Research Article

Received: 13 May 2024 Revised: 5 August 2024 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.13904

Foliar application of biostimulants improves nutritional and bioactive quality of walnuts

Liege Aguiar Pascoalino,[a,b] Tânia CSP Pires,[a,b] José Pinela,[a,b] Manuel Ângelo Rodrigues,[a,b] Isabel CFR Ferreira,[a,b] Lillian Barros,[a,b] João CM Barreira[a,b] and Filipa S Reis[a,b*]

Abstract

BACKGROUND: Owing to their health benefits, walnuts are attracting interest as a good option for nutritious meals, thereby promoting their production. Furthermore, the adoption of ecologically and environmentally friendly agriculture strengthens biostimulant use as a sustainable complement to traditional fertilizers. This study evaluated the effects of different foliar-applied biostimulants in walnut tree orchards, in northeastern Portugal, on walnuts' chemical composition and bioactivity.

RESULTS: Walnut samples were rich in fat (particularly the polyunsaturated linoleic acid), dietary fiber and protein. Sucrose was the most prevalent soluble sugar, followed by glucose and fructose. Studied samples also showed an antioxidant activity comparable (or superior) to that of Trolox. Some plant biostimulants (e.g. Sprint Plus®) had a positive impact on the nutritional composition of walnuts, more specifically by boosting tocopherol levels, besides improving the bioactivity of walnut extracts against specific bacteria.

CONCLUSION: Overall, this research demonstrated that important quality traits of walnuts can be improved using sustainable agricultural bioproducts and practices.

Keywords: *Juglans regia* L.; plant biostimulants; nutritional value; chemical composition; bioactivity

INTRODUCTION

Walnut tree (*Juglans regia* L.), belonging to the Juglandaceae family, produces one of the nuts frequently present in Mediterranean diets and is widespread in Portugal, where its production exceeds 6000 tons per year.[1] Recently, walnuts have been considered as a natural functional food of elevated economic significance due to their nutritional and medicinal advantages.[2,3]

Nuts' unsaturated fatty acid composition has been associated with improving digestion, accelerating wound healing, strengthening the immune system and improving blood circulation. Furthermore, it has been reported that their characteristic lipid profile is correlated with a reduced risk of cardiovascular diseases, particularly due to the levels of mono- and polyunsaturated fatty acids, which contribute to decreasing low-density lipoprotein cholesterol, usually known as 'bad cholesterol'. Furthermore, short-term trials have shown that nuts may be associated with increasing high-density lipoprotein, normally referred to as 'good cholesterol', besides strengthening the antioxidant defense system.[4,5]

Several studies have explored the traditional applications, phytochemistry and pharmacology of walnuts.[6] Additionally, investigations have delved into the antioxidant properties of both the fruits and leaves of this tree,[7] as well as the chemical characterization and nutritional properties of walnuts.[8]

Traditionally, the challenge imposed by increasing food requirements was approached by an intensification of synthetic fertilizers and pesticide use. However, only a fraction of the applied nutrients are absorbed by plants leading to disruptions in microbial populations in addition to contamination of water resources.[9] Hence, there is an urgent need for a transition from contemporary agricultural methods to eco-friendly practices, aiming to reduce or substitute fertilizers and pesticides, specifically by using biostimulants. Plant biostimulants can be any material or combination of compounds from natural sources or microorganisms that enhance crop productivity and quality without causing environmental impact.[10] Plant biostimulants do not fall under the category of fertilizers given that they do not directly provide nutrients to plants; therefore, they could be considered as a complement since they support the absorption of nutrients, increase tolerance to biotic/abiotic stresses and stimulate plant growth.[11,12] Studies report that marine bioactive compounds extracted from algae, for example, have numerous beneficial results regarding increased quality and yield.[13,14]

* *Correspondence to: FS Reis, Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus Sta Apolónia, Bragança, Portugal. E-mail: freis@ipb.pt*

a *Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Bragança, Portugal*

b *Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Bragança, Portugal*

Indeed, the work reported here aimed to evaluate the influence of using plant biostimulants on the chemical profile, nutritional composition and bioactivity of walnuts. Interestingly, it is important to mention that the biostimulants investigated were administered via leaves, a method potentially more effective due to the limited accessibility of nutrients when applied to the soil and the inefficiency in their translocation from root system to fruits. Numerous studies have demonstrated the effectiveness of applying biostimulants through foliar application techniques in different crops.[14-18] Thus, the study offers useful information on the potential of several plant biostimulants to be used in sustainable agriculture. It will also promote the consumption of plant foods obtained with these techniques and their inclusion in a healthy and equilibrated diet.

MATERIALS AND METHODS

Foliar application of biostimulants and sample preparation

Walnut samples were collected from a field experiment conducted in a walnut orchard of the Franquette cultivar, located in Vale da Porca (41°54′34″ N, –6°88′91″ W, 583 m above sea level) within the municipality of Macedo de Cavaleiros, in northeastern Portugal. The trees, which are 20 years old, are planted at a spacing of 7 m × 7 m and are irrigated using a drip irrigation system. Soil management involves a natural vegetation cover that is mowed twice annually. In the field trial, six treatments were established based on the application of five plant biostimulants and a control treatment, namely: (i) Basfoliar® K Premium; (ii) Tradebor®; (iii) Sprint Plus®; (iv) Fitoalgas Green®; (v) Stimulus™; and (vi) control (sprayed only with water).

Basfoliar® K Premium is a high-concentration liquid foliar fertilizer, containing potassium (25% (w/v) K_2O), enhanced with 3% seaweed extract (*Scklonia maxima*) and 10 g L^{-1} of boron. It is recommended for application close to harvest to increase fruit size and quality. The product was applied at a rate of 5 L ha^{-1} in two separate applications. Tradebor® is a foliar fertilizer containing boron in the form of ethanolamine (15.4% w/v), which offers enhanced mobility within the plant. It was applied at a rate of 1.5 L ha^{-1} across three applications. Sprint Plus® is a plant biostimulant composed of free amino acids, which act rapidly on the plant. It contains 28.8% (w/v) free amino acids, 10.8% (w/v) total nitrogen and 67.4% organic matter. It was applied at a rate of 1.5 L ha^{-1} in three applications. Fitoalgas Green® contains a pure extract of *Ascophyllum nodosum*, obtained through a cold extraction process. It is designed to promote flowering, fruit set and fruit quality. The product contains 16.5% (w/v) *A. nodosum* expressed as dry weight. It was applied at a rate of 2.5 L ha^{-1} in three applications. Stimulus™ contains a pure extract of *A. nodosum*, obtained through a cold extraction process. The commercial product comprises 6.6% (w/v) *A. nodosum* expressed as dry weight, 10.6% (w/v) free amino acids, 4% (w/v) nitrogen and 18.9% (w/v) organic matter. It was applied at a rate of 2.5 L ha^{-1} in three applications. Basfoliar® K Premium was applied only twice, close to the harvest period, while the other products were applied three times, following a schedule as close as possible to the manufacturers' recommendations. The application dates were 21 June and 9 and 26 July. All these products are usually approved for use in organic farming.

The experiment was planned as a randomized design with three replicates. Once in the laboratory, dried walnut fruit samples were ground in a food chopper (A327R1, Moulinex, Spain), homogenized, kept shielded from moisture and light and analyzed in triplicate.

