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Original article

Recovery of bioactive compounds from walnut (*Juglans regia* L.) green husk by supercritical carbon dioxide extraction

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Summary   In this work, the use of supercritical carbon dioxide, a nontoxic solvent, was proposed to extract bioactive compounds from *Juglans regia* L. green husk and was compared to other traditional techniques based on solvents such as ethanol, methanol and water. Supercritical CO_2 was combined with ethanol as an organic modifier at a rate of 20% of the total flow to achieve greater extraction of polar compounds. The extracts were characterised in terms of extraction yields, antioxidant activity, total polyphenol content, phenolic acids, juglone, volatile organic compounds and antifungal activity. The results showed that the extracts obtained with supercritical CO_2 were rich in polyphenols (10750 mg GAE/100 g) and juglone (1192 mg/100 g) and exerted high antioxidant activity and antifungal activity compared with the tested fungi. Walnut green husk is not just a food industry waste but also an important economic source of bioactive compounds that could be used for food active packaging.

Keywords   Antifungal activity, juglone, polyphenols, volatile organic compounds.

Introduction

Walnut (*Juglans regia* L.) is mainly cultivated for its wood, which is processed principally by the furniture industry, and for nuts that are rich in lipids, polysaccharides, unsaturated fatty acids, phospholipids and proteins (Vergano *et al.*, 1993). The annual global production of walnut drupes reached 3662507 tons in 2018 (FAO, 2018). In Italy, the region traditionally most linked to the cultivation of walnut is Campania, accounting for 59.6% of national production. The nut is the edible inner part of the walnut, composed of two fleshy and oil-rich cotyledons, covered with a dry and bitter film called 'kernel'. The green husk is the outer epicarp (mesocarp) of the walnut fruit and represents, on average, 55% of the walnut drupe (Yilmaz *et al.*, 2017). It is the basic material for traditional walnut liqueur (Stampar *et al.*, 2006), but it often represents industrial processing waste that is hard to dispose of. Various studies have demonstrated that it can be used in both the cosmetic and pharmaceutical industries

(Pereira *et al.*, 2007; Ribeiro *et al.*, 2015). The green husk, however, should be re-evaluated because it is recognised as a potential source of phenolic compounds and bioactive compounds (Soto-Maldonado *et al.*, 2019) particularly phenolic acids and juglone, the most abundant phenolic compound of walnut. Phenolics can reduce the risk of cardiovascular and degenerative diseases by preventing oxidative stress and the oxidation of biological macromolecules. Moreover, they can scavenge-free radicals and possess metal-chelating properties in addition to their described anticancer activities (Jahanban-Esfahlan *et al.*, 2019).

Cosmulescu *et al.*, (2010) found that juglone, vanillic acid, myricetin, syringic acid, coumaric acid and ferulic acid were the phenolics most commonly present in five *cultivars* of walnut analysed.

Ferulic acid is a phenolic compound used as an antioxidant and food preservative with anti-inflammatory, antidiabetic and cardioprotective properties. Studies on the absorption and metabolic fate of this compound showed that after oral administration (5.15 mg kg^{-1} of body weight), ferulic acid and its metabolites were quickly detected in plasma with a peak of concentration found 30 min after ingestion

(Rondini *et al*., 2002). The lethal dose (LD_{50}) value is 2445 mg kg^{-1} body weight in male rats and 2113 mg kg^{-1} body weight in female rats (Alam, 2019). Ferulic acid was added to active packaging films in low-density polyethylene and ethylene-vinyl acetate copolymer (GilakHakimabadi *et al*., 2019).

O-cumaric acid intake can be beneficial for the reduction of dyslipidaemia, hepatosteatosis and oxidative stress in rats fed with a diet supplemented with 100 mg kg^{-1} for 8 weeks (Hsu *et al*., 2009). Furthermore, *in vitro* studies showed the anticarcinogenic activity of o-coumaric acid, but precautions must be taken before using this compound (Sen *et al*., 2013).

Syringic acid and vanillic acid could suppress hepatic fibrosis in chronic liver injury in rats at a dose of 10 mg kg^{-1} of body weight (Itoh *et al*., 2010), and syringic acid was shown to have antidiabetic activity in experimental diabetes mouse models when administered at an oral dose of 50 mg kg^{-1} of body weight (Muthukumaran *et al*., 2013). In food packaging, the addition of syringic acid in films based on chitosan may contribute to extending the shelf life of food (Yang *et al*., 2019).

The oral administration of vanillic acid at a dose of 50 mg kg^{-1} of body weight for 8 weeks controlled diabetic hypertension by reducing blood glucose, insulin and blood pressure and combating oxidative stress by activating of tissue antioxidants in rats (Vinothiya & Ashokkumar, 2017).

Juglone (5-hydroxy-1,4-napthoquinone) is an organic compound found naturally in the leaves, roots and skins of plants in the *Juglandaceae* family. It is an allelopathic (Topal *et al*., 2007), antimicrobial and antifungal compound (Clark *et al*., 1990). Latos *et al*., (2019) found that juglone added to biodegradable aliphatic polyesters could be used as a natural dye and as an indicator of the ageing time of biodegradable polyesters. Furthermore, *in vivo* studies showed that intravenous injection of juglone significantly decreased the mean arterial blood pressure in normotensive and hypertensive rats at doses ranging from 0.003 to 3 mg kg^{-1} (Ahmad *et al*., 2020). Wang *et al*., (2019) showed that the intraperitoneal injection of 4 mg kg^{-1} juglone significantly inhibited the growth of liver cancer, while abdominal injection at different doses (2.5 and 5 mg kg^{-1}) inhibited colon cancer proliferation in mice (Liu *et al*., 2017). An LD_{50} of 0.25 mg juglone/ 100 g of body weight in mice and rats has been reported (Strugstad & Despotovski, 2013).

The antioxidant potential of nut-based extracts (Stampar *et al*., 2006) and their antimicrobial activity (Clark *et al*., 1990) have been demonstrated, but specific information about walnut green husks is currently very limited.

Solvents of varying polarity, such as water and aqueous mixtures of ethanol, methanol and acetone (Jakopic & Veberic, 2009), are most frequently used for extracting bioactive compounds from plant matter. Jakopic & Veberic (2009) studied the extraction of phenolic compounds from walnut green fruits in methanol and ethanol and found that for the extraction of total phenolics and certain individual phenols, such as juglone and chlorogenic acid, methanol was more efficient than ethanol. In contrast, ellagic and sinapic acid extraction was more favourable when using ethanol. To optimise the extraction and concentration of bioactive components from walnut green husks, it is possible to use supercritical carbon dioxide (CO_2), a nonpolar solvent with a low critical temperature and intermediate critical pressure. The dissolution power of supercritical CO_2 can be adjusted by small additions of organic modifiers of differing polarity to improve the extraction of polar compounds. This is an environmentally friendly extraction technique because CO_2 is a nontoxic, nonflammable, recyclable and inexpensive solvent that is easy to remove from extracts (Aiello *et al*., 2020). It has been demonstrated in other matrices, such as tomato waste and hemp (*Cannabis sativa* L.) seeds, that supercritical CO_2 extraction allows higher quantities of polyphenols to be obtained compared to traditional extraction (Aiello *et al*., 2020; Romano *et al*., 2020).

