; a^* (from green to red) and b^* (from blue to yellow) are ranging from -120 to 120. Using the below mentioned equation, the WI (Whiteness index) was calculated.

$$(WI) = 100-[(100-L^*)^2+(a^*)^2+(b^*)^2]^{1/2}$$

Browning Index

For determination of the browning index of walnut samples, the method as described by Lee *et al*., (2016) was followed with certain modifications. About 15g of each sample were homogenised in 90% methanol in a lab blender for about two minutes and then centrifuged at 8000rpm for 20 min at 4°C. Whatman no.2 filter paper was used to filter the supernatants. Immediately, the absorbance was measured at 420nm and greater values of absorbance indicate advanced browning of the tissue.

Proximate composition

Ash, moisture, protein, fat contents of the grounded walnut kernels were evaluated according to Association of Analytical Chemists (AOAC, 1998) protocol. For each component, the samples were examined in triplicate. The carbohydrate content was established by comparing the differences in other components, rather than by using

an analytical method. Each sample's mineral content was determined using the Association of Analytical Chemists' method (AOAC, 1990). Zinc (Zn), Iron (Fe), Magnesium (Mg), Manganese (Mn) and Copper (Cu) were analysed by AAS (Atomic Absorption Spectrophotometer) whereas, sodium (Na) and potassium (K) by Flame Photometer. All absorption measurements were performed on an Analytik Jena AAS Vario-6 Graphite Furnace Spectrometer (Made in Germany), which was equipped with a PC-controlled 6-piece lamp turret with hollow cathode lamps mounted as line radiators, as well as a deuterium hollow cathode lamp for background absorption compensation and argon supply.

Total fat

Walnut grinds were transferred to a glass extraction beaker following centrifugation. Fat content was measured by the Soxhlet extraction method. Petroleum ether was used as an extraction solvent for all of the grinds and fat. Each sample received a total of 150 mL of petroleum ether. Total fat extraction was carried out using an automatic Soxhlet device (Gerhardt's Soxtherm® SE-416 MK). The solvent was removed and recycled after the extraction. The sum of the fat utilised for fatty acid analysis and the fat extracted with the Soxtherm device was used to compute total fat.

Total phenolic content

Total phenolic content in the extracts was determined colorimetrically with Folin-Ciocalteu reagent by using the method described by Vasco *et al*., (2008) with slight modifications. About 0.5 mL of walnut extract of each sample was added to 2.5 mL Folin-Ciocalteu reagent (previously diluted with distilled water 1:10v/v). Stirred and set aside for 5 minutes to allow for response. The reaction mixture was then given 2 mL of 7.5 % sodium carbonate (Na_2CO_3). The tubes were then vortexed and incubated for 2 hours at the room temperature (approx. 25°C) with intermittent shaking. A spectrophotometer was used to measure the absorbance of the mixtures at 765 nm. The standard curve was calibrated with gallic acid in the range of 0-400mg/l (r^2 = 0.9988). Milligram gallic acid equivalents per gram of dry material (mgGAE/g) were used to calculate the final values. All the determinations were carried in triplicates.

Total flavonoid and flavonol content

With slight modifications, the total flavonoids were determined using the Aluminium chloride colorimetric assay (Ebrahimzadeh *et al*., 2009). Briefly, 0.5mL of each extract was mixed with 1 mL of 2% Aluminium chloride solution and followed by 1.5ml of 60% methanol. 6mL of 5% potassium acetate was added to the mixture. For the development of yellow colour, the mixtures were left at room temperature for 40 minutes, demonstrating the presence of flavonoid. The absorbance of the reaction mixture was measured at 415nm using spectrophotometer. Standard solution of Quercetin in concentrations 0, 25, 50, 100, 200mg l^{-1} were prepared in 96% methanol and used for standard curve calibration (r^2 = 0.9794).

For flavonol measurement, the same method was used, except the incubation period was 150 min instead of 40 minutes, and the absorbance was measured at 440nm. (r^2 = 0.9941). The results were expressed as milligrams of Quercetin equivalents per gram of dry sample (mgQE/g). All the determinations were carried in triplicates.

Total antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity

Total antioxidant activity was measured using the radical scavenging capability of the DPPH free radical (Trandafir *et al*., 2016) method with slight modifications. Walnut extract (2.0 mL) of each sample were mixed with 2.0mL of DPPH-methanolic

solution (4×10^{-4}M). The mixture was gently shaken, and allowed to react in dark at room temperature for 30 min. After the incubation period of 30 min at room temperature, absorbance was measured using spectrophotometer at 517nm. The antioxidant activity was calculated by comparing the decrease in DPPH absorbance, caused by the addition of test samples, to the control. Standards of ascorbic acid with various concentrations were used for calibration of the standard curve (r^2 = 0.9844). Antioxidant capacity was expressed in mg ascorbic acid equivalents per gram of dry sample (mg AAE/g). The scavenging activity was calculated using the formula;

scavenging activity (%) = [1-(A - As)/A]×100

where, the absorbance of the sample is As, whereas the absorbance of the control is A. A mixture of methanol and DPPH (ratio, 1:1) was taken as blank, and the mixture of standard (Ascorbic acid) and DPPH (ratio, 1:1) served as control. Samples were analysed in triplicates. Observations were taken in triplicate in all cases and means were used for making graphs. Duncan mean range test was done through SPSS software for analysis data on DPPH, TPC, Flavonols and Flavonoids.