Standards and reagents

Chemicals and reagents were of analytical purity (Fisher Scientific, Lisbon, Portugal), except for the solvents used in high-performance liquid chromatography (HPLC), which were of HPLC grade. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Centesimal composition analysis

The centesimal composition (ash, crude fat, crude protein and total fiber) was analyzed following AOAC procedures.[19] Crude protein was determined by the macro-Kjeldahl method with an automatic distillation and titration unit (Pro-Nitro-A; Selecta, Barcelona, Spain); ash was evaluated by incineration at 550 °C and crude fat was determined by Soxhlet extraction with petroleum ether. Total dietary fiber (TDF) was calculated through an enzymatic–gravimetric method (AOAC 993.19), with α-amylase, protease and amyloglucosidase. Results were expressed as grams per 100 g of dry weight (dw). The available carbohydrate content (g (100 g)$^{-1}$ dw) was estimated by difference using the formula: 100 − (g fat + g ash + g proteins + g fiber), and the energy value (kcal (100 g)$^{-1}$ dw) was estimated according to the Regulation (EC) No. 1169/2011 as follows: 4 × (g protein + g available carbohydrate) + 2 × (g fiber) + 9 × (g fat).

Chemical composition analysis

The chemical composition of walnuts encompassed tocopherols and fatty acids as lipophilic constituents and soluble sugars and organic acids as hydrophilic constituents.

Tocopherols

Tocopherols were determined using an HPLC system (Knauer, Smartline System 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA).[14] The identification was performed by chromatographic comparisons with commercial standards, and the quantification using the internal standard method (IS, tocol). The results were expressed in mg (100 g)$^{-1}$ dw.

Fatty acids

Fatty acid profile was determined by gas–liquid chromatography with flame ionization detection/capillary column, after the extraction and derivatization to fatty acid methyl esters (FAMEs).[14] The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a Macherey-Nagel (GC 1000, Düren, Germany) column (50% cyanopropylmethyl–50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm df), and a flame ionization detector. The identification was made by comparing the relative retention times of FAME peaks with commercial standards and the results were expressed as the relative percentage (Clarity DataApex 4.0.1.7 software).

Lipid quality indices

The polyunsaturated fatty acid (PUFA)/saturated fatty acid (SFA) ratio was calculated from the relative percentages. The atherogenicity (AI), hypocholesterolemic (HI) and thrombogenicity (TI) health indices of the walnut fatty acids were calculated as previously described.[20]

Soluble sugars

Soluble sugars profile was determined by HPLC and consisted of an integrated system with a pump (Knauer, Smartline System 1000, Berlin, Germany), an auto-sampler (AS-2057, Jasco, Easton, MD, USA), a degasser system (Smartline Manager 5000) and a refractive index detector (Knauer Smartline 2300, Berlin, Germany). Freeze-dried samples were extracted with a water–ethanol mixture (80:20) after being spiked with melezitose (IS, 5 mg mL^{-1}).[21] Compounds were identified by comparison with commercial standards and quantified using the IS method (Clarity 2.4 software, DataApex, Prague, Czech Republic). Results are expressed in g $(100\ g)^{-1}$ dw.

Organic acids

Samples were extracted at room temperature by adding 25 mL of 4.5% metaphosphoric acid and under stirring for 20 min. Extracts were filtered twofold (Whatman No. 4 paper + 0.22 µm nylon filters) and moved to HPLC vials. The organic acids were determined by ultrafast liquid chromatography coupled to a photodiode array detector (UFLC-PDA, Shimadzu 20A Series, Kyoto, Japan). The quantification was performed by association of the peak area recorded at 215 nm (245 nm to ascorbic acid) with calibration curves from commercial standards. The results were expressed in mg $(100\ g)^{-1}$ dw.

Bioactivity evaluation

Sample preparation

For obtaining extracts, 1 g of ground sample was suspended in 50 mL of ethanol–water (80:20, v/v) at 25 °C by stirring for 1 h, and then filtered through Whatman No. 4 paper. The residue was subsequently re-extracted under identical parameters. Lastly, ethanol was evaporated from the combined extracts with a rotary evaporator (Büchi R-210, Flawil, Switzerland), and the aqueous phase was lyophilized to afford a dried extract (FreeZone 4.5, Labconco, Kansas City, MO, USA). The lyophilized hydroethanolic extracts were redissolved to perform the bioactivity assays.

Antioxidant activity

TBARS assay. Porcine (*Sus domesticus*) brains were dissected and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mmol L^{-1}, pH 7.4) to obtain a 1:2 (w/v) brain tissue homogenate that was centrifuged (3000 × *g*, 10 min). An aliquot of the supernatant was incubated with the extracts at diverse concentrations with $FeSO_4$ (10 µmol L^{-1}; 0.1 mL) and ascorbic acid (0.1 mmol L^{-1}M; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by adding trichloroacetic acid (28% w/v, 0.5 mL) and thiobarbituric acid (2% w/v, 0.38 mL), heated at 80 °C for 20 min. After centrifugation (3000 × *g*, 10 min), the supernatant was measured by its absorbance at 532 nm. The results were expressed as EC_{50} values (µg mL^{-1}), calculated by interpolation from the TBARS formation inhibition percentage graph *versus* sample concentration.

OxHLIA assay. An erythrocyte solution (2.8% v/v; 200 µL) prepared in PBS was mixed with extract solution (0.03–1 mg mL^{-1} in PBS); PBS (negative control); distilled water (baseline); or Trolox (positive control; 7.81–250 µg mL^{-1} in PBS). Following pre-incubation (at 37 °C, 10 min) with shaking, 200 µL of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH; 160 mmol L^{-1} in PBS) was added, and the optical density was measured at 690 nm (ELx800 microplate reader, Bio-Tek Instruments, Winooski, VT, USA) over time until complete hemolysis. IC_{50} values (µg mL^{-1}) for a Δt of 60 min were obtained by comparing the extract concentration to the Δt values (min), which resulted from the half hemolysis time (Ht_{50} values) obtained from the hemolytic curves of each extract concentration minus the Ht_{50} value of the negative control.

Anti-inflammatory activity

Dexamethasone (50 µmol L^{-1}) was used as a positive control. The mouse macrophage-like cell line RAW 264.7 (Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures GmbH), stimulated with lipopolysaccharide (LPS), was used in the assay. The nitric oxide (NO) produced was determined by reading absorbances at 540 nm (Bio-Tek Instruments, Inc., Winooski, VT, USA), and the quantification was done using a standard calibration curve. Results were expressed as IC_{50} values (µg mL^{-1}) equivalent to the sample concentration corresponding to 50% inhibition of NO production.

Antimicrobial activity

The antimicrobial potential of the extracts was evaluated against a panel of different pathogenic bacteria and fungi.

Antibacterial activity. The extracts were tested on five Gram-negative and three Gram-positive bacterial strains frequently associated with foodborne diseases, namely *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 9027) and *Yersinia enterocolitica* (ATCC 8610) (Gram-negative bacteria) and *Listeria monocytogenes* (ATCC 19111), *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC 25923) (Gram-positive bacteria). All these microorganisms were purchased at Frilabo, Porto, Portugal. Regarding the clinical bacterial strains, these were clinical isolates from the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). The strains included five Gram-negative bacteria: *Escherichia coli* (VRU12881), *Proteus mirabilis* (VRU17684), *Klebsiella pneumoniae* (VRI17214), *Pseudomonas aeruginosa* (VRU14123) and *Morganella morganii* (VRU14272); and three Gram-positive bacteria: *Enterococcus faecalis* (VRU14041), *Listeria monocytogenes* (VRU17684) and methicillin-resistant *Staphylococcus aureus* (MRSA) (VRI17654). The results acquired by the microdilution method were expressed as MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration).