In this work, walnut green husks were subjected to different extraction systems: conventional systems with water, ethanol and methanol and innovative systems with supercritical carbon dioxide. The extracts were characterised in terms of polyphenols, volatile organic compounds, antioxidant capacity and antifungal activity.

Materials and method

Materials

Juglans regia L. 'Sorrento' variety drupes obtained from orchards located in Campania, Italy, were used to conduct this study. The green husks were manually separated from the walnuts and freeze-dried to prevent their deterioration. The average moisture content was found to be $80 \pm 0.3\%$. Subsequently, they were ground with a knife mill (Grindomix M200, Retsch Italia, Verdere Scientific Srl, Bergamo, Italy) at 9000× g for 3 min and sieved to obtain particles with ø ≤ 1 mm. These operations allowed maximisation of the surface for exchange with the solvent.

Chemicals

The carbon dioxide (assay 99.9%) used was provided by SOL Spa (Naples, Italy). All solvents and reagents used were purchased from Sigma-Aldrich Co. (Milano, Italy).

Conventional extraction

Conventional extractions were accomplished using water, ethanol and methanol separately. For each type of extraction, approximately 500 mg of minced matrix with ø ≤ 1 mm was added to a 50-mL centrifuge tube to which 20 mL of water, ethanol or methanol was added. The tube was vigorously mixed for 2 min and then centrifuged for 15 min at $4000 \times g$ (centrifuge PK 131, ALC International Srl, Milano, Italy). The upper layer was filtered through filter paper and recovered. The ethanol and methanol solvents were removed with a rotary evaporator, while the water was removed by freeze-drying. The extraction operations were repeated five times, and the extract was stored at −20 °C until analysis (Aiello *et al.*, 2020 with some modifications).

Supercritical carbon dioxide extraction

The experiments were performed using an SFC 4000 extractor (JASCO International Co., Ltd., Tokyo, Japan) equipped with a 50-mL volume extractor vessel. The extraction with supercritical carbon dioxide was completed following the method developed by Popovici *et al.* (2012), with appropriate modifications. Approximately 15 g of minced matrix with ø ≤ 1 mm was loaded into the extraction vessel. The extractive parameters were as follows: P = 300 bar, T = 50 °C, flow = 10 mL min^{-1}, 20% ethanol used as a cosolvent and extraction time 195 min. Ethanol was chosen as the cosolvent to improve polar compound extraction and for its lower toxicity. In the final extract, ethanol was removed under nitrogen flow.

Antioxidant activity

DPPH assay

Antioxidant activity by the scavenging effect of radicals on 2,2-diphenyl-1-picrylhydrazyl (DPPH) was performed according to Gogoi *et al.*, (2020), with some modifications. A 6×10^{-5} M DPPH solution in methanol was prepared. The solid extracts were dissolved in water to prepare solutions at known concentrations in the range of 0.2–1.2 mg mL^{-1}. The procedure consisted of adding 9 mL of DPPH solution to 1 mL of each extract solution and then maintaining solutions in the dark for 60 min (until stable absorption values were obtained). The absorbance of the purple was measured at 517 nm using a Hitachi UV–VIS U-2900 spectrometer (Hitachi, Japan). The DPPH scavenging effect was expressed as a percentage of decolouration of DPPH compared to a control solution:

$$\% \text{scavenging effect} = [(\text{ADPPH} - \text{AS})/(\text{ADPPH})] \times 100.$$

where

ADPPH = absorbance of DPPH control solution.
AS = absorbance of extract solutions.

The EC$_{50}$ values (mg mL^{-1} of extract), that is the concentration that allows a 50% reduction (discolouration) of a solution with a known titre of DPPH, were then determined.

ABTS assay

This method is based on the suppression of the absorbance of radical cations of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) by antioxidants in the test sample. The antioxidant activity, measured as the Trolox equivalent antioxidant capacity (TEAC), was determined according to Pycia *et al.*, 2019 with some modifications. Briefly, 50 mg of each extract was diluted with 2 mL of methanol. The obtained solution was further diluted with methanol (1:40). An aliquot of 0.5 mL of each solution was added to a working solution containing ABTS. A Trolox calibration curve in the range of 10–150 μM was prepared under the same conditions as the samples. The (%) decrease in absorbance measured at 734 nm after 6 min of incubation of the sample was calculated for the uninhibited radical cation solution. The antioxidant capacity was expressed as mmol Trolox equivalents (TE) g^{-1} of extract.

FRAP assay

The ferric reducing/antioxidant power (FRAP) procedure was conducted according to the modified method of Tabaraki *et al.*, 2014. Briefly, 50 mg of each extract was diluted with 2 mL of methanol. The obtained solution was further diluted with methanol (1:40). An aliquot of 0.3 mL of each solution was added to the FRAP reagent. The Trolox calibration curve was prepared in the range of 10-150 μM. After 10 min of incubation at room temperature in the dark, the absorbance was recorded at 593 nm. The antioxidant capacity was expressed as mmol Trolox equivalents (TE) g^{-1} of extract.

Total polyphenols

The determination of total phenol content was performed with the Folin–Ciocalteu method described by Gogoi *et al.*, (2018) with some modifications. Standards and samples were prepared in the same way: 1 mL of solution containing polyphenols (extract or standard solution) was supplemented with 1 mL of Folin–Ciocalteu in a 10-mL flask. Standard solutions were in the range of 10–50 mg L^{-1} gallic acid and were used to construct the calibration line ($R^2 = 0.9997$). After 3 min of incubation to allow the reaction between the Folin–Ciocalteu reagent and the polyphenols, 1 mL of a saturated sodium carbonate

solution was added, and then, the mixture was brought to a volume of 10 mL with deionised water. The solutions were incubated in the dark at room temperature for 90 min and showed a blue colour. The blank was prepared by adding 1 mL of deionised water to the same reagents. The measurements were performed with a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 750 nm. The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of dried extract.