Results

Morphological characterisation of walnut

A morphological study was conducted out based on a few key qualitative and quantitative characteristics. In this paper, the morphological characteristics of four different walnut genotypes are described in depth. Twenty important quality traits were studied and were found significant variable among genotypes (P=0.05). The majority of the nuts were ovate in shape, with only a few being round (Table 2). The genotypes also differed in terms of shell strength. Shell surface showed variations from rough to smooth; shell colour from light to medium. The data revealed that the nut weight ranged between 11.70 to 14.51g; nut diameter between 9.60 to 14.27mm; nut length between 17.40 to 20.51mm; kernel weight ranged between 2.13 to 7.21g; kernel recovery between 14.6 to 61.57% and kernel colour from light to amber (Table 3).

Table 2. Nut shape, Shell strength, shell integrity, Kernel plumpness and other physical parameters of different walnut genotypes.

Physical Characteristics:	CS	TM	KW	KG
Nut Shape	Round	Ovate	Ovate	Ovate
Shape in cross section	Oblate	Round	Round	Round
Shape of base perpendicular to suture	Rounded	Truncate	Rounded	Rounded
Shape of Apex perpendicular to suture	Truncate	Rounded	Rounded	Rounded
Prominence of apical tip	Medium	Medium	Weak	Medium
Position of pad on suture	On whole length	On upper 2/3 of nut	On whole length	On upper 2/3 of nut
Prominence of pad on suture	Strong	Medium	Strong	Strong
Shell surface	Rough	Smooth	Rough	Moderately smooth
Shell colour	Light	Medium	Medium	Medium
Shell seal	Strong	Strong	Intermediate	Intermediate
Shell strength	Intermediate	Strong	Weak	Strong
Shell integrity	Strong	Strong	Strong	Strong
Shell thickness	Medium	Medium	Medium	Medium
Ease of removal of kernel halves	Easy	Moderate	Moderate	Moderate
Kernel plumpness	Plumpy	Plumpy	Moderate	Moderate

Phytochemical determinations

Total Phenolic content, Flavonoids and Flavonols

Antioxidant function is mediated by phenolic chemicals, which are significant plant ingredients having redox potential (Soobrattee *et al*., 2005). The extract's hydroxyl groups are responsible for aiding radical scavenging. Walnuts have a higher TPC than other nuts and are abundant in phenolics (Kornsteiner

et al., 2006, Reddy *et al*., 2010). According to Habibi *et al*., (2019) as a result of coating the fresh kernels, the values of total phenols, colour, and sensory qualities were largely retained. Folin-Ciocalteu reagent was used to calculate total phenolic content. By graphing the absorbance against the gallic acid concentrations, the calibration equation for estimating total phenolic was created (in triplicate). The total phenolic content of four walnut genotypes ranged from 19 mg gallic acid equivalent/g to 50.49 mgGAE/g. The walnut genotype *KW* (Kupwara) showed the highest phenolic content (50.49 mgGAE/g). The total phenolic content of the extracts was calculated using the calibration curve's equations as milligram gallic acid equivalent (mg GAE/g) dry weight of the plant extract: $y = 0.0271x - 0.0421$; $r^2 = 0.9988$, where

y and x correspond to absorbance and the gallic acid equivalent in mg/g respectively (Fig.1).

Table 3. Proximate compositions, Mineral content and Physical properties of different Genotypes of Walnut.

Proximate composition	TM	KG	CS	KW
Moisture (g /100 g kernel)	6.53±0.22	6.98±0.21	7.33±0.23	5.65±0.21
Protein (g/ 100 g kernel)	12.8±0.32	14.1±0.43	14.6±0.42	15.8±0.44
Fat (g /100 g kernel)	64.7±0.72	62.5±0.71	61.3±0.65	67.8±0.64
Ash (g /100 g kernel)	1.76±0.02	1.65±0.01	1.55±0.03	1.82±0.05
Carbohydrate (g/ 100 g kernel)	14.21±1.43	14.77±1.75	15.22±1.08	8.93±1.37
Mineral content (milligrams/100 gram of kernel weight)				
Ca	175.2±12.01	174.9±12.12	178.5±14.32	180.4±14.21
Mg	138.8±9.75	151.6±10.45	146.3±9.87	150.8±12.08
K	251.39±29.0	268.99±30.0	276.83±25.0	280.82±26.0
Na	1.97±0.6	2.18±0.8	2.1±0.5	2.99±0.9
Zn	2.51±0.06	2.35±0.03	2.33±0.08	2.97±0.10
Cu	3.04±0.01	2.76±0.04	2.61±0.02	2.79±0.04
Mn	16.5±0.13	20.9±0.12	25.35±0.21	25.15±0.20
Fe	2.53±0.3	2.58±0.4	2.34±0.8	3.17±0.5
Physical parameters				
Nut weight(g)	13.31±0.70	14.51±0.71	12.46±0.80	11.71±0.76
Kernel weight(g)	4.22±0.65	2.13±0.54	5.16±0.47	7.21±0.45
Kernel recovery (%)	31.7±2.34	14.6±3.43	41.4±3.67	61.5±4.12
Nut length(mm)	17.4±0.34	17.66±0.56	18.95±0.23	20.51±0.78
Nut diameter at cheek(mm)	10.63±0.76	12.5±0.13	14.27±0.36	9.6±0.62
Nut diameter at suture(mm)	9.58±0.65	11.82±0.35	11.31±0.34	10.66±0.42
Kernel colour				
L*	51.69±3.10	51.55±3.12	52.72±3.56	50.76±3.11
a*	6.81±0.09	6.38±0.12	7.65±0.16	9.18±0.08
b*	23.15±2.43	24.85±2.75	25.77±2.38	26.39±2.17
Whiteness index(WI)	46.03±1.54	45.17±1.63	45.62±1.32	43.38±1.59
Browning index(BI)	0.37±0.01	0.51±0.02	0.44±0.01	0.81±0.01