Antifungal activity. *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404) strains (Frilabo, Porto, Portugal) were utilized. Extract samples were prepared by dissolving them in a mixture of 5% (v/v) dimethylsulfoxide and 95% autoclaved distilled water to achieve a stock concentration of 20 mg mL^{-1}. Subsequently, 90 µL of this solution was added to the microplate in duplicate, along with 100 µL of malt extract broth (MEB). MIC determinations were conducted using the microdilution technique, with the smallest concentration showing no visible growth defined as the MIC. Minimum fungicidal concentration (MFC) was determined by subculturing 2 µL of the extract in MEB for 72 h at 26 °C, with the smallest concentration showing no visible growth considered the MFC, indicating 99.5% killing of the original inoculum. The commercial fungicide ketoconazole was used as the positive control.

Statistical analysis

To compare the influence of the different biostimulants applied, a one-way analysis of variance was performed after preliminary

assumptions were confirmed: homoscedasticity of variances (Levene's test), normality of distributions (Shapiro–Wilk test) and the existence of statistically significant differences (Welch's statistics). When statistical significance differences were recognized, the dependent variable was compared using Tukey's honestly significant difference (HSD) or the Games–Howell test when homoscedasticity was verified or not, respectively.

The Statistical Package for the Social Sciences (SPSS) version 24 (IBM *et al*., USA) was used. All results were expressed as mean value ± standard deviation, maintaining the decimal places allowed by the magnitude of the standard deviation. All tests were performed with a 5% significance level, three samples were mixed to have a representative pool and all tests were performed in triplicate.

RESULTS AND DISCUSSION

This preliminary study evaluated potential changes in the expression of different nutrients and bioactive compounds in walnuts due to the biostimulant foliar treatment applied to this crop. Other factors such as crop location, edaphoclimatic conditions, irrigation system or tree density can also influence walnut chemical profiles and should be considered for complementary studies.

Centesimal composition

The centesimal composition of Franquette walnuts submitted to different treatments is presented in Table 1. Fat was the prevalent macronutrient, ranging from 56 g (100 g)$^{-1}$ in the Basfoliar® K Premium treatment to 61 g (100 g)$^{-1}$ in the control, without significant differences between the treatments. The TDF ranged from 32.4 g (100 g)$^{-1}$ in both Basfoliar® K Premium and Stimulus™ to 40 g (100 g)$^{-1}$ in the control. The total protein ranged from 13.3 g (100 g)$^{-1}$ in Tradebor® to 14.7 g (100 g)$^{-1}$ in Fitoalgas Green®. Ash content varied from 1.97 to 2.37 g (100 g)$^{-1}$.

The high fat content explained the walnuts' high energy value. Walnut consumption used to be limited because of this. Nevertheless, this perspective has undergone reassessment due to the absence of a resultant net increase in body mass following its consumption.[22] Indeed, moderate walnut consumption is advised since its fat is considered 'healthy' and has cardioprotective properties,[23] among other benefits. Also, walnuts' generous dietary fiber value makes them a 'source of dietary fiber' according to the European Regulation (EC) No. 1924/200633, as their TDF content is higher than 3 g (100 g)$^{-1}$.[24]

Numerous walnuts grown in New Zealand,[25] Portugal[26] and Spain[27] showed a nutritional profile like that observed in this study. Thus, considering the application of different biostimulants and despite some significant differences between treatments, it is possible to declare that using plant biostimulants does not compromise the nutritional composition of walnuts.

Lipophilic constituents

Table 2 summarizes the results obtained for the composition of tocopherols and fatty acids. Data presented show that walnuts are a great source of tocopherols and exhibit three vitamers, the α-, γ- and δ-tocopherol isoforms, with γ-tocopherol being the predominant one. The total tocopherol values varied from 20.9 mg (100 g)$^{-1}$ (Basfoliar® K Premium) to 24.6 mg (100 g)$^{-1}$ (Sprint Plus®). These values are in line with those described by Miraliakbari and Shahidi for walnuts in Canada (14.91–26.72 mg (100 g)$^{-1}$)[28] and by Abdallah *et al*. for six walnut varieties (18.6–43.6 mg (100 g)$^{-1}$).[2] Crews *et al*. reported comparable results, this encompassing the lack of β-tocopherol found in walnuts harvested across various countries.[29] All these works reported γ-tocopherol as the principal tocopherol in walnuts. These outcomes emphasize the slight variability of tocopherol contents in walnuts based on the geographical origin and cultivar. The results of this work show that the different biostimulants had little effect on tocopherol content, except when Sprint Plus® was used, with a slight increase compared to the control. Other studies proved that the use of biostimulants could improve the yield and increase the concentrations of important functional constituents like γ-tocopherol and β-tocopherol.[18,30,31]

Regarding fatty acid composition, no significant alterations in total monounsaturated fatty acids (MUFAs) and PUFAs were observed among the applied biostimulants. Nevertheless, a small decrease in SFA contents was observed in samples submitted to the Basfoliar® K Premium and Tradebor® treatments. According to the literature,[5,26,32,33] the primary unsaturated fatty acids in walnuts are linoleic, α-linolenic and oleic acids, and the primary SFAs are palmitic and stearic acids. The results presented in Table 2 include only the main fatty acids found; although, other fatty acids were identified in trace amounts (>2%): myristic (C14:0), palmitoleic (C16:1), marginal (C17:0), arachidonic (C20:0), eicosenoic (C20:1) and docosanoic (C22:0) acids.

Linoleic acid (C18:2n6) was the most abundant fatty acid, ranging between 58% and 59.6%, followed by oleic (C18:1n9c) and α-linolenic (C18:3n3) acids, with contents of 16.8–15.8% and 13.1–12.1%, respectively. The total of C16:0 and C18:0 was 10.01–9.32% and 2.9–2.67%, respectively. These ranges were similar to those described by other works.[5,29]

Because of these results, walnut is claimed to be a healthy source of lipids for human nutrition since it is rich in MUFA (oleic

Table 1. Centesimal composition (g (100 g)$^{-1}$) and energy value (kcal (100 g)$^{-1}$) of walnuts from trees submitted to foliar treatments with different biostimulants

	Basfoliar® K Premium	Tradebor®	Sprint Plus®	Fitoalgas Green®	Stimulus™	Control
Protein	13.7 ± 0.6^{b}	13.3 ± 0.7^{b}	14.8 ± 0.3^{a}	14.7 ± 0.5^{a}	13.5 ± 0.4^{b}	14.2 ± 0.6^{ab}
Ash	2.0 ± 0.1^{c}	2.4 ± 0.1^{a}	2.1 ± 0.1^{b}	2.37 ± 0.05^{a}	2.1 ± 0.1^{bc}	2.0 ± 0.1^{bc}
Fat	56 ± 2^{a}	57 ± 5^{a}	60 ± 3^{a}	59 ± 4^{a}	58 ± 4^{a}	61 ± 2^{a}
TDF	6.5 ± 0.1^{a}	6.4 ± 0.3^{a}	6.5 ± 0.1^{a}	6.5 ± 0.2^{a}	6.5 ± 0.2^{a}	6.5 ± 0.2^{a}
Carbohydrates	22 ± 2^{a}	21 ± 5^{a}	16 ± 3^{a}	18 ± 4^{a}	20 ± 4^{a}	17 ± 3^{a}
Energy	683 ± 12^{a}	688 ± 23^{a}	705 ± 15^{a}	697 ± 19^{a}	695 ± 19^{a}	708 ± 11^{a}

Results are presented as mean ± standard deviation. Different letters in each column indicate statistically significant differences between samples ($P < 0.05$).