Phenolic acids and juglone by UHPLC

After determining the total phenol content, the juglone and the main phenolic acids of the husk were identified and quantified according to Nour *et al.*, (2013) with appropriate modifications. An LC-4000 series UHPLC system equipped with a PDA detector (MD-4010, JASCO International Co., Ltd., Tokyo, Japan) was used. As the mobile phase, a 2% acetic acid solution in water (Solvent A) and 50:50 water-acetonitrile with 0.5% acetic acid (Solvent B) were used. The flow was 0.8 mL min^{-1}. The chromatographic conditions were set as follows: 0 min, 20% B; 5 min, 22% B; 8 min, 23% B; 12 min, 24% B; 14 min, 24.5% B; 22 min, 28% B; 28 min, 30% B; 30 min, 35% B; 40 min, 50% B; 50 min, 60% B; and 58 min, 20% B. A total of 5 µl of this solution were injected into a C18 Water Spherisorb ODS2, 5-µm, 250 × 4.5 mm analytical column. The wavelengths were set at 280 and 420 nm to detect phenolic acids and juglone, respectively. The phenolic acids analysed were hydroxybenzoic acid, vanillic acid, chlorogenic acid, caffeic acid, syringic acid, ferulic acid and o-coumaric acid. The results were expressed as mg/100 g of extract.

Volatile organic compounds (VOCs)

The analysis of the VOCs in the extracts was carried out with the solid-phase microextraction technique (SPME) as reported by Buttery *et al.*, (2000), with some modifications. In a 10-mL vial, 5 mL of appropriately dissolved extract was mixed with 122 µL of 2-methyl-3-heptanone as an internal standard (IS) at a concentration of 408 ppm. The vial was sealed, and the sample with the standard was continuously stirred with a magnetic stirrer for 15 min at 50 °C. Subsequently, a Supelco divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre was introduced into the vial, and stirring continued for another 60 min at 50 °C. Finally, the desorption of the VOCs was carried out in an Agilent 6890 N GC equipped with an Agilent 5973 N MS. The analytes were separated on an HP-5MS capillary column (30 m × 0.25 mm ID × 0.25 µm, Agilent Technologies, Santa Clara, USA). The gas carrier was helium with a flow of 1 mL min^{-1}. The injector was set at 200 °C in splitless mode. The operating conditions of the oven were as follows: 40 °C for 5 min; increase of 5 °C min^{-1} up to 180 °C; increase of 3 °C min^{-1} up to 240 °C held for 5 min. A NIST MS search 2.0 database was used for VOC identification. The results were expressed as mg kg^{-1} of extract.

Fatty acid composition of supercritical CO$_2$ + ethanol extract

The extract obtained with supercritical CO$_2$ + ethanol was subjected to fat extraction and fatty acid determination. The fat was extracted using hexane. Five millilitres of hexane was added to approximately 150 mg of walnut husk extract five times. After centrifugation, the upper layer was recovered and filtered through bleaching coals to eliminate the pigments. Levels of fatty acid methyl esters (FAMEs) were determined after transesterification of a 1% solution of fat in hexane, adding 300 µL of 2 N KOH in methanol to 1 mL of this solution (EEC, 1991). After vigorous mixing, phase separation was achieved. A total of 1 µL of the upper layer, containing the FAMEs, were injected into an Agilent Technologies 6890N gas chromatograph equipped with a capillary column (100 m × 0.25 mm inner diameter, film thickness of 0.20 µm) with a polystationary phase (90% biscyanopropyl/10% cyanopropylphenyl siloxane) (Supelco, Bellafonte, PA, USA), hydrogen flame ionisation detector (FID) and programmed temperature vaporiser (PTV). The carrier gas used was helium with a flow rate of 1 mL min^{-1}. The oven temperature programme was as follows: 140 °C × 5 min, 4 °C min^{-1} ramp to 175 °C for 20 min, and 3 °C min^{-1} ramp to 240 °C for 20 min. The chromatogram peaks were identified using an external 37-component standard (Supelco TM 37 component FAME mix) by comparing the retention times of the standards with those of the samples under the same operating conditions. The results were expressed as % w/w.

Antifungal activity

The antifungal activity was assessed qualitatively and quantitatively. The qualitative procedure used is that described by Torrijos *et al.*, (2019) with appropriate changes. The walnut husk extract was suspended in PDB (potato dextrose broth) at a concentration of 50 mg mL^{-1} and then added to PDA (potato dextrose agar) culture media inoculated with the spores of the target fungi. The plates obtained were incubated for 3 days at 25 °C. After three days of incubation, the growth of the fungi was assessed, and the diameter of the inhibition halo was measured. Antifungal activity was considered positive only if the diameter exceeded 8 mm. The effectiveness was assessed against the

following fungal species: *Penicillium commune* (151); *Penicillium verrucosum* (V49 and V47); *Penicillium digitatum* (99); *Aspergillus parasiticus* (96); *Aspergillus flavus* (146); *Aspergillus niger* (96); *Aspergillus carbonarius* (147); *Fusarium graminearum* (841); and *Fusarium verticillioides* (883 and 884). The two fungal strains of *P. verrucosum* V49 and V47 were provided by the collection of the Technical Research Center of Finland (VTT), and the other strains used in the study were provided by the collection of the Institute of Science of Food Production (ISPA-CNR) of Italy. For each test, four replicates were completed.

Statistical analysis

All analytical determinations were repeated three times for each extraction method, and the reported results represent the averages of the values obtained. The data were subjected to one-way analysis of variance (ANOVA) and Tukey's test ($P \leq 0.05$) using XLSTAT software (Addinsoft, New York, USA).

Results and discussion

Extraction yields and antioxidant capacity

Table 1 shows the yields for the four different types of extraction. The data were expressed as g of extract per 100 g of dry weight (DW). The extraction by supercritical CO_2 with ethanol showed an extractive yield (27.18 g/100 g DW) comparable to those obtained with ethanol and methanol extractions (36.13 and 39.27 g/100 g DW). This is possible due to the use of high pressures and due to the intermediate characteristics that CO_2 exhibits in the supercritical state (Aladić et al., 2014). Supercritical CO_2 without ethanol was not used because it did not allow any extraction yield to be obtained.

In order to evaluate the antioxidant capacity of the extracts, DPPH, ABTS and FRAP assays were used (Table 1). The antioxidant capacities of extracts depend not only on the extract composition but also on the conditions of the test used; therefore, more than one type of antioxidant capacity measurement needs to be performed to take into account the various modes of action of antioxidants. The assays used in this work were based on electron transfer and measured the capacity of an antioxidant to reduce an oxidant, which changes colour when reduced. The ABTS assay is particularly interesting in plant extracts because the absorption wavelength at 734 nm eliminates colour interference.