The mean and standard deviation (n=3) are used to calculate the values. L* is a lightness chromatic component, while a* and b* are two chromatin components that range from green to red and blue to yellow, respectively.

Data on the flavonoid and flavonol content of different extracts of walnut kernels ranged from 188.5 to 815 mgQE/100g and 3.46 to 7.77mgQE/g respectively (Figs.2 and 3). Significantly high flavonoid content 815 mgQE/100g was displayed by *KW* (Kupwara) walnut genotype (Table 4). The following formulas were established on the calibration curve to obtain total flavonoid content as Quercetin equivalent (mg/g): $y = 0.0236x + 0.191$; $r^2 = 0.9794$, where y and x implies the absorbance and the Quercetin equivalent in mg/g respectively.

Table 4. Phytochemical determination.

Samples	TPC	TF	DPPH	TOFL
KW	50.4^{c}	781.1^{c}	97.1^{c}	7.7^{c}
CS	32.4^{b}	476.1^{b}	95.3^{c}	6.7^{c}
TM	19.8^{a}	188.5^{a}	92.2^{b}	5.2^{b}
KG	38.7^{b}	815^{c}	82.6^{a}	3.4^{a}

Genotypes showing similar superscript values are statistically,non-significant at 5% level of significance means followedby the same letters within the columns are not significantly different using Duncan's multiple range test (p=0.05).

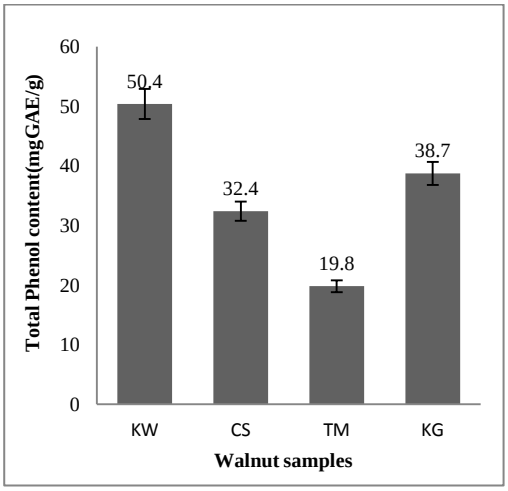

Fig.1. Total Phenolic content (mgGAE/g) of different Walnut genotypes. Data are shown as mean ±SD (Standard Deviation) of three replicates.

Flavonols are a type of polyphenols, a broader group of natural compounds found in plants. These compounds play a protective role for plants and are known to exhibit antioxidant effect. In the present study, the highest flavonol content was observed in *KW* (7.77mgQE/g). Total flavonols was determined as quercetin equivalent (mg g^{-1}) functioning as the standard compound, via the subsequent formulae from the calibration curve: $y = 0.0071x + 0.0539$; $r^2=0.994$, where y is the absorbance and x is the quercetin equivalent in mg g^{-1}. The variation in the total flavonoid and flavonol content in the genotypes may be due to genetic diversity, biological, environmental, seasonal variations that significantly affect the flavonoid content of the nuts, as reported in the literature (Kumar *et al*.,2018).

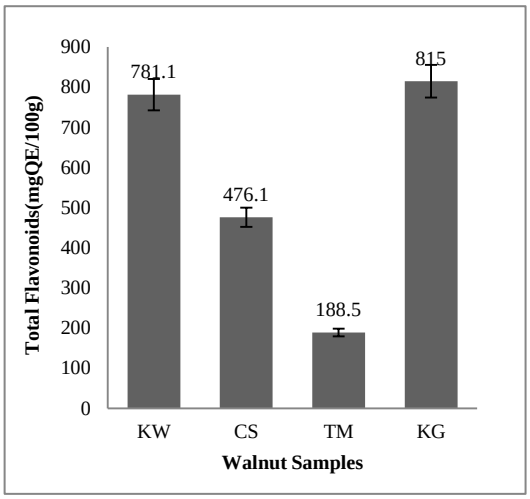

Fig.2. Total Flavonoids (mgQE/100g) of different Walnut genotypes. Data are shown as mean ±SD (Standard Deviation) of three replicates.