Table 2. Tocopherol (mg (100 g)$^{-1}$) and main fatty acid (relative %) composition of walnuts from trees submitted to foliar treatments with different biostimulants

	Basfoliar® K Premium	Tradebor®	Sprint Plus®	Fitoalgas Green®	Stimulus™	Control
Tocopherols						
α-Tocopherol	0.44 ± 0.02^{ab}	0.30 ± 0.02^{d}	0.47 ± 0.02^{a}	0.43 ± 0.02^{bc}	0.41 ± 0.02^{c}	0.46 ± 0.02^{a}
γ-Tocopherol	19 ± 1^{d}	19 ± 1^{cd}	22 ± 1^{a}	20.8 ± 0.7^{bc}	19.5 ± 0.5^{cd}	21.5 ± 0.7^{ab}
δ-Tocopherol	1.4 ± 0.1^{d}	1.60 ± 0.05^{abc}	1.71 ± 0.07^{a}	1.58 ± 0.04^{bc}	1.49 ± 0.06^{cd}	1.6 ± 0.1^{ab}
Total	21 ± 1^{d}	21 ± 1^{d}	25 ± 1^{a}	23 ± 1^{bc}	21.4 ± 0.6^{cd}	23.6 ± 0.7^{ab}
Fatty acids						
C16:0	9.3 ± 0.2^{b}	9.6 ± 0.2^{ab}	9.5 ± 0.3^{ab}	9.7 ± 0.3^{ab}	9.5 ± 0.1^{ab}	10.0 ± 0.2^{a}
C18:0	2.7 ± 0.1^{a}	2.79 ± 0.04^{a}	2.8 ± 0.2^{a}	2.81 ± 0.06^{a}	2.83 ± 0.05^{a}	2.90 ± 0.03^{a}
C18:1n9c	16.4 ± 0.5^{a}	16.8 ± 0.2^{a}	16 ± 1^{a}	15.9 ± 0.4^{a}	16.6 ± 0.5^{a}	16 ± 1^{a}
C18:2n6c	60 ± 1^{a}	58 ± 1^{a}	59 ± 3^{a}	59.2 ± 0.3^{a}	60 ± 1^{a}	58.1 ± 0.7^{a}
C18:3n3	12.1 ± 0.2^{a}	12.8 ± 0.4^{a}	12.8 ± 0.3^{a}	12.4 ± 0.4^{a}	12.1 ± 0.6^{a}	13.1 ± 0.6^{a}
Fatty acid class						
SFA	12.0 ± 0.3^{ab}	12.4 ± 0.3^{ab}	12.3 ± 0.5^{ab}	12.5 ± 0.3^{ab}	12.3 ± 0.1^{ab}	12.9 ± 0.3^{a}
MUFA	16.4 ± 0.5^{a}	16.8 ± 0.2^{a}	16 ± 1^{a}	15.9 ± 0.4^{a}	16.6 ± 0.5^{a}	16 ± 1^{a}
PUFA	72 ± 1^{a}	70.8 ± 0.3^{a}	72 ± 2^{a}	71.6 ± 0.6^{a}	71.1 ± 0.5^{a}	71 ± 1^{a}
Lipid quality indices						
PUFA/SFA	5.98	5.70	5.85	5.71	5.77	5.51
AI	0.11	0.11	0.11	0.11	0.11	0.12
TI	0.16	0.16	0.16	0.17	0.17	0.17
HI	9.41	9.05	9.16	8.97	9.20	8.67

Results are presented as mean ± standard deviation. Different letters in each line indicate statistically significant differences between samples ($P < 0.05$). nd, not detected; tr, trace amounts.
C16:0, palmitic acid; C18:0, stearic acid; C18:1n9c, oleic acid; C18:2n6c, linoleic acid; C18:3n3, α-linolenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; AI, atherogenicity index; TI, thrombogenicity index; HI, hypocholesterolemic index.

acid) and PUFA (linoleic and α-linoleic acids).[34] These liposoluble compounds have been recognized for their role in preventing dyslipidemia[35] and reducing low-density lipoprotein cholesterol levels.[36] As presented in Table 2, the PUFA/SFA ratio in walnut samples ranged from 5.51 in the control to 5.85 in the Sprint Plus® treated samples, indicating a positive impact on cardiovascular health, with higher ratios suggesting greater benefits. Additionally, the low AI and TI values of walnut samples suggested a potential to inhibit plaque accumulation and reduce cholesterol levels. The high HI values further confirm the cardiovascular benefits of walnuts, with studied samples showing a favorable nutritional quality index. In general, walnut samples treated with biostimulants tended to present better lipid quality, characterized by higher PUFA/SFA and HI values, along with lower AI and TI values, in comparison to the control sample.

Hydrophilic constituents

The studied walnuts contained 8.1 to 10.7 g (100 g)$^{-1}$ of soluble sugars (Table 3). The most abundant sugar was sucrose, and significantly lower concentrations of fructose and glucose were determined. The monosaccharides fructose and glucose were not identified in the samples treated with Basfoliar® K Premium and Sprint Plus®. Nonetheless, the samples treated with Fitoalgas Green® showed a slight rise in fructose and glucose content compared to the control samples. Comparable results were reported by Brufau *et al.* and Wojdyło *et al.*, with sucrose as the primary sugar.[37,38] It is well known that the low quantity of sugars in walnuts and the high protein content make them a suitable food for inclusion in healthy diets.[39] The treatments applied in this study did not alter this tendency.

Concerning the organic acids (Table 3), oxalic acid, quinic acid, malic acid, succinic acid and trace amounts of fumaric acid were detected. The most considerable quantities recorded of succinic and quinic acids were in samples treated with Tradebor® and Sprint Plus®, with 1858 and 1859 mg (100 g)$^{-1}$ of total organic acids. A study by Erdoğan *et al.*, considering different genotypes of Turkish *Juglans regia*, also revealed that the most prevalent organic acids in walnuts were succinic, malic and oxalic acids. However, wide fluctuations were recorded in the profiles according to the genotype variety.[40] When the organic acid content is related to the biostimulant used in the production of walnuts, it is possible to conclude that all the samples showed higher concentrations of organic acids than the control. Given this, the use of biostimulants intricately intertwines with mechanisms enhancing tolerance to biotic and abiotic stress, improving quality characteristics and increasing nutrient availability, making them a valuable alternative to fertilizers. Moreover, they bolster plant metabolic processes and associated microbiota, aiding in nutrient acquisition and overall plant health. However, their impact on the production of biologically active compounds introduces complexity, as stimulating such compounds may indicate stress in crops but also offer nutritional and bioactive advantages. Hence, if the crop integrity is maintained, it is interesting to stimulate the production of desired compounds.