The extract obtained by supercritical CO_2 with ethanol had the greatest antioxidant capacity measured by DPPH and ABTS (EC_{50} 0.306 mg mL^{-1} and 0.229 mmol TE g^{-1}, respectively), as reported in Table 1. The lowest values of EC_{50} represent the highest antioxidant capacity and vice versa. When measured by FRAP assay, the antioxidant capacity was highest in the water extract (0.459 mmol TE g^{-1}). The different antioxidant activity levels obtained from the assays may reflect a relative difference in the ability of antioxidant compounds in the extracts to reduce ABTS*+, the DPPH-free radical and ferric iron *in vitro* systems. The extracts obtained by methanol, water and ethanol showed EC_{50} values lower than that found in extract by supercritical CO_2 (0.593, 0.568 and 0.704 mg mL^{-1}, respectively), but higher than those found by Yang et al., (2014) in walnut shell extracts. The antioxidant activities of the extracts performed with the same assay are different, mainly due to the extraction techniques, the solubility of the extract in a particular solvent and the cultivars and harvesting periods of raw material. Akbari et al., (2012) found lower EC_{50} values in different parts of walnut fruit as a function of genotype. Wenzel et al., (2017) observed DPPH EC_{50} values ranging from 0.35 to 0.59 mg mL^{-1}, based on the variety of walnut and the maximum antioxidant potential of 0.0270 mmol TE g^{-1} measured by the FRAP assay in dried walnut (*Juglans nigra*) husks extracted by supercritical CO_2 at 68 °C and with 20% ethanol. The ABTS and FRAP values in all the analysed extracts were higher than those found by Pycia et al., 2019 in various walnut varieties.

Polyphenol content

The total polyphenol content in walnut green husk extracts depended on the extraction solvent system

Table 1 Extraction yields of different solvents (g/100 g DW) and antioxidant properties of the extracts expressed as DPPH EC_{50} (mg mL^{-1}), ABTS (mmol TE g^{-1}) and FRAP (mmol TE g^{-1}) values

Extraction method	Yield	DPPH (EC_{50})	ABTS	FRAP
Water	6.49 ± 1.77[b]	0.568 ± 0.032[b]	0.226 ± 0.005[a]	0.459 ± 0.005[a]
Ethanol	36.14 ± 5.20[a]	0.704 ± 0.014[a]	0.128 ± 0.001[b]	0.099 ± 0.011[c]
Methanol	39.28 ± 7.01[a]	0.593 ± 0.013[b]	0.102 ± 0.001[c]	0.083 ± 0.005[c]
Supercritical CO_2 + Ethanol	27.18 ± 1.34[a]	0.306 ± 0.015[c]	0.229 ± 0.002[a]	0.139 ± 0.007[b]

a–c: different letters in the same column indicate statistically significant differences ($P < 0.05$).

(Table 2) and was positively correlated with the antioxidant capacity. In fact, the extract obtained by supercritical CO_2 with ethanol, which showed the highest antioxidant activity measured by DPPH and ABTS assays, also showed the highest phenol content (10750 mg GAE/100 g of extract), followed by the extract obtained with methanol (5809 mg GAE/100 g of extract). The extracts obtained by water (3038 mg GAE/100 g of extract) and ethanol (3640 mg GAE/ 100 g of extract) did not show statistically significant differences between them. As reported by Spigno *et al.*, (2007) and Chew *et al.*, (2011), systems with two types of solvents (binary solvents) are capable of extracting many more phenolic compounds than single-solvent extraction systems. Considering the supercritical CO_2 with ethanol system to be a double solvent system, its combination with high pressure, can explain the greater extraction of polyphenols by this method. The addition of 20% ethanol to supercritical CO_2 was chosen because it is the best condition for the extraction of polyphenols, as reported also by Wenzel *et al.*, (2017).

The extract obtained by supercritical CO_2 + ethanol also showed the highest contents of ferulic acid (986.96 mg/100 g of extract), syringic acid (631.78 mg/ 100 g of extract), o-coumaric acid (77.03 mg/100 g of extract), vanillic acid (27.88 mg/100 g of extract) and juglone (1192.04 mg/100 g of extract), as reported in Table 2. Juglone was found, as reported in the literature, to be the most abundant phenolic compound for all the extraction methods (Table 2). Supercritical CO_2 was more selective towards these compounds than ethanol and more selective towards 4-hydroxybenzoic acid (274.18 mg/100 g of extract) and methanol, which principally extracted caffeic acid (121.77 mg/100 g of extract). Stampar *et al.*, (2006) found the following range of values, expressed as mg/ 100 g of dry weight, in walnut green husk extracts at

different dates of sampling: chlorogenic acid 3.89–15.20; vanillic acid 1.18–21.0; caffeic acid 1.00–1.87; syringic acid 13.10–17.3; ferulic acid 0.91–21.30; and juglone 218–1404.

Volatile organic compounds

The aromatic composition varied according to the extraction method (Table 3). Many of these compounds have also been identified by Buttery *et al.*, (2000) at similar concentrations. The extract obtained with supercritical CO_2 + ethanol was the richest in volatile compounds and the most varied with regard to its type. In fact, in this type of extract, we found compounds such as alkanes, alkenes, alcohols, esters, terpenes and aldehydes. The water extract showed mostly terpenes and esters. The methanol extract mainly contained alkanes and aldehydes. The extract in ethanol was the most similar in terms of the types of compounds to the supercritical CO_2 extract. The molecules contained in the supercritical CO_2 extract appeared to be present in higher concentrations than the other types, especially alkenes, aldehydes and terpenes. In particular, among alkenes (representing 39.70% of total VOCs), 1-tetradecene and 1-hexadecene were the most abundant (74.40 and 73.79 mg kg^{-1}, respectively). 1-Tetradecene in walnut husk extracts obtained with supercritical CO_2 was also found by Seabra *et al.*, (2019). Among aldehydes (representing 30.40% of total VOCs), decanal was the most abundant (58.14 mg kg^{-1}); this molecule is present in walnut fruit at very low concentrations (Elmore *et al.*, 2005; San Román *et al.*, 2015). The terpenes identified represented 2.20% of the total VOCs and were caryophyllene and geranylacetone (3.56 and 6.28 mg kg^{-1}, respectively); caryophyllene was also found in the leaf essential oil of walnut trees (Verma *et al.*, 2013), and geranylacetone was also identified in

Table 2 Total polyphenol contents (mg GAE/100 g extract) and individual phenolic compounds (mg/100 g extract) in the different extracts

	Water	Ethanol	Methanol	Supercritical CO_2 + Ethanol
Total polyphenols	3038.32 ± 1.26[c]	3640.25 ± 3.24[c]	5809.12 ± 0.89[b]	10750.03 ± 5.00[a]
Hydroxybenzoic acid	7.23 ± 0.91[c]	274.18 ± 16.61[a]	113.99 ± 1.30[b]	103.89 ± 11.26[b]
Vanillic acid	12.45 ± 2.66[b]	10.78 ± 0.30[bc]	6.42 ± 1.30[c]	27.88 ± 0.91[a]
Chlorogenic acid	4.71 ± 0.74[c]	≤LOQ*	23.27 ± 0.38[a]	16.16 ± 1.31[b]
Caffeic acid	≤LOQ*	10.74 ± 0.50[bc]	121.77 ± 5.66[a]	15.34 ± 1.12[b]
Syringic acid	161.07 ± 7.06[c]	254.64 ± 6.47[b]	1.07 ± 0.013[d]	631.78 ± 39.70[a]
Ferulic acid	11.72 ± 1.34[c]	14.67 ± 1.16[c]	64.26 ± 5.76[b]	986.96 ± 16.80[a]
o-coumaric acid	40.23 ± 1.05[b]	16.39 ± 1.05[c]	3.65 ± 0.14[d]	77.03 ± 5.06[a]
Juglone	360.34 ± 5.03[c]	324.04 ± 16.18[c]	475.55 ± 7.62[b]	1192.04 ± 17.26[a]

a–d: different letters in the same line indicate statistically significant differences ($P < 0.05$).
LOQ, 0.1 mg/100 g.