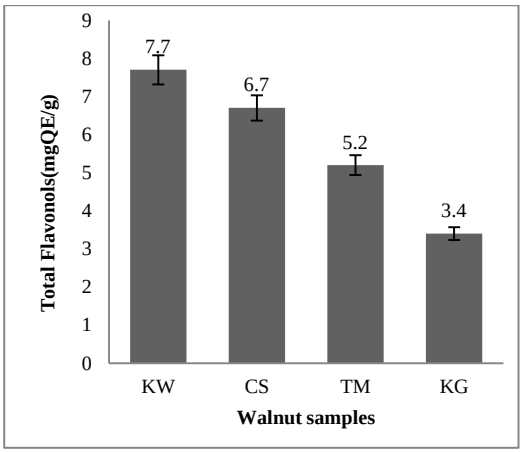

Fig.3. Total Flavonols (mgQE/g) of different Walnut genotypes. Data are shown as mean ±SD (Standard Deviation) of three replicates.

Antioxidant potential of walnut samples

Antioxidant activity (DPPH free radical scavenging activity)

The antioxidative potential of walnut extracts was determined by detecting changes in the absorbance of the DPPH radical at 517 nm, due to their free radical scavenging effects. DPPH assay results revealed the antioxidant activities of different extracts of walnut kernels. In the current study, free radical scavenging activity (DPPH) assay ranged from 82 to 97.1% among the four extracts (Fig.4). Maximum free radical scavenging activity (97.19%) was observed in *KW* (Kupwara) extract followed by 95.35%, 92.23% and 82.60% in *CS* (Char-e-Sharief), *TM* (Tangmarg) and *KG* (Kulgam) respectively.

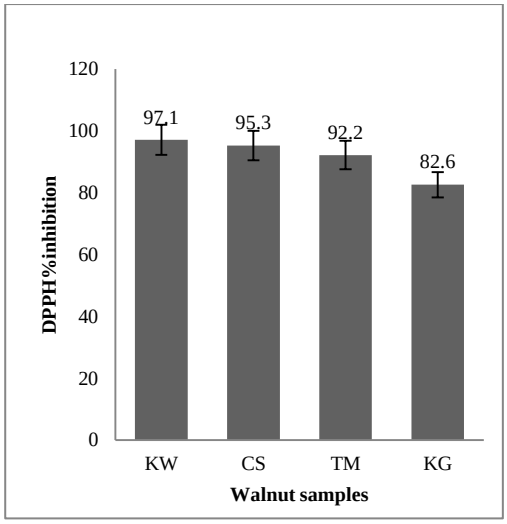

Fig. 4. DPPH %inhibition (mgQE/100g) of different Walnut genotypes. Data are shown as mean ±SD (Standard Deviation) of three replicates.

Discussion

In the first part of the current study, the maximum variability was observed in nut weight, kernel weight, nut length, nut diameter and kernel recovery. Similar variations were also reported by various workers (Pandey *et al*., 2004, Ozkan and Koyuncu, 2005, Rana *et al*., 2007, Aryapak and Ziarati, 2014). Furthermore, earlier research has shown that higher PPO enzyme activity causes higher browning and a lower whitening index in walnut pellicle (Habibie *et al*., 2021). In breeding programmes, nuts with smooth, strong, thin shells, with a tight seal and light kernels are most desirable (McGranahan and Leslie, 1990).

Phenolic and Flavonoids impart major contribution to the antioxidant activity of plant extract. The types and amounts of phenolic compounds may play a role in the antioxidant activity of the extracts under investigation (Aryal *et al*., 2019). The result in the nuts under investigation correspond rather well with the observations of Pereira *et al*., (2008) wherein, the TPC of walnuts was estimated to be 58.8 to 95.1mgGAE/g and 3.3 to 31mgGAE/g as was recalculated by Bakkalbasi *et al*., (2012). Comparing the works of literature, the highest TPC of the walnut was reported to be 58 mgGAE/g (Tapia *et al*., 2013). The extraction procedures and solvents are in charge of dissolving the plant's endogenous components (Siddhuraju *et al*., 2003). Furthermore, plant components might be either polar or non-polar. Due to the presence of a hydroxyl group, phenolic compounds are more soluble in polar organic solvents. As a result, methanol was chosen as the extraction solvent (Wang *et al*., 2006). The total phenolic content of the four genotypes under investigation showed variation. In summary, *Juglans regia* L. samples under study exhibited statistically significant differences in TPC. This may be due to different climatic conditions, different altitudes, geographical variations, which may alter the amount of phenolic compounds. Flavonoids are water-soluble poly phenolic compounds with a large number of aromatic rings that are found in abundance in plants as glycosides. These secondary metabolites protect lipids from oxidation by scavenging free radicals, chelating metals, activating antioxidant enzymes, reducing tocopherol radicals, and blocking oxidation-causing enzymes (Heim *et al*., 2002). The efficacy of flavonoid's antioxidant action is determined by the number and position of free OH groups (Panche *et al*., 2016). The results in the present study regarding flavonoids and flavanols have been found to be in close proximity with the earlier findings (Yang *et al*., 2009). The variation in the total flavonoid and flavonol content in the genotypes may be due to genetic diversity, biological, environmental, seasonal variations that significantly affect the flavonoid content of the nuts, as reported in the literature (Kumar *et al*.,2018). The DPPH method is a