Bioactivity properties

It is acknowledged that the primary aim of employing biostimulants is to foster a more sustainable and environmentally friendly production system, devoid of risks to human health and with minimal alteration to crops' nutritional attributes. Therefore, a critical aspect entails analyzing the nutritional and chemical

Table 3. Soluble sugars (g (100 g)$^{-1}$) and organic acids (mg (100 g)$^{-1}$) composition of walnuts from trees submitted to foliar treatments with different biostimulants

	Basfoliar® K Premium	Tradebor®	Sprint Plus®	Fitoalgas Green®	Stimulus™	Control
Soluble sugars						
Fructose	nd	0.25 ± 0.01^{b}	nd	0.44 ± 0.02^{a}	0.14 ± 0.01^{c}	0.14 ± 0.01^{c}
Glucose	nd	0.31 ± 0.01^{b}	nd	0.57 ± 0.01^{a}	0.19 ± 0.01^{c}	0.15 ± 0.01^{d}
Sucrose	8.1 ± 0.3^{c}	8.7 ± 0.5^{b}	9.3 ± 0.3^{b}	8.9 ± 0.2^{b}	8.8 ± 0.2^{b}	10.4 ± 0.4^{a}
Total	8.1 ± 0.3^{d}	9.3 ± 0.5^{c}	9.3 ± 0.3^{c}	9.9 ± 0.2^{b}	9.1 ± 0.2^{c}	10.7 ± 0.4^{a}
Organic acids						
Oxalic acid	43 ± 4^{b}	32.3 ± 0.2^{c}	30 ± 1^{cd}	57 ± 5^{a}	24 ± 2^{d}	9.2 ± 0.5^{e}
Quinic acid	623 ± 50^{a}	599 ± 53^{a}	590 ± 28^{a}	631 ± 26^{a}	611 ± 46^{a}	623 ± 41^{a}
Malic acid	216 ± 6^{bc}	187 ± 12^{cd}	261 ± 15^{a}	176 ± 8^{d}	249 ± 10^{a}	244 ± 11^{ab}
Succinic acid	954 ± 30^{ab}	1040 ± 75^{a}	977 ± 78^{a}	808 ± 44^{bc}	948 ± 45^{ab}	705 ± 49^{c}
Fumaric acid	tr	tr	tr	tr	tr	tr
Total	1835 ± 40^{a}	1858 ± 85^{a}	1859 ± 66^{a}	1673 ± 40^{ab}	1832 ± 78^{a}	1582 ± 92^{b}

Results are presented as mean ± standard deviation. Different letters in each line indicate statistically significant differences between samples ($P < 0.05$). nd, not detected; tr, trace amounts.

characteristics. Additionally, to enrich the investigation, the bioactive properties of the samples were assessed to elucidate the impact of biostimulants on these parameters.

Antioxidant activity

The *in vitro* antioxidant potential of the walnut sample extracts is presented in Table 4. Control sample showed the smallest EC_{50} value for TBARS (10.7 ± 0.6 μg mL^{-1}), followed by samples treated with Stimulus™ (11.3 ± 0.6 μg mL^{-1}). To the best of the authors' knowledge, there are no results concerning the antioxidant potential of the walnut kernel using this kind of assay. Santos *et al*. reported the antioxidant activity for lipid peroxidation inhibition of walnut leaves, presenting an EC_{50} of 20.4 ± 0.8 μg mL^{-1}.[41] Moreover, Vieira *et al*. also studied walnut leaves and obtained an EC_{50} of 26.8 ± 0.2 μg mL^{-1}.[42]

In the OxHLIA assay, the inhibition of erythrocyte membrane damage stimulated by the free radical generator AAPH and other subsequent radicals produced in the system was achieved with an IC_{50} of 18 ± 1 μg mL^{-1}, with samples treated with both Fitoalgas Green® and control. It should be highlighted that this concentration was lower than that of Trolox (21.8 ± 0.3 μg mL^{-1}), which is a commercial antioxidant compound well known for its high capacity to neutralize free radicals. The anti-hemolytic activity of walnut leaves was evaluated by Carvalho *et al*., who achieved IC_{50} values of 60 μg mL^{-1} after 3 h of reaction,[43] and by Vieira *et al*., who reported an IC_{50} value of 32 μg mL^{-1}.[42]

The IC_{50}/EC_{50} values of Trolox (21.8 ± 0.3 and 5.4 ± 0.6 μg mL^{-1} for OxHLIA and TBARS assays, respectively) were in most cases lower than those obtained with the walnut extracts, indicating that Trolox displays higher anti-peroxidation and anti-hemolytic potential than the natural extracts, as was somewhat expected. Nonetheless, Trolox is a pure antioxidant, while the extracts are multifaceted mixtures of numerous compounds with diverse bioactive mechanisms of action. In general, walnut extracts showed lower EC_{50} values for the TBARS assay compared with OxHLIA. In fact, the IC_{50} values of the extracts are 0.8–2.9 times higher than that of Trolox ([sample]/[Trolox]) in the OxHLIA assay, compared with the 1.9–5.4 times difference found for the TBARS assay. Upon examination of the outcomes in the context of the investigated biostimulants, the samples treated with Stimulus™ exhibited superior antioxidant capacity, closely resembling the control sample, which was particularly evident in the TBARS assay. On the other hand, in the OxHLIA assay, samples treated with Fitoalgas Green® demonstrated the most promising antioxidant results.

Table 4. Antioxidant activity via TBARS formation and oxidative hemolysis (OxHLIA) inhibition of walnuts from trees submitted to leaf treatments with different biostimulants

Sample/treatment	TBARS (EC_{50}, μg mL^{-1})	OxHLIA (IC_{50}, μg mL^{-1})
Basfoliar® K Premium	21 ± 1^{b}	35 ± 4^{b}
Tradebor®	30 ± 2^{a}	63 ± 3^{a}
Sprint Plus®	14.3 ± 0.5^{c}	35 ± 4^{b}
Fitoalgas Green®	26 ± 2^{a}	18 ± 2^{c}
Stimulus™	11.3 ± 0.6^{c}	64 ± 2^{a}
Control	10.7 ± 0.6^{c}	18 ± 1^{c}
Trolox	5.4 ± 0.3^{d}	21.8 ± 0.3^{c}

Results are presented as mean ± standard deviation. Different letters in each column indicate statistically significant differences between the samples ($P < 0.05$). Lower EC_{50} and IC_{50} values indicate greater antioxidant capacity.