Table 3 Main volatile organic compounds (mg kg^{-1}) in the different extracts

	Compound	Water	Ethanol	Methanol	Supercritical CO_2 + Ethanol
Alkanes	Decan	0.18 ± 0.03[b]	5.20 ± 0.65[ab]	5.10 ± 0.59[ab]	8.55 ± 2.91[a]
	Dodecan	n.d.	12.68 ± 2.78[a]	10.20 ± 1.01[a]	10.07 ± 3.49[a]
	Tetradecan	n.d.	17.47 ± 2.59[a]	2.53 ± 0.25[b]	18.13 ± 1.00[a]
	Hexadecan	n.d.	22.63 ± 0.67	n.d.	n.d.
	Heptadecan	n.d.	7.85 ± 0.45[a]	6.04 ± 0.90[a]	n.d.
Alkenes	3-Dodecene	n.d.	n.d.	n.d.	6.20 ± 0.53
	1-Tetradecene	n.d.	n.d.	n.d.	74.40 ± 4.82
	1 Hexadecene	n.d.	n.d.	n.d.	73.79 ± 7.77
	E-7-Octadecene	n.d.	n.d.	n.d	22.80 ± 5.18
Alcohols	4 Hexen-3-Ol-2,5-Dimethyl	10.19 ± 1.34[a]	7.48 ± 0.22[a]	n.d.	n.d.
	3-Methyl-2-Heptanol	2.47 ± 0.04	n.d	n.d	n.d
Aldehydes	Nonanal	3.56 ± 0.06[c]	49.79 ± 0.49[a]	n.d	20.33 ± 2.10[b]
	2-Nonenal	n.d	n.d	n.d	17.63 ± 7.2
	Decanal	1.59 ± 0.04[c]	24.67 ± 0.32[b]	8.63 ± 1.1[c]	58.14 ± 5.14[a]
	Dodecanal	n.d.	n.d.	n.d.	7.00 ± 4.1
	Tetradecanal	n.d.	n.d.	n.d.	7.01 ± 2.72
	7 Hexadecenal (Z)	n.d.	n.d.	n.d.	25.76 ± 0.08
Esters	Octanoic Acid Ethyl Ester	n.d.	n.d.	n.d.	9.09 ± 0.47
	Nonanoic Acid Ethyl Ester	n.d.	n.d.	n.d.	7.90 ± 0.39
	Ethyl Ester Palmitic Acid	n.d.	n.d.	n.d.	31.19 ± 5.58
Terpenes	Caryophyllene	n.d.	0.90 ± 0.18[b]	n.d.	3.56 ± 1.07[a]
	Geranylacetone	0.86 ± 0.05[b]	5.92 ± 1.04[b]	2.63 ± 0.58[b]	6.28 ± 0.92[a]
	Beta-Ionone Methyl	4.39 ± 0.61	n.d.	n.d.	n.d.
	Beta-Farnesene	1.99 ± 0.05	n.d.	n.d	n.d.
	Limonene	1.42 ± 0.05	n.d.	n.d.	n.d.
Others	1,3-Di-Tert-Butylbenzene	3.09 ± 0.02[c]	24.80 ± 0.14[b]	21.95 ± 4.76[b]	38.46 ± 3.73[a]
	1,4 Naphtalendione	n.d.	n.d.	3.31 ± 0.81	n.d.
	Phenol 2,4 Bis 1,1 Dimetiletil	35.77 ± 1.09[a]	n.d.	5.69 ± 1.59[b]	n.d.

a-d: different letters in the same line indicate statistically significant differences ($P < 0.05$).

n.d., not detected.

the essential oil of walnut leaves (Bou Abdallah *et al.*, 2016). Seabra *et al.*, (2019) showed that in extracts obtained with supercritical CO_2 alkanes, terpenes, oxygenated terpenes and alkaloids were the most abundant compounds. The differences could be derived from different variety locations and extraction parameters.

In the ethanol extract, the aldehydes represented 41.50% of the total VOCs and nonanal was the most abundant compound (49.79 mg kg^{-1}), which was present at a greater concentration than in extracts obtained with water and supercritical CO_2. This compound was also found in different walnut varieties (Lee *et al.*, 2011; Abdallah *et al.*, 2015).

In the water extract, there were other interesting compounds, such as phenol 2,4 bis 1,1-dimethylethyl (35.77 mg kg^{-1}), limonene (1.42 mg kg^{-1}), beta-ionone methyl (4.39 mg kg^{-1}) and beta-farnesene (1.99 mg kg^{-1}). Limonene was present in walnuts at concentrations between 0.13 and 0.6 mg kg^{-1} depending on walnut treatment (Hao *et al.*, 2020), in walnut oil and in walnut leaves (Grosso *et al.*, 2018; Farag, 2008); beta-

farnesene was also found in walnut leaves (Farag, 2008).

Fatty acid composition

Due to the nonpolar nature of carbon dioxide allowing fat extraction, fat content and fatty acid composition were also analysed in samples obtained with supercritical CO_2 + ethanol. The amount of fat was 35.40 g/100 g of extract. To the best of our knowledge, this is the first time that the fatty acid composition of extracts from walnut husks has been reported, allowing us to better understand their high nutritional value. In fact, this extract was richer in unsaturated fatty acids than saturated fatty acids (Table 4). In particular, C18: 2n6c (33.83%) and C20: 1 (21.90%) were the most abundant unsaturated fatty acids, while C16: 0 (27.16%) was the most abundant saturated fatty acid. Furthermore, docosahexaenoic acid (DHA) C22:6n3 was found in even lower quantities (0.31%). Polyunsaturated fatty acids have beneficial and preventive effects against cardiovascular diseases