simple practical and sensitive assay which has been widely used to detect active antioxidants. The ability of antioxidants to react with DPPH, which is a stable free radical, and its ability to detect active antioxidants with scavenging capacity, even in low concentrations (Pinelo *et al*., 2004). It is a discolouration assay in which an antioxidant is added to a DPPH solution in methanol and the results are examined. The findings of this study are consistent with those of earlier research (Pereira *et al*., 2008) reporting 90.2 to 92.6% DPPH scavenging activity. Their findings also revealed that walnut kernel extracts have high free radical scavenging potential, which can significantly contribute to its antioxidant properties. Thus walnut extracts particularly those having higher free radical scavenging ability can have potential chemo-preventive roles when compared to untreated walnuts (Chatrabnous *et al*., 2018).

A linear relationship between total phenolic and flavonoid concentration and antioxidant capability has been reported in several studies (Shrestha *et al*., 2006). Total phenolic component concentration of walnut micro shoots was found to have a significant linear connection with increasing antioxidant activity (Chenainy *et al*., 2013). Phenolic compounds are a class of antioxidant-active secondary metabolites commonly used in the food business. Scientists have recently become interested in extracting natural phenolic compounds from agricultural goods and waste and using them instead of synthetic chemicals (Habibi *et al*., 2021). The TPs in dried walnuts have been extensively studied. The reported outcomes, on the other hand, were mixed. According to Pereira *et al*., (2008), the TPs of six different walnut cultivars growing in Portugal ranged from 58.9 to 95.1mgGAE/g. Another study looked at total polyphenols in four dry walnut cultivars (*Juglans regia* L. cultivars *Serr*, *Hartley*, *Chandler*, and *Howard*), with the *Howard* having the greatest TPs content (58.2 mg GAE/g) (Tapia *et al*., 2013). Several findings, on the other hand, revealed substantially lower levels of TPs in dry walnuts (Chatrabnous *et al*., 2018), ranging from 15.8 to 16.9mgGAE/g (Anderson *et al*., 2001, Chen and Blumberg, 2008) or 10.7 to 16.0mgGAE/g as determined by Arranz *et al*., (2008). In our analysis, the phenolic compounds were higher than the TPs in all investigations. These differences in TPs concentration could be due to the usage of different cultivars or the variable phases of kernel development during harvest. Walnut products have an antioxidant effect due to phenolic components and phytochemicals, which help to prevent the detrimental effects of free radicals. By lowering the scavenging activity power of DPPH radicals, the antioxidant potential of fresh walnut kernel sample was determined in this study. Through the correlation analysis of phytochemical contents and antioxidant ability of the walnut extracts, the phenolic and flavonoid contents have exhibited excellent association with DPPH. According to Erkan *et al*., (2008), total phenolic content of extracts from various natural sources and radical scavenging activity are closely related. Correlation coefficient estimates the levels of association among two or more parameters. The present investigation highlights a significant relation between total phenolic and flavonoid content with free radical scavenging capacity of the extracts. Significant positive correlation was found among TP-DPPH (r^2 = 0.833) and TF-DPPH, (r^2 = 0.794) were observed. It is feasible to conclude that the antioxidant potential of the extracts is mostly related to the presence of phenolic and flavonoids in them by comparing the correlation coefficients (R values). The total flavonoid and total phenolic content were perfectly synchronised. It was successfully demonstrated that samples with a high phenolic content also contain a large number of flavonoids. The extracts with high content of total phenolics and total flavonoids also exhibited higher antioxidant activities. Thus, the contents of total phenolics and flavonoids were confirmed as largely responsible for the antioxidant activity of walnut kernels.

From the foregoing discussion, it is clear that the secondary metabolites were examined in different

samples of *Juglans regia* L. in order to highlight their total phenolic and flavonoid content and antioxidant potential. The obtained results indicate that all the four genotypes reveal different levels of TPC and flavonoids and free radical scavenging activity. However, the extracts from *KW* (Kupwara) and *CS* (Char-e-Sharief) presented higher TPC, total flavonoids followed by *TM* (Tangmarg) and *KG* (Kulgam). Therefore, the fact that highest antioxidant activity, among the genotypes, analysed in the present study, has been found in *KW* (Kupwara) extract, owing to the greater accumulation of phenolics in its kernels. According to several studies, the antioxidant activity of walnut kernels can be linked to the existence of such phenolic compounds, primarily because high TPC and total flavonoids levels indicate high DPPH levels (Shi *et al*., 2017). Our findings also point to a strong positive relationship between antioxidant activity and total phenolic and flavonoid concentration in walnut kernels. The same results have been reported in wild vegetable extracts (Aryal *et al*., 2019), *Lantana camara* (Kumar *et al*., 2014), *Trifolium pratense* extract (Esmaeili *et al*., 2015). Antioxidants do play a role in preventing diseases including obesity, cardiovascular disease, and neurodegenerative disease by counteracting the detrimental effects of oxidative processes (He *et al*., 2018, Chen *et al*., 2016 and Srinivasulu *et al*., 2018). Due to the presence of those compounds, in the extracts under investigation, indicate how useful and beneficial these kernels are to human health.

