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 CO2 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 CO2 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 (LD₅₀) 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-cumaric 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-naphthoquinone) 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 LD₅₀ 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 (CO₂), a nonpolar solvent with a low critical temperature and intermediate critical pressure. The dissolution power of supercritical CO₂ 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 CO₂ 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 CO₂ 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 ± 0.3%. Subsequently, they were ground with a knife mill (Grindomix M200, Retsch Italia, Verdere Scientific Srl, Bergamo, Italy) at 9000 x 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 (assy 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 \( \phi \leq 1 \text{ mm} \) 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 \(-20^\circ \text{C}\) 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 \( \phi \leq 1 \text{ mm} \) was loaded into the extraction vessel. The extractive parameters were as follows: \( P = 300 \text{ bar} \), \( T = 50^\circ \text{C} \), 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

**DPHH assay**

Antioxidant activity by the scavenging effect of radicals on 2,2-diphenyl-1-picrylhydrazyl (DPHH) was performed according to Gogoi et al., (2020), with some modifications. A \( 6 \times 10^{-5} \text{ M} \) DPHP 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 DPHP 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 DPHP scavenging effect was expressed as a percentage of decolouration of DPHP compared to a control solution:

\[
\%\text{scavenging effect} = \left( \frac{\text{ADPPH} - \text{AS}}{\text{ADPPH}} \right) \times 100.
\]

where

\( \text{ADPPH} = \) absorbance of DPPH control solution.

\( \text{AS} = \) 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 DPHP, 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 \( \mu \text{g mL}^{-1}\) 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 \( \mu \text{g mL}^{-1}\). 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 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.

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 trans-esterification 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% 2-bicyclopropyl/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 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 2) and was positively correlated with the antioxidant capacity. In fact, the extract obtained by supercritical CO\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 the literature, to be the most abundant phenolic compound for all the extraction methods (Table 2). Supercritical CO\textsubscript{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\textsubscript{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\textsubscript{2} extract. The molecules contained in the supercritical CO\textsubscript{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\textsuperscript{−1}, respectively). 1-Tetradecene in walnut husk extracts obtained with supercritical CO\textsubscript{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\textsuperscript{−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 geranylactone (3.56 and 6.28 mg kg\textsuperscript{−1}, respectively); caryophyllene was also found in the leaf essential oil of walnut trees (Verma et al., 2013), and geranylactone was also identified in

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**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\textsubscript{2} + Ethanol |
|------------------|----------------|----------------|-----------------|--------------------------------------------|
| Total polyphenols| 3038.32 ± 1.26\textsuperscript{a} | 3640.25 ± 3.24\textsuperscript{a} | 5809.12 ± 0.89\textsuperscript{a} | 10750.03 ± 5.00\textsuperscript{a} |
| Hydroxybenzoic acid | 7.23 ± 0.91\textsuperscript{a} | 274.18 ± 16.61\textsuperscript{a} | 113.99 ± 1.30\textsuperscript{a} | 103.89 ± 11.26\textsuperscript{a} |
| Vanillic acid    | 12.45 ± 2.68\textsuperscript{a} | 10.78 ± 0.30\textsuperscript{b} | 6.42 ± 1.30\textsuperscript{b} | 27.88 ± 0.91\textsuperscript{c} |
| Chlorogenic acid | 4.71 ± 0.74\textsuperscript{a} | ≤LOQ\textsuperscript{a} | 23.27 ± 0.38\textsuperscript{b} | 16.16 ± 1.31\textsuperscript{b} |
| Caffeic acid     | ≤LOQ\textsuperscript{a} | 10.74 ± 0.50\textsuperscript{b} | 121.77 ± 5.66\textsuperscript{c} | 15.34 ± 1.12\textsuperscript{b} |
| Syringic acid    | 161.07 ± 7.06\textsuperscript{b} | 254.64 ± 6.47\textsuperscript{b} | 1.07 ± 0.013\textsuperscript{b} | 631.78 ± 39.70\textsuperscript{a} |
| Ferulic acid     | 11.72 ± 1.34\textsuperscript{a} | 14.67 ± 1.16\textsuperscript{a} | 64.26 ± 5.76\textsuperscript{b} | 986.96 ± 16.80\textsuperscript{a} |
| o-Coumaric acid  | 40.23 ± 1.95\textsuperscript{b} | 16.39 ± 1.05\textsuperscript{b} | 3.65 ± 0.14\textsuperscript{b} | 77.03 ± 5.06\textsuperscript{a} |
| Juglone          | 360.34 ± 5.03\textsuperscript{c} | 324.04 ± 16.18\textsuperscript{c} | 475.55 ± 7.62\textsuperscript{c} | 1192.04 ± 17.26\textsuperscript{c} |

\(\text{LOQ}, 0.1 \text{mg/100 g.}\)
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%). Polysaturated fatty acids have beneficial and preventive effects against cardiovascular diseases.