Table 4 Fatty acid composition of the supercritical CO_2 + ethanol extract

Fatty acids	% w/w
C14: 0	0.97 ± 0.02
C16: 0	27.16 ± 0.56
C16:1	1.68 ± 0.00
C17: 0	0.63 ± 0.07
C18: 0	4.16 ± 0.05
C18:1n9	6.77 ± 0.03
C18:2n6	33.83 ± 0.21
C20: 0	0.27 ± 0.00
C20:1n9	21.90 ± 0.19
C18:3n3	0.20 ± 0.00
C22: 0	0.22 ± 0.02
C20:3n6	0.25 ± 0.01
C20:3n3	0.15 ± 0.00
C20:4n6	0.15 ± 0.00
C23: 0	0.10 ± 0.00
C24: 0	1.24 ± 0.02
C22:6n3 (DHA)	0.31 ± 0.01
SFA	34.76 ± 0.45
MUFA	30.35 ± 0.22
PUFA	34.88 ± 0.23
MUFA + PUFA/SFA	1.88 ± 0.04

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

(Sokoøa-Wysoczańska et al., 2018). The fatty acid composition is qualitatively comparable to that of walnut oil but not quantitatively comparable. Although the fatty acid composition depends on the walnut cultivar (Dogan & Akgul, 2005; Martínez & Maestri, 2008), generally the most abundant fatty acid present in walnut oil is linoleic acid (C18: 2n6c), as

reported by Amaral et al., (2003). In walnut oil, while the linoleic acid and linolenic contents are higher than those in green husk extract, ranging from 49.93% (Dogan & Akgul, 2005) to 62.50% (Amaral et al., 2003) and 10.48%–12.04% (Gharibzahedi et al., 2014), respectively, the eicosenoic content is lower (approximatively 0.20%).

Antifungal activity

After three days of incubation at 25 °C, fungal growth was assessed by measuring the diameter of the inhibition zone (Table 5). The water extract was the most versatile and showed inhibition zones against *Penicillium verrucosum*, *Penicillium digitatum*, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium graminearum* and *Fusarium verticilliodes*. Interesting results were also obtained with the CO_2 and methanol extracts against *Penicillium digitatum* (15 mm for both), *Fusarium graminearum* (8 mm) and *Fusarium verticilliodes* (9 and 10 mm). The antifungal activity of the extracts obtained with methanol and supercritical CO_2 + ethanol is mainly due to the juglone (Wianowska et al., 2016) that is present in high amounts in these two types of extracts. The water extract had the greatest antifungal activity, despite the lowest concentration of juglone, which may be due to other compounds with antifungal properties, such as phenol 2,4-bis (1,1-dimethylethyl), also called 2,4-di-tert-butyl phenol (Varsha et al., 2015), which was in fact more concentrated in the water extract (35.77 mg kg^{-1}) than in other extracts. Additionally, limonene, present only in water extract, may contribute to antifungal activity to a lesser extent (Chee et al., 2009).

Table 5 Antifungal activity on solid medium and minimum inhibition concentration (ppm) of the different extracts

Strain	Genus and species	Water	MIC	Ethanol	MIC	Methanol	MIC	Supercritical CO_2 + Ethanol	MIC
V49	Penicillium verrucosum	++	100	-	nd	-	nd	-	nd
99	Penicillium digitatum	+++	50	-	nd	+++	50	+++	50
V47	Penicillium verrucosum	+++	50	-	nd	-	nd	-	nd
151	Penicillium commune	-	nd	-	nd	-	nd	-	nd
96	Aspergillus parasiticus	-	nd	-	nd	-	nd	-	nd
146	Aspergillus flavus	+	150	-	nd	-	nd	-	nd
153	Aspergillus niger	+	150	-	nd	-	nd	-	nd
147	Aspergillus carbonarius	-	nd	-	nd	-	nd	-	nd
838	Fusarium graminearum	++	100	-	nd	++	100	++	100
841	Fusarium graminearum	+	150	-	nd	-	nd	-	nd
883	Fusarium verticillioides	+	150	-	nd	++	100	++	100
884	Fusarium verticillioides	+	150	-	nd	-	nd	-	nd

Inhibition diameter + <8 mm.
Inhibition diameter ++ = 8–10 mm.
Inhibition diameter +++ > 10 mm.
nd, not detected.

Conclusions

The extracts obtained by supercritical CO_2 proved to be rich in bioactive compounds, such as phenolic acids and juglone, and to exert antifungal activity. Moreover, the nonpolar nature of carbon dioxide allows fat extraction from walnut green husks. This type of extract is rich in unsaturated fatty acids that have beneficial and preventive effects against cardiovascular diseases. Despite the high costs, due to the complexity of the machinery that must support the use of high pressure, this extraction technique allows a lower environmental impact, as the solvent used does not leave traces and can be recovered and reused for further extractions. The use of this technique allows transformation of the biomass obtained from the walnut production process from waste to resources, guaranteeing benefits for companies such as reduction in disposal costs, possible biopesticide applications and improved reputation. Extracts from walnut green husks, rich in bioactive compounds, could be used to prepare active packaging for the food industry and biopesticides or in the cosmetic industry and as pharmaceutical agents after proper validation.

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Author contribution

Raffaele Romano: Conceptualization (equal); Funding acquisition (lead); Methodology (equal); Project administration (lead); Resources (equal); Supervision (equal). **Alessandra Aiello:** Data curation (equal); Formal analysis (equal); Methodology (equal). **Lucia De Luca:** Data curation (equal); Formal analysis (equal); Methodology (equal). **Giuseppe Meca:** Investigation (equal); Supervision (equal). **Fabiana Pizzolongo:** Conceptualization (equal); Writing-original draft (equal); Writing-review & editing (equal). **Paolo Masi:** Resources (equal); Visualization (equal).

Conflicts of interest

The authors declare no conflicts of interest.

Ethical approval

Ethics approval was not required for this research.

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References

Abdallah, I.B., Tlili, N., Martinez-Force, E. *et al.* (2015). Content of carotenoids, tocopherols, sterols, triterpenic and aliphatic alcohols, and volatile compounds in six walnuts (*Juglans regia* L.) varieties. *Food Chemistry*, **173**, 972–978.

Ahmad, T., Khan, T., Alamgeer & Shah, A.J. (2020). Juglone as antihypertensive agent acts through multiple vascular mechanisms. *Clinical and Experimental Hypertension*, **42**, 335–344.

Aiello, A., Pizzolongo, F., Scognamiglio, G., Romano, A., Masi, P. & Romano, R. (2020). Effects of supercritical and liquid carbon dioxide extraction on hemp (*Cannabis sativa* L.) seed oil. *International Journal of Food Science & Technology*, **55**, 2472–2480.

Akbari, V., Jamei, R., Heidari, R. & Esfahlan, A.J. (2012). Antiradical activity of different parts of Walnut (*Juglans regia* L.) fruit as a function of genotype. *Food chemistry*, **135**, 2404–2410.