Conclusions

It may be concluded, based on the findings of this investigation, that genotypes *KW* (Kupwara), *CS* (Char-e-Sharief), *TM* (Tangmarg) and *KG* (Kulgam) were found rich in mineral composition especially Mg, Mo, Mn. Although other elements like Zn, Cu, Co, Fe were also found in significantly higher concentration. The results also suggested a strong correlation between the total phenolic, flavonoid content and antioxidant potential. The four walnut genotypes (*KW, CS, TM* and *KG*), in the present study, highlighted varying TPC, flavonoids and antioxidant activities. However, the possible reason for the variations could be difference in altitude, temperature, soil, UV, humidity. Since these variables are key determinants of metabolism and secondary metabolite accumulation (Connor *et al*., 2005, Ahuja *et al*., 2010). Nonetheless, biochemical characteristics differed between walnut genotypes from various places. To put it another way, environmental conditions appear to have a major impact on biochemical properties (Sarikhani *et al*., 2021). Owing to maximum nut and kernel weight as well as nut recovery and higher phenolic profile of *KW* (Kupwara) extract illuminate the exploitation potential of this genotype for commercial cultivation for market. Based on the good fruit quality, rich mineral composition, higher total phenolic and flavonoid content and better free radical scavenging properties, this genotype seems to serve as a potential and promising one for production of walnut on large scale to avoid non uniformity. More research is needed to identify the active chemicals responsible for the kernel's high antioxidant capacity.

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Conflict of interest

The authors declare no conflict of interest.

References

Ahad T, Gull A, Nisar J, Masoodi l, Rather AH (2020) Effect of storage temperatures, packaging materials and storage periods on antioxidant activity and non-enzymatic browning of antioxidant treated walnut kernels. Journal of Food Science and Technology. 57(10),

3556–3563. doi: 10.1007/s13197-020-04387-5

Ahuja I, De Vos RC, Bones AM, Hall RD (2010) Plant molecular stress responses face climate change. Trends in plant science. 15(12), 664-674.

Anderson KJ, Teuber SS, Gobeille A, Cremin P, Waterhouse AL, Steinberg FM (2001) Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation. The Journal of Nutrition. 131, 2837-2842.

Arranz S, Perez-Jimenez J, Saura-Calixto F (2008) Antioxidant capacity of walnut (*Juglans regia* L.): Contribution of oil and defatted matter. European Food Research and Technology. 227, 425-431.

Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurungand R, Koirala N (2019) Total phenolic content, Flavonoid content and Antioxidant potential of wild vegetables from Western Nepal. Plants. 8, 96. doi:10.3390/plants8040096. www.mdpi.com/journal/plants.

Aryapak S, Ziarati P (2014) Nutritive value of Persian walnut (*Juglans regai* L.) orchards. American –Eurasian Journal of Agriculture and Environmental Science. 14(11), 1228-35.

Bakkalbasi E, Yilmaz OM, Javidipour I, Artik N (2012) Effects of packaging materials, storage conditions and variety on oxidative stability of shelled walnuts. LWT-Food Science and Technology. 46(1), 203-209. http://dx.doi.org/10.1016/j.Iwt.2011.10.006.

Chatrabnous N, Yazdani N, Vahdati K (2018) Determination of nutritional value and oxidative stability of fresh walnut. Journal of Nuts. 9(1), 11-20.

Chatrabnous N, Yazdani N, Tavallali V, Vahdati K (2018) Preserving quality of fresh walnuts using plant extracts. LWT. 91, 1-7.

Chen C (2016) Sinapic acid and its derivatives as medicine in oxidative stress-induced diseases and ageing. Journal of Oxidative Medicine and Cellular Longevity. http://doi.org/10.1155/2016/3571614(2016)

Chen CYO, Blumberg JB (2008) In vitro activity of almond skin polyphenols for scavenging free radicals and inducing quinone reductase. Journal of Agricultural and Food Chemistry. 56, 4427-4434.

Cheniany M, Ebrahimzadeh H, Masoudi-Nejad A, Vahdati K, Leslie CA (2010) Effect of endogenous phenols and some antioxidant enzyme activities on rooting of Persian walnut (*Juglans regia* L.). African Journal of Plant Science. 4(12), 479-487.

Cheniany M, Ebrahimzadeh H, Vahdati K, Preece J, Masoudinejad A, Mirmasoumi M (2013) Content of different groups of phenolic compounds in micro shoots of *Juglans regia* cultivars and studies on antioxidant activity. Acta Physiologia Plantarum. 35, 443–450.