Anti-inflammatory activity

The walnut samples, tested up to a maximum concentration of 400 μg mL^{-1}, did not show anti-inflammatory activity with the methodology used. Fizeşan *et al*. investigated a bioactive compound-rich *Juglans regia* L. extract and reported its antioxidant and anti-inflammatory effects through biochemical assays and histopathological analysis.[44] Moreover, Ryu *et al*. investigated the anti-inflammatory reaction of LPS-activated macrophages (RAW 264.7 murine cell line) to a *J. regia*-containing extract. Their results revealed no significant anti-inflammatory response at concentrations up to 400 μg mL^{-1}. However, cell survival markedly decreased at 800 μg mL^{-1} ($P < 0.001$). Additionally, exposure to the extract (10–800 μg mL^{-1}) did not notably reduce NO

Table 5. Antibacterial activity of walnuts from trees submitted to foliar treatments with different biostimulants

Foodborne bacteria	Basfoliar® K Premium MIC	Tradebor® MIC	Sprint Plus® MIC	Fitoalgas Green® MIC	Stimulus™ MIC	Control MIC	Streptomycin MIC	Streptomycin MBC	Methicillin MIC	Methicillin MBC	Ampicillin MIC	Ampicillin MBC
Enterobacter cloacae	2.5	2.5	2.5	2.5	2.5	2.5	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	5	5	5	10	10	10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterocolitica	2.5	2.5	2.5	2.5	5	5	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	1.25	1.25	0.6	0.3	2.5	2.5	0.007	0.007	n.t.	n.t.	0.15	0.15
Bacillus cereus	5	2.5	10	10	10	10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	5	2.5	2.5	2.5	5	5	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	0.6	0.3	0.6	0.6	1.25	0.6	0.007	0.007	0.007	0.007	0.15	0.15

Clinical isolates	MIC	MIC	MIC	MIC	MIC	MIC	Ampicillin MBC	Ampicillin MBC	Imipenem MIC	Imipenem MBC	Vancomycin MIC	Vancomycin MBC
Escherichia coli	2.5	2.5	2.5	1.25	1.25	1.25	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	10	10	10	10	10	10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	5	5	5	5	10	10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	5	5	2.5	5	5	5	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Enterococcus faecalis	5	5	5	10	5	5	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	5	5	10	5	10	10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	2.5	5	10	5	5	2.5	<0.15	<0.15	n.t.	n.t.	0.25	0.5

MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; n.t., not tested. All walnut extracts showed MBC values greater than 10 mg mL^{-1}.

production in LPS-activated RAW 264.7 cells associated with the control group.[45]

Besrour *et al.* explored walnut leaves and proved that a concentration of 109 ± 5 μg mL^{-1} inhibited half of the NO produced by macrophage cells (RAW 264.7).[46] Yan *et al.* also reported an EC_{50} value of 319 μg mL^{-1} for a hydroethanolic extract of *J. regia* leaves.[47] Moreover, other studies have shown that different parts of *J. regia*, such as its aqueous seed extract[48] and hydroethanolic husk extract,[49] present anti-inflammatory properties in different *in vitro* and *in vivo* assays.

Antimicrobial activity

The antibacterial properties of the samples are presented in Table 5. Regarding food bacteria, it is possible to conclude that the samples treated with Fitoalgas Green® displayed the best ability to inhibit the development of *Y. enterocolitica*, followed by the extract treated with Sprint Plus®, both with MIC values of 0.3 and 0.6 mg mL^{-1}. Subsequently, all the extracts, except the Stimulus™ one, showed better results in inhibiting the growth of *S. enterica* and *L. monocytogenes* than the control, with MIC equal to 2.5 mg mL^{-1}. In all cases, the MBC was higher than 10 mg mL^{-1}. Concerning the clinical bacteria, the extracts treated with biostimulants presented similar results to those of the control, standing out that the extract treated with Sprint Plus® could inhibit the growth of *Proteus mirabilis* with a MIC value of 2.5 mg mL^{-1}.

Overall, the walnuts treated with Fitoalgas Green® showed better bacteriostatic activity against *Y. enterocolitica*, *S. aureus* and *E. coli*. The foodborne bacterium *P. aeruginosa* was more resistant to the tested walnut extracts (MIC and MBC >10 mg mL^{-1}). Also, all samples demonstrated high values against *S. aureus* and these results agree with the literature.[50] The antibacterial capacity of Portuguese walnuts was earlier evaluated by Oliveira and coworkers, who tested various *J. regia* cultivars *versus* different Gram-negative and Gram-positive bacteria. The samples showed positive results inhibiting the growth of *S. aureus*, *B. cereus* and *P. aeruginosa*.[50] On the other hand, Sharma *et al.* estimated the antibacterial capacity of walnut husk extracts made with different solvents. Despite using a different methodology, the authors concluded that ethanolic extract usually showed a higher inhibition diameter against the studied bacteria (*E. coli*, *K. pneumoniae* and *S. aureus*).[51] Vieira *et al.* also studied hydroethanolic extract of walnut husk and got the lowest MIC (higher antibacterial potential) for the MRSA strain.[49] To the extent of our understanding, no studies were found evaluating the antimicrobial potential of walnuts treated with biostimulants, indicating a novel aspect of research in this area.

The results regarding the antifungal capacity of the walnut samples were not extraordinary. Although practically all samples inhibited the fungal growth of the examined pathogenic fungal species (*A. brasiliensis* and *A. fumigatus*), the MIC was 10 mg mL^{-1} (the maximum concentration tested). However, these data show that applying biostimulants does not induce the production of specific antifungal agents, as the same results were verified for control and treatment samples, which validates their use regarding this parameter.

Ara *et al.* studied the antifungal potential of *J. regia* leaves. They reported the significant antifungal activity of walnut leaves against *C. glabrata*, *C. albicans* and *C. tropicalis*.[52] Bennacer *et al.* also investigated the antifungal activity of *J. regia* leaves cultivated in Algeria and the extract presented higher antifungal potential on *A. terreus*, *A. ochraceus* and *A. brasilliensis*.[53]

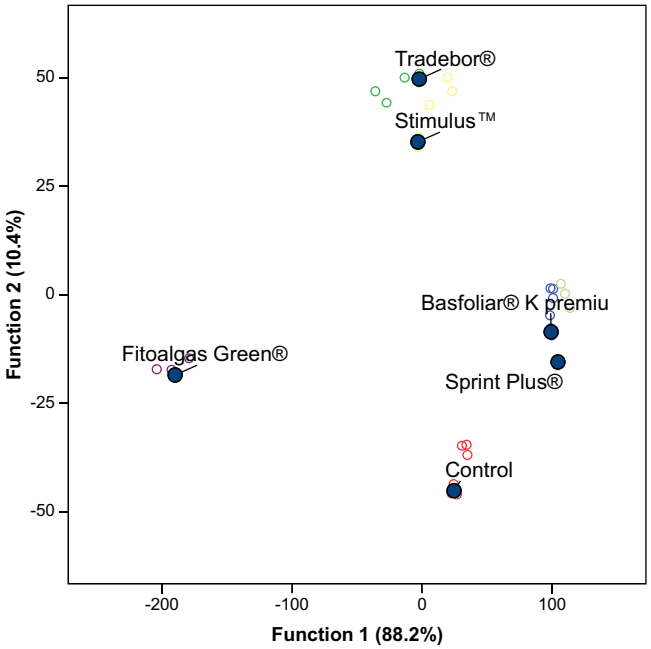

Figure 1. Spatial distribution of markers set by the canonical discriminant function coefficients.

Overall effect on walnut quality

A linear discriminant analysis (LDA) was conducted to evaluate the impact of the applied biostimulants on the global quality of walnuts, while simultaneously considering all the parameters discussed above. High percentages of correct classification were obtained after the use of stepwise LDA. The selected variables were glucose, sucrose, γ-tocopherol, α-tocopherol, malic acid, oxalic acid, OxHLIA and TBARS. The model defined four discriminant functions. The first function accounted for 88.2% of the total variance and separated the samples in the biplot based on glucose, oxalic acid and malic acid levels. Meanwhile, the second function accounted for 10.4%, with separation mainly attributed to OxHLIA (Fig. 1). Consequently, the biplot effectively distinguished Fitoalgas Green® treated samples, exhibiting higher levels of glucose and oxalic acid and lower levels of malic acid, along with reduced TBARS activity. Control samples stood out for their elevated γ-tocopherol levels and increased TBARS and OxHLIA activity. In contrast, samples treated with Tradebor® and Stimulus™ displayed lower bioactivity and levels of α-tocopherol and oxalic acid. Sprint Plus® treated samples showcased more malic acid and α- and γ-tocopherols, with no detectable glucose, thus presenting a somewhat interesting nutritional composition.