Aladić, K., Jokić, S., Moslavac, T. *et al.* (2014). Cold pressing and supercritical CO2 extraction of hemp (*Cannabis sativa*) seed oil. *Chemical and Biochemical Engineering Quarterly*, **28**, 481–490.

Alam, M.A. (2019). Anti-hypertensive effect of cereal antioxidant ferulic acid and its mechanism of action. *Frontiers in Nutrition*, **6**, 121.

Amaral, J.S., Casal, S., Pereira, J.A., Seabra, R.M. & Oliveira, B.P. (2003). Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Portugal. *Journal of Agricultural and Food Chemistry*, **51**, 7698–7702.

Bou Abdallah, I., Baatour, O., Mechrgui, K. *et al.* (2016). Essential oil composition of walnut tree (*Juglans regia* L.)'leaves from Tunisia. *Journal of EssEntial oil rEsEarch*, **28**, 545–550.

Buttery, R.G., Light, D.M., Nam, Y., Merrill, G.B. & Roitman, J.N. (2000). Volatile components of green walnut husks. *Journal of Agricultural and Food Chemistry*, **48**, 2858–2861.

Chee, H.Y., Mm, H. & Lee, M.H. (2009). In vitro antifungal activity of limonene against Trichophyton rubrum. *Mycobiology*, **37**, 243–246.

Chew, K.K., Khoo, M.Z., Ng, S.Y., Thoo, Y.Y., Aida, W.W. & Ho, C.W. (2011). Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of Orthosiphon stamineus extracts. *International Food Research Journal*, **18**, 1427.

Clark, A.M., Jurgens, T.M. & Hufford, C.D. (1990). Antimicrobial activity of juglone. *Phytotherapy Research*, **4**, 11–14.

Cosmulescu, S.N., Trandafir, I., Achim, G. (2010). Phenolics of green husk in mature walnut fruits. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, **38**, 53-56.

Dogan, M. & Akgul, A. (2005). Fatty acid composition of some walnut (*Juglans regia* L.) cultivars from east Anatolia. *Grasas y Aceites*, **56**, 328–331.

EEC Commission Regulation. (1991). No. 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Official Journal. No. L*, **248**, 1–83.

Elmore, J.S., Nisyrios, I. & Mottram, D.S. (2005). Analysis of the headspace aroma compounds of walnuts (*Juglans regia* L.). *Flavour and Fragrance Journal*, **20**, 501–506.

FAOSTAT. (2018). FAO global statistical yearbook. Retrieved from http://www.fao.org/faostat/en/#data/QC.

Farag, M.A. (2008). Headspace analysis of volatile compounds in leaves from the Juglandaceae (walnut) family. *Journal of Essential Oil Research*, **20**, 323–327.

Gharibzahedi, S.M.T., Mousavi, S.M., Hamedi, M. & Khodaiyan, F. (2014). Determination and characterization of kernel

biochemical composition and functional compounds of Persian walnut oil. *Journal of Food Science and Technology*, **51**, 34–42.

GilakHakimabadi, S., Ehsani, M., Khonakdar, H.A., Ghaffari, M. & Jafari, S.H. (2019). Controlled-release of ferulic acid from active packaging based on LDPE/EVA blend: Experimental and modeling. *Food Packaging and Shelf Life*, **22**, 100392.

Gogoi, R., Loying, R., Sarma, N., Munda, S. & Pandey, S.K. (2018). A comparative study on antioxidant, anti-inflammatory, genotoxicity, anti-microbial activities and chemical composition of fruit and leaf essential oils of Litsea cubeba Pers from North-east India. *Industrial Crops and Products*, **125**, 131–139.

Gogoi, R., Sarma, N., Begum, T., Pandey, S.K. & Lal, M. (2020). North-East Indian Chromolaena odorata (L. King Robinson) aerial part essential oil chemical composition, pharmacological activities - neurodegenerative inhibitory and toxicity study. *Journal of Essential Oil Bearing Plants*, **23**, 1173–1191.

Grosso, A.L., Asensio, C.M., Nepote, V. & Grosso, N.R. (2018). Antioxidant activity displayed by phenolic compounds obtained from walnut oil cake used for walnut oil preservation. *Journal of the American Oil Chemists' Society*, **95**, 1409–1419.

Hao, J., Xu, X.L., Jin, F., Regenstein, J.M. & Wang, F.J. (2020). HS-SPME GC–MS characterization of volatiles in processed walnuts and their oxidative stability. *Journal of Food Science and Technology*, **57**, 2693–2704.

Hsu, C.L., Wu, C.H., Huang, S.L. & Yen, G.C. (2009). Phenolic compounds rutin and o-coumaric acid ameliorate obesity induced by high-fat diet in rats. *Journal of Agricultural and Food Chemistry*, **57**, 425–431.

Itoh, A., Isoda, K., Kondoh, M. *et al.* (2010). Hepatoprotective effect of syringic acid and vanillic acid on CCl4-induced liver injury. *Biological and Pharmaceutical Bulletin*, **33**, 983–987.

Jahanban-Esfahlan, A., Ostadrahimi, A., Tabibiazar, M. & Amarowicz, R. (2019). A comparative review on the extraction, antioxidant content and antioxidant potential of different parts of walnut (*Juglans regia* L.) Fruit and tree. *Molecules*, **24**, 2133.

Jakopic, J. & Veberic, R. (2009). Extraction of phenolic compounds from green walnut fruits in different solvents. *Acta Agriculturae Slovenica*, **93**, 11.

Latos, M., Masek, A. & Zaborski, M. (2019). The potential of juglone as natural dye and indicator for biodegradable polyesters. *Proceedings of the Institution of Mechanical Engineers, Part L: Journal of Materials: Design and Applications*, **233**, 276-285.

Lee, J., Vázquez-Araújo, L., Adhikari, K., Warmund, M. & Elmore, J. (2011). Volatile compounds in light, medium, and dark black walnut and their influence on the sensory aromatic profile. *Journal of Food Science*, **76**, C199–C204.

Liu, T., Li, Y. & Li, N. (2017). Effects and mechanism of juglone in combination with 5-FU on colon cancer CT-26 cells in vivo. *BIO Web of Conferences*, **8**, 01013.

Martínez, M.L. & Maestri, D.M. (2008). Oil chemical variation in walnut (*Juglans regia* L.) genotypes grown in Argentina. *European Journal of Lipid Science and Technology*, **110**, 1183–1189.

Muthukumaran, J., Srinivasan, S., Venkatesan, R.S., Ramachandran, V. & Muruganathan, U. (2013). Syringic acid, a novel natural phenolic acid, normalizes hyperglycemia with special reference to glycoprotein components in experimental diabetic rats. *Journal of Acute Disease*, **2**, 304–309.