Connor AM, Finn CE, Alspach, PA (2005) Genotypic and environmental variation in antioxidant activity and total phenolic content among blackberry and hybridberry cultivars. Journal of the American Society for Horticulture Science. 130(4), 527-533.

Ebrahimzadeh MA, Nabavi SM, Nabavi SF (2009) Corelation between the in vitro iron chelating activity and polyphenol and flavonoid contents of some medicinal plants. Pakistan Journal of Biological Sciences. 12(12), 934-938.

Erkan N, Ayranci G, Ayranci E (2008) Antioxidant activities of rosemary (*Rosmarinus Officinalis* L.) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosemarinic acid and sesamol. Journal of Food Chemistry. 110(1), 76-82.

Esmaeili AK, Taha RM, Mohajer S, Banisalam B (2015) Antioxidant activity and total phenolic and Flavonoid content of various solvent Extracts from In Vivo and In Vitro grown *Trifolium pratense* l. (Red Clover). Hindawi Publishing Corporation, BioMed Research International. http://dx.doi.org/10.1155/2015/643285

Gao P, Liu R, Jin Q, Wang X (2019) Comparative study of chemical compositions and antioxidant capacities of oils obtained from two species of walnuts: *Juglans regia* and *Juglans sigillata*. Journal of Food Chemistry. 279-287.

Gharibzahedi SMT, Mousavi SM, Hamedi M, Khodaiyan F (2014) Determination and characterization of kernel biochemical composition and functional compounds of Persian walnut oil. Journal of Food Science Technology. 51(1), 34–42. doi: 10.1007/s13197-011-0481-2

Gursul S, Karabulut I, Durmaz G (2019) Antioxidant efficacy of thymol and carvacrol in microencapsulated walnut oil triacylglycerols. Journal of Food Chemistry. 278, 806-810.

Habibi A, Yazdani N, Saba MK, Vahdati K (2019) Ascorbic acid incorporated with walnut green husk extract for preserving the postharvest quality of cold storage fresh walnut kernels. *Scientia Horticulturae.* 245, 193-199.

Habibie A, Yazdani N, Chatrabnous N, Koushesh Saba M, Vahdati K (2021) Inhibition of Browning via aqueous gel solution of Aleo vera: a new method for preserving fresh fruits as a case study on fresh kernels of Persian walnut. Journal of Food Science and Technology. 1-10.

Habibi A, Yazdani N, Koushesh Saba M, Vahdati K (2021) Ascorbic acid preserved phenolic compounds of cold stressed fresh walnut kernels. Acta Horticulturae. 1315, 665-668.

He GR, Wang SB, Du GH (2018) Natural Small Molecule Drugs from Plants Part VII. Springer Singapore. 703-707.

Heim KE, Tagliaferro AR, Bobilya DJ (2002) Flavonoid antioxidants: chemistry, metabolism and structure activity relationships. Journal of Biochemistry. 13, 572-584.

Jahanbani R, Ghaffari SM, Salami M, Vahdati K, Sepehri H, Namazi Sarvestani N, Sheibani N, Moosavi-Movahedi AA (2016) Antioxidant and anticancer activities of walnut (*Juglans regia* L.) protein hydrolysates using different proteases. Plant Foods and Human Nutrition. 71, 402-409.

Jahanbani R, Ghaffari SM, Vahdati K, Salami M, Khalesi MR, Sheibani N, Moosavi-Movahedi AA (2018) Kinetics study of protein hydrolysis and inhibition of angiotensin converting enzyme by peptides hydrolysate extracted from walnut. International Journal of Peptide Research and Therapeutics. 24(1), 77-85.

Khodadadi F, Tohidfar M, Mohayeji M, Dandekar AM, Leslie CA, Kluepfel D, Butterfield T, Vahdati K (2016) Induction of polyphenol oxidase in walnut and its relationship to the pathogenic response to bacterial blight. Journal of the American Society for Horticultural Science. 141(2), 119-124.

Khodadadi F, Tohidfar M, Vahdati K, Dandekar AM, Leslie CA (2020). Functional analysis of walnut polyphenol oxidase gene (*JrPPO1*) in transgenic tobacco plants and PPO induction in response to walnut bacterial blight. Plant Pathology. 69, 756-764.

Kornsteiner M, Wagner KH, Elmadfa I (2006) Tocopherols and total phenolics in 10 different nut types. Journal of Food Chemistry. 98(2), 381-387.

Kumar S, Sandhir R, Ojha, S (2014) Evaluation of antioxidant activity and total phenol in different varieties of *Lantana camara* leaves. BioMed Central Research Notes 7, 560. https://www.biomedcentral.com/1756-0500/7/560

Kumar V, Roy BK (2018) Population authentication of the Traditional medicinal plant *Cassia tora* L. based on ISSR markers and FTIR analysis. Journal of Scientific Reports. 8, 10714. [CrossRef]

Lee B, Seo JD, Rhee JK, Kim CY (2016) Heated apple juice supplemented with onion has greatly improved nutritional quality and browning index. Journal of Food Chemistry. 201315-319.

McGranahan GH, Leslie C (1990) Walnuts (*Juglans*). Journal of Acta Horticulturae. 290, 905-51.