CONCLUSIONS

Biostimulants have emerged as pivotal components in modern agriculture, offering a multifaceted solution to address pressing demands for alternative products while mitigating ecological concerns. In this study, the most notable positive effects on walnut composition were obtained with the Sprint Plus® treatment, which effectively improved the nutritional profile, translated into higher levels of malic acid and α- and γ-tocopherols and the absence of glucose. These results were valuable in helping select the optimal biostimulant to be used in walnut production. This study also contributes to increasing the comprehension of walnut composition and cultivation practices since, to the best of the authors' knowledge, it was the first time that this set of experiments was implemented. Finally, judicious application and further research are necessary to optimize the utilization of biostimulants in agriculture, ensuring sustainable intensification while minimizing environmental impact.

ACKNOWLEDGEMENTS

This work was supported by national funds through FCT/MCTES (PIDDAC): CIMO, UIDB/00690/2020 (DOI: 10.54499/UIDB/00690/2020) and UIDP/00690/2020 (DOI: 10.54499/UIDP/00690/2020); and SusTEC, L.A. Pascoalino/0007/2020 (DOI:10.54499/LA/P/0007/2020). The authors also thank the National funding by FCT – Foundation for Science and Technology, through the institutional scientific employment program, contract with L.Barros, and the individual scientific employment program, contract with JP (DOI: 10.54499/CEECIND/01011/2018/CP1578/CT0002), F.S. Reis (2021.03728.CEECIND) and J.C.M. Barreira (CEECIND/04479/2017). LAP thanks FCT for her individual research grant (UI/BD/153745/2022).

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

REFERENCES

1 FAO, Food and Agriculture Organization (FAO) [Internet] Available: https://www.fao.org/faostat/en/#data/QCL [6 May 2024].
2 Abdallah IB, Tlili N, Martinez-Force E, Rubio AGP, Perez-Camino MC, Albouchi A *et al.*, Content of carotenoids, tocopherols, sterols, triterpenic and aliphatic alcohols, and volatile compounds in six walnuts (*Juglans regia* L.) varieties. *Food Chem* **173**:972–978 (2015).
3 Bouabdallah I, Bouali I, Martinez-Force E, Albouchi A, Perez Camino MC and Boukhchina S, Composition of fatty acids, triacylglycerols and polar compounds of different walnut varieties (*Juglans regia* L.) from Tunisia. *Nat Prod Res* **28**:1826–1833 (2014).
4 Ni ZJ, Zhang YG, Chen SX, Thakur K, Wang S, Zhang JG *et al.*, Exploration of walnut components and their association with health effects. *Crit Rev Food Sci Nutr* **62**:5113–5129 (2022).
5 Verardo V, Riciputi Y, Sorrenti G, Ornaghi P, Marangoni B and Caboni MF, Effect of nitrogen fertilisation rates on the content of fatty acids, sterols, tocopherols and phenolic compounds, and on the oxidative stability of walnuts. *LWT Food Sci Technol* **50**:732–738 (2013).
6 Bhagat Singh Jaiswal MT, *Juglans regia*: A Review of Its Traditional Uses Phytochemistry and Pharmacology (2017). Available: https://zenodo.org/record/1036380 [14 March 2024].
7 Jahanban-Esfahlan A, Ostadrahimi A, Tabibiazar M, Amarowicz R, Pellati F, Mercolini L *et al.*, 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 (2019).
8 Maestri D, Cittadini MC, Bodoira R and Martínez M, Tree nut oils: chemical profiles, extraction, stability, and quality concerns. *Eur J Lipid Sci Technol* **122**:1900450 (2020).
9 Mandal S, Anand U, López-Bucio J, Radha, Kumar M, Lal MK *et al.*, Biostimulants and environmental stress mitigation in crops: a novel and emerging approach for agricultural sustainability under climate change. *Environ Res* **233**:116357 (2023).
10 du Jardin P, Plant biostimulants: definition, concept, main categories and regulation. *Sci Hortic* **196**:3–14 (2015).