Nour, V., Trandafir, I. & Cosmulescu, S. (2013). HPLC determination of phenolic acids, flavonoids and juglone in walnut leaves. *Journal of Chromatographic Science*, **51**, 883–890.

Pereira, J.A., Oliveira, I., Sousa, A. *et al.* (2007). Walnut (*Juglans regia* L.) leaves: Phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. *Food and Chemical Toxicology*, **45**, 2287–2295.

Popovici, C., Gîtin, L. & Alexe, P. (2012). Characterization of walnut (*Juglans regia* L.) green husk extract obtained by supercritical carbon dioxide fluid extraction. *Journal of Food and Packaging Science, Technique and Technologies*, **1**, 5–9.

Pycia, K., Kapusta, I., Jaworska, G. & Jaworska, A. (2019). Antioxidant properties, profile of polyphenolic compounds and tocopherol content in various walnut (*Juglans regia* L.) varieties. *European Food Research and Technology*, **245**, 607–616.

Ribeiro, A.S., Estanqueiro, M., Oliveira, M.B. & Sousa Lobo, J.M. (2015). Main benefits and applicability of plant extracts in skin care products. *Cosmetics*, **2**, 48–65.

Romano, R., Aiello, A., Pizzolongo, F., Rispoli, A., De Luca, L. & Masi, P. (2020). Characterization of oleoresins extracted from tomato waste by liquid and supercritical carbon dioxide. *International Journal of Food Science & Technology*, **55**, 3334–3342.

Rondini, L., Peyrat-Maillard, M.N., Marsset-Baglieri, A. & Berset, C. (2002). Sulfated ferulic acid is the main in vivo metabolite found after short-term ingestion of free ferulic acid in rats. *Journal of Agricultural and Food Chemistry*, **50**, 3037–3041.

San Román, I., Bartolomé, L., Gee, W.S., Alonso, R.M. & Beck, J.J. (2015). Comparison of ex situ volatile emissions from intact and mechanically damaged walnuts. *Food Research International*, **72**, 198–207.

Seabra, I.J., Braga, M.E., Oliveira, R.A. & de Sousa, H.C. (2019). Two-step high pressure solvent extraction of walnut (*Juglans regia* L.) husks: scCO2+ CO2/ethanol/H2O. *Journal of CO2 Utilization*, **34**, 375–385.

Sen, S., Atmaca, P., Terzioglu, G. & Arslan, S. (2013). Anticarcinogenic effect and carcinogenic potential of the dietary phenolic acid: o-coumaric acid. *Natural Product Communications*, **8**, 1934578X1300800922.

Sokoøa-Wysoczańska, E., Wysoczański, T., Wagner, J. *et al.* (2018). Polyunsaturated fatty acids and their potential therapeutic role in cardiovascular system disorders—a review. *Nutrients*, **10**, 1561.

Soto-Maldonado, C., Vergara-Castro, M., Jara-Quezada, J. *et al.* (2019). Polyphenolic extracts of walnut (*Juglans regia*) green husk containing juglone inhibit the growth of HL-60 cells and induce apoptosis. *Electronic Journal of Biotechnology*, **39**, 1–7.

Spigno, G., Tramelli, L. & De Faveri, D.M. (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering*, **81**, 200–208.

Stampar, F., Solar, A., Hudina, M., Veberic, R. & Colaric, M. (2006). Traditional walnut liqueur–cocktail of phenolics. *Food Chemistry*, **95**, 627–631.

Strugstad, M. & Despotovski, S. (2013). A summary of extraction, synthesis, properties, and potential uses of juglone: A literature review. *Journal of Ecosystems and Management*, **13**, 1–16.

Tabaraki, R. & Rastgoo, S. (2014). Comparison between conventional and ultrasound-assisted extractions of natural antioxidants from walnut green husk. *Korean Journal of Chemical Engineering*, **31**, 676–683.

Topal, S., Kocacaliskan, I., Arslan, O. & Tel, A.Z. (2007). Herbicidal effects of juglone as an allelochemical. *Phyton*, **46**, 259–269.

Torrijos, R., Nazareth, T.M., Pérez, J., Mañes, J. & Meca, G. (2019). Development of a bioactive sauce based on oriental mustard flour with antifungal properties for pita bread shelf life improvement. *Molecules*, **24**, 1019.

Varsha, K.K., Devendra, L., Shilpa, G., Priya, S., Pandey, A. & Nampoothiri, K.M. (2015). 2, 4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated Lactococcus sp. *International Journal of Food Microbiology*, **211**, 44–50.

Vergano, G., Botta, R. & Radicati, L. (1993). Preservation of *Juglans regia* L. nuts under different storage conditions. In *International Symposium on Quality of Fruit and Vegetables: Influence of Pre-and Post-Harvest Factors and Technology* 379 (451-458).

Verma, R.S., Padalia, R.C., Chauhan, A. & Thul, S.T. (2013). Phytochemical analysis of the leaf volatile oil of walnut tree (*Juglans regia* L.) from western Himalaya. *Industrial Crops and Products*, **42**, 195–201.

Vinothiya, K. & Ashokkumar, N. (2017). Modulatory effect of vanillic acid on antioxidant status in high fat diet-induced changes in diabetic hypertensive rats. *Biomedicine & Pharmacotherapy*, **87**, 640–652.

Wang, P., Zhang, S.D., Jiao, J. *et al.* (2019). ROS-mediated p53 activation by juglone enhances apoptosis and autophagy in vivo and in vitro. *Toxicology and Applied Pharmacology*, **379**, 114647.

Wenzel, J., Storer Samaniego, C., Wang, L. (2017). Antioxidant potential of *Juglans nigra*, black walnut, husks extracted using supercritical carbon dioxide with an ethanol modifier. *Food science & Nutrition*, **5**, 223-232.

Wianowska, D., Garbaczewska, S., Cieniecka-Roslonkiewicz, A., Dawidowicz, A.L. & Jankowska, A. (2016). Comparison of antifungal activity of extracts from different Juglans regia cultivars and juglone. *Microbial Pathogenesis*, **100**, 263–267.

Yang, J., Chen, C., Zhao, S., Ge, F. & Liu, D. (2014). Effect of solvents on the antioxidant activity of walnut (*Juglans regia* L.) shell extracts. *Journal of Food and Nutrition Research*, **2**, 621–626.

Yang, K., Dang, H., Liu, L. *et al.* (2019). Effect of syringic acid incorporation on the physical, mechanical, structural and antibacterial properties of chitosan film for quail eggs preservation. *International Journal of Biological Macromolecules*, **141**, 876–884.

Yilmaz, S., Akça, Y. & Saçlik, S. (2017). Green husk and inshell biomass production capabilities of six walnut cultivars. *Journal of International Scientific Publications Agriculture and Food*, **5**, 389–397.