Oliveira I, Sousa A, Ferreira ICFR et al (2008) Total phenols, antioxidant potential and antimicrobial activity of Walnut (*Juglans regia* L.) green husks. Journal of Food and Chemical Toxicology. 46, 2326-2331.

Ozkan G, Koyuncu MA (2005) Physical and chemical composition of some walnut (*Juglans regia* L.) genotypes grown in Turkey. Grasas y Aceites. 56(2), 141-46.

Panche AN, Diwan AD, Chandra SR (2016) Flavonoids an overview. Journal of Nutritional Science. 5e47. [CrossRef].

Panday G, Tripathi AN, Verma MK, Sofi AA (2004) Collection, characterisation and evaluation of walnut (*Juglans regia* L.) germplasm from North western Himalyas. Indian journal of plant genetic Resources. 17(1), 77-88.

Pakrah S, Rahemi M, Nabipour A, Zahedzadeh F, Kakavand F, Vahdati K (2021) Sensory and nutritional attributes of Persian walnut kernel influenced by maturity stage, drying method, and cultivar. Journal of Food Processing and Preservation, e15513.

Pereira JA, Oliviera I, Sousa A, Ferreira IC, Bento A, Estevinho I (2008) Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars. Journal of Food and Chemical Toxicology. 46(6), 2103-2111. http://dx.doi.ogr/10.1016/j.fct.2008.02.002.

Pinelo M, Rubilar M, Sineiro J, Nunez MJ (2004) Extraction of antioxidant phenolics from almond hulls (*Prunus amygdalus*) and pine sawdust (*Pinus pinaster*). Journal of Food Chemistry. 85, 267-273.

Rana JC, Singh D, Yadav SK, Verma MK, Kumar K, Pradheep K (2007) Genetic diversity collected and observed in Persian walnuts (*Juglans regia* L.) in the Western Himalayas region of India. Plant Genetic Resources Newsletter. 151, 63-68.

Reddy CVK, Sreeramulu D, Raghunath M (2010) Antioxidant activity of fresh and dry fruits commonly consumed in India. Journal of Food Research International. 43(1), 285-288.

Sarikhani S, Vahdati K, Ligterink W (2021) Biochemical properties of Superior Persian Walnut Genotypes originated from Southwest of Iran. International Journal of Horticultural Science and Technology. 8(1), 13-24.

Shi B, Zhang W, Li X, Pan X (2017) Seasonal variations of phenolic profiles and antioxidant activity of walnut (*Juglans sigillata* Dode) green husks. International Journal of Food Properties. 20, S2635-S2646. doi.org/ 10. 1080/ 10942912. 2017.1381706

Shrestha PM, Dhillon SS (2006) Diversity and traditional knowledge concerning wild food species in a locally managed forest in Nepal. Journal of Agroforestry Systems. 66, 55-63. [CrossRef].

Siddhuraja P, Becker K (2003) Antioxidant properties of various solvent extracts of total phenolic constituents from three different

agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. Journal of Agriculture and Food Chemistry. 51, 2144-2155. [CrossRef]

Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T (2005) Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. Mutation Research – Fundamental and Molecular Mechanisms of Mutagenesis. 579, 200-213. [CrossRef]

Srinivasulu C, Ramgopal M, Ramanjaneyulu G, Anuradha CM, Kumar CS (2018) Syringic acid (SA)-A Review of its occurrence, Biosynthesis, Pharmacological and Industrial importance. Journal of Biomedicine and Pharmacotherapy. 108, 547-557. http://doi.org/10.1016/j.biopha.2018.09.069.

Tapia MI, Sanchez-Morgado JR, Garcia-Parra J, Ramirez R, Hernandez T, Gonzalez-Gomez D (2013) Comparative study of the nutritional and bioactive compounds content of four walnut (*Juglans regia* L.) cultivars. Journal of Food Composition and Analysis.31(2),232-237. http://dx.doi.org/10.1016/j.jfca.2013.06.004.

Trandafir I, Cosmulescu S, Botu M, Nour V (2016) Antioxidant activity, phenolic and mineral contents of the walnut kernel (*Juglans regia* L.) as a function of the pellicle colour. Journal of Fruits. 71(3), 177-184. doi:10.1051/fruits/2016006.

Vahdati K (2014) Traditions and folks for walnut growing around the silk road. *Acta Horticulturae.* 1032, 19-24.

Vasco C, Ruales J, Kamal-Eldin A (2008) Total phenolic compounds and antioxidant capacities of major fruits from *Ecuador*. Journal of Food Chemistry. 111, 816-823.

Wang L, Weller CL (2006) Recent advances in extraction of nutraceuticals from plants. Trends in Food Science and Technology. 17, 300-312. [CrossRef]

Yang J, Chen C, Zhao S, Ge F, Liu D (2014) Effects of Solvents on the antioxidant activity of Walnut (*Juglans regia* L.) Shell Extracts. Journal of Food and Nutritional Research. 2, 621-626.

Yang J, Liu RH, Halim L (2009) Antioxidant and anti-proliferative activities of common edible nut shell. LWT- Food Science and Technology. 42, 1-8.