11 Drobek M, Frąc M and Cybulska J, Plant biostimulants: importance of the quality and yield of horticultural crops and the improvement of plant tolerance to abiotic stress – a review. *Agronomy* **9**:335 (2019).
12 European Parliament and the Council of the European Union, Regulation (EU)/Laying down rules on themaking available on the market of EU fertilising products and amending Regulations (EC) No. 1069/2009 and (EC) No. 1107/2009 and repealing Regulation (EC) No. 2003/2003 (2019).
13 Blunden G, Agricultural uses of seaweeds and seaweed extracts (1991).
14 Pascoalino LA, Reis FS, Barros L, Rodrigues MÂ, Correia CM, Vieira AL *et al*., Effect of plant biostimulants on nutritional and chemical profiles of almond and hazelnut. *Appl Sci (Switzerland)* **11**:7778 (2021).
15 Tekaya M, Dahmen S, Ben Mansour M, Ferhout H, Chehab H, Hammami M *et al*., Foliar application of fertilizers and biostimulant has a strong impact on the olive (*Olea europaea*) rhizosphere microbial community profile and the abundance of arbuscular mycorrhizal fungi. *Rhizosphere* **19**:100402 (2021).
16 Toscano Adamo ML, Yonny ME, Villalba GF and Nazareno MA, Natural biostimulants foliar application as sustainable mitigation strategy of drought stress damage on the melon crop (*Cucumis melo* L.). *Sci Hortic* **323**:112471 (2024).
17 Al-Karaki GN and Othman Y, Effect of foliar application of amino acid biostimulants on growth, macronutrient, total phenol contents and antioxidant activity of soilless grown lettuce cultivars. *S Afr J Bot* **154**:225–231 (2023).
18 Roque J, Carvalho A, Rodrigues M, Correia C and Lima-Brito J, Assessing the effect of plant biostimulants and nutrient-rich foliar sprays on walnut nucleolar activity and protein content (*Juglans regia* L.). *Horticulturae* **10**:314 (2024).
19 AOAC, *Official methods of analysis of AOAC International*. Association of Official Analysis Chemists International, Rockville, MD (2016).
20 Pinela J, Fuente BDL, Rodrigues M, Pires TCSP, Mandim F, Almeida A *et al*., Upcycling fish by-products into bioactive fish oil: the suitability of microwave-assisted extraction. *Biomolecules* **13**:1 (2022).
21 Pascoalino LA, Ayuso M, Pereira E, Ferreira E, Ferreira ICFR and Barros L, Physical, nutritional, and chemical profile of innovative bakery products. *Austin J Nutri Food Sci [Internet]* **11**:1172 (2023) Available: https://austinpublishinggroup.com/nutrition-food-sciences/fulltext/ajnfs-v11-id1172.php.
22 Sabaté J, Nut consumption and body weight. *Am J Clin Nutr* **78**:647S–650S (2003).
23 Ros E, Nuts and novel biomarkers of cardiovascular disease. *Am J Clin Nutr [Internet]* **89**:1649S–56S (2009) Available: https://pubmed.ncbi.nlm.nih.gov/19321561/.
24 Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods [Internet]. *Off J Eur Union* L404/09-L404/25 (2006). Available: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1924&from=DE
25 Savage GP, Chemical composition of walnuts (*Juglans regia* L.) grown in New Zealand. *Plant Foods Hum Nutr (Dordrecht, Netherlands)* **56**:75–82 (2001).
26 Amaral JS, Casal S, Pereira JA, Seabra RM and Oliveira BPP, Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Portugal. *J Agric Food Chem* **51**:7698–7702 (2003).
27 Tapia MI, Sánchez-Morgado JR, García-Parra J, Ramírez R, Hernández T and González-Gómez D, Comparative study of the nutritional and bioactive compounds content of four walnut (*Juglans regia* L.) cultivars. *J Food Compos Anal* **31**:232–237 (2013).
28 Miraliakbari H and Shahidi F, Antioxidant activity of minor components of tree nut oils. *Food Chem* **111**:421–427 (2008).
29 Crews C, Hough P, Godward J, Brereton P, Lees M, Guiet S *et al*., Study of the main constituents of some authentic walnut oils. *J Agric Food Chem* **53**:4853–4860 (2005).
30 Distefano M, Steingass CB, Leonardi C, Giuffrida F, Schweiggert R and Mauro RP, Effects of a plant-derived biostimulant application on quality and functional traits of greenhouse cherry tomato cultivars. *Food Res Int* **157**:111218 (2022).
31 Keshavarz K, Vahdati K, Samar M, Azadegan B and Brown PH, Foliar application of zinc and boron improves walnut vegetative and reproductive growth. *HortTechnology* **21**:181–186 (2011).
32 Bada JC, León-Camacho M, Prieto M, Copovi P and Alonso L, Characterization of walnut oils (*Juglans regia* L.) from Asturias, Spain. *J Am Oil Chem Soc* **87**:1469–1474 (2010).
33 Maguire LS, O'Sullivan SM, Galvin K, O'Connor TP and O'Brien NM, Fatty acid profile, tocopherol, squalene and phytosterol content of walnuts, almonds, peanuts, hazelnuts and the macadamia nut. *Int J Food Sci Nutr* **55**:171–178 (2004).
34 Rébufa C, Artaud J and Le Dréau Y, Walnut (*Juglans regia* L.) oil chemical composition depending on variety, locality, extraction process and storage conditions: a comprehensive review. *J Food Compos Anal* **110**:104534 (2022).
35 Alasalvar C, Salvadó JS and Ros E, Bioactives and health benefits of nuts and dried fruits. *Food Chem* **314**:126192 (2020).
36 Coates A, Hill A and Tan S, Nuts and cardiovascular disease prevention. *Curr Atheroscler Rep* **20**:1–9 (2018).
37 Brufau G, Boatella J and Rafecas M, Nuts: source of energy and macronutrients. *Br J Nutr* **96**:S24–S28 (2006).
38 Wojdyło A, Turkiewicz IP, Tkacz K, Nowicka P and Bobak Ł, Nuts as functional foods: variation of nutritional and phytochemical profiles and their in vitro bioactive properties. *Food Chem: X* **15**:100418 (2022).
39 Kim Y, Keogh JB and Clifton PM, Benefits of nut consumption on insulin resistance and cardiovascular risk factors: multiple potential mechanisms of actions. *Nutrients* **9**:1271 (2017).
40 Erdoğan Ü, Argin S, Turan M, Çakmakçı R and Olgun M, Biochemical and bioactive content in fruits of walnut (*Juglans regia* L.) genotypes from Turkey. *Fresen Environ Bull* **30**:6713–6727 (2021).
41 Santos A, Barros L, Calhelha RC, Dueñas M, Carvalho AM, Santos-Buelga C *et al*., Leaves and decoction of *Juglans regia* L.: different performances regarding bioactive compounds and in vitro antioxidant and antitumor effects. *Ind Crops Prod* **51**:430–436 (2013).
42 Vieira V, Pereira C, Pires TCSP, Calhelha RC, Alves MJ, Ferreira O *et al*., Phenolic profile, antioxidant and antibacterial properties of *Juglans regia* L. (walnut) leaves from the northeast of Portugal. *Ind Crops Prod* **134**:347–355 (2019).
43 Carvalho M, Ferreira PJ, Mendes VS, Silva R, Pereira JA, Jerónimo C *et al*., Human cancer cell antiproliferative and antioxidant activities of *Juglans regia* L. *Food Chem Toxicol* **48**:441–447 (2010).
44 Fizeşan I, Rusu ME, Georgiu C, Pop A, Ştefan MG, Muntean DM *et al*., Antitussive, antioxidant, and anti-inflammatory effects of a walnut (*Juglans regia* L.) septum extract rich in bioactive compounds. *Antioxidants.* **10**:119 (2021).
45 Ryu HY, Lee H, Kong HJ and Kang JH, Anti-inflammatory effects of complex extract including Eucommia ulmoides in LPS-induced RAW 264.7 cells. *J Acupunct Res* **36**:256–263 (2019).
46 Besrour N, Oludemi T, Mandim F, Pereira C, Dias MI, Soković M *et al*., Valorization of *Juglans regia* leaves as cosmeceutical ingredients: bioactivity evaluation and final formulation development. *Antioxidants* **11**:677 (2022).
47 Yan M, Chen M, Zhou F, Cai D, Bai H, Wang P *et al*., Separation and analysis of flavonoid chemical constituents in flowers of *Juglans regia* L. by ultra-high-performance liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry. *J Pharm Biomed Anal* **164**:734–741 (2019).
48 Przekora A, Belcarz A, Kowalczyk K, Wójcik M, Wojciechowska K and Ginalska G, UVB protective, anti-aging, and anti-inflammatory properties of aqueous extract of walnut (*Juglans regia* L.) seeds. *Acta Pol Pharm-Drug Res* **75**:1167–1176 (2018).
49 Vieira V, Pereira C, Abreu RMV, Calhelha RC, Alves MJ, Coutinho JAP *et al*., Hydroethanolic extract of *Juglans regia* L. green husks: a source of bioactive phytochemicals. *Food Chem Toxicol* **137**:111189 (2020).
50 Pereira JA, Oliveira I, Sousa A, Valentão P, Andrade PB, Ferreira ICFR *et al*., Phenolic profile in the quality control of walnut (*Juglans regia* L.) leaves. *Food Chem Toxicol* **46**:783–789 (2008).
51 Sharma P, Ravikumar G, Kalaiselvi M, Gomathi D and Uma C, In vitro antibacterial and free radical scavenging activity of green hull of *Juglans regia*. *J Pharm Anal* **3**:298–302 (2013).
52 Ara T, Shafi S, Ghazwani M, Mir JI, Shah AH, Qadri RA *et al*., In vitro potent anticancer, antifungal, and antioxidant efficacy of walnut (*Juglans regia* L.) genotypes. *Agronomy* **13**:1232 (2023).
53 Bennacer A, Sahir-Halouane F, Aitslimane-Aitkaki S, Oukali Z, Oliveira IV, Rahmouni N *et al*., Structural characterization of phytochemical content, antibacterial, and antifungal activities of *Juglans regia* L. leaves cultivated in Algeria. Biocatalysis and agricultural. *Biotechnology* **40**:102304 (2022).