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**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$^{ab}$ | 8.58 ± 2.91$^*$               |
| 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.     | 6.20 ± 0.53 |                               |
| 1-Tetradecene                | n.d.   | n.d.     | 74.40 ± 4.82 |                               |
| 1-Hexadecene                 | n.d.   | n.d.     | 73.79 ± 7.77 |                               |
| E-7-Octadecane               | n.d.   | n.d.     | 22.80 ± 5.18 |                               |
| Alcohols                     |        |          |          |                               |
| 4 Hexen-3-01,2,5-Dimethyl    | 10.19 ± 1.34$^a$ | 7.48 ± 0.22$^a$ | n.d.     | n.d.                          |
| 3-Methyl-2-Heptanal          | 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.     | 17.63 ± 7.2  |                               |
| Decanal                      | 1.59 ± 0.04$^c$ | 24.67 ± 0.32$^a$ | 8.63 ± 1.1$c$ | 58.14 ± 5.14$^a$             |
| Dodecanal                    | n.d.   | n.d.     | 7.00 ± 4.1  |                               |
| Tetradecanal                 | n.d.   | n.d.     | 7.01 ± 2.72 |                               |
| 7 Hexadecen (Z)              | n.d.   | n.d.     | 25.76 ± 0.08 |                               |
| Esters                       |        |          |          |                               |
| Octanoic Acid Ethyl Ester    | n.d.   | n.d.     | 9.09 ± 0.47 |                               |
| Nonanoic Acid Ethyl Ester    | n.d.   | n.d.     | 7.90 ± 0.39 |                               |
| Ethyl Ester Palmitic Acid    | 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.   | 3.31 ± 0.81 | n.d.      | n.d.                          |
| Phenol 2,4 Bis 1,1 Dimetileti | 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).

d.n., not detected.
Table 4 Fatty acid composition of the supercritical CO2 + 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 |
| C18:2n6   | 33.83 ± 0.21 |
| C20:0      | 0.27 ± 0.00 |
| C20:1n9   | 21.90 ± 0.19 |
| C20:3n6   | 0.25 ± 0.01 |
| C20:6n3   | 0.15 ± 0.00 |
| C20:4n6   | 0.15 ± 0.00 |
| C20:3n3   | 0.22 ± 0.02 |
| C20:1n6   | 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.

(Sokońska-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 (C18:2n6), 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 (approximately 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 verticillioides. Interesting results were also obtained with the CO2 and methanol extracts against Penicillium digitatum (15 mm for both), Fusarium graminearum (8 mm) and Fusarium verticillioides (9 and 10 mm). The antifungal activity of the extracts obtained with methanol and supercritical CO2 + 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 CO2 + Ethanol MIC |
|--------|-------------------------|-----------|-------------|--------------|--------------------------------|
| V49    | Penicillium verrucosum  | ++ 100    | -           | nd           | nd                            |
| 99     | Penicillium digitatum    | +++ 50    | -           | nd           | +++ 50                        |
| V47    | Penicillium verrucosum  | +++ 50    | -           | nd           | ++                            |
| 151    | Penicillium commune      | -         | nd          | -            | -                             |
| 96     | Aspergillus parasiticus  | -         | nd          | -            | -                             |
| 146    | Aspergillus flavus       | +         | 150         | -            | nd                            |
| 153    | Aspergillus niger        | +         | 150         | -            | nd                            |
| 147    | Aspergillus carbonarius  | -         | nd          | -            | -                             |
| 838    | Fusarium graminearum     | ++ 100    | -           | nd           | ++ 100                        |
| 841    | Fusarium graminearum     | +         | 150         | -            | -                             |
| 883    | Fusarium verticillioides | +         | 150         | -            | ++ 100                        |
| 884    | Fusarium verticillioides | +         | 150         | -            | -                             |

Inhibition diameter + < 8 mm.
Inhibition diameter ++ = 8–10 mm.
Inhibition diameter +++ > 10 mm.
nd, not detected.
Conclusions
The extracts obtained by supercritical CO₂ 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 unadsorbed 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.

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Ethics approval was not required for this research.

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