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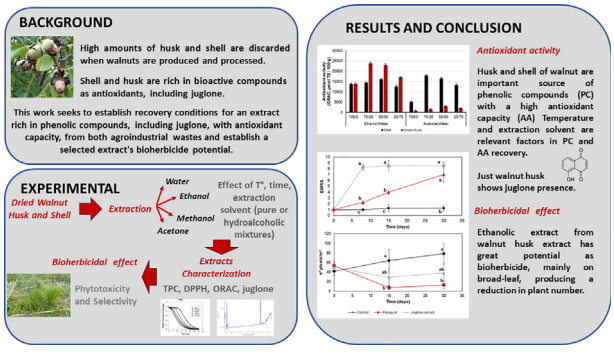
Research Article

Potential of solid wastes from the walnut industry: Extraction conditions to evaluate the antioxidant and bioherbicidal activities

Carmen Soto-Maldonado [a,b,*], Eduardo Caballero-Valdés [a,b], Julissa Santis-Bernal [b], John Jara-Quezada [b], Lida Fuentes-Viveros [a], María Elvira Zúñiga-Hansen [a,b]

[a] Centro Regional de Estudios en Alimentos Saludables, Av. Universidad 330, Curauma-Placilla, Valparaíso, Chile
[b] Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Av. Brasil 2085, Valparaíso, Chile

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Walnut production (*Juglans regia*) generates a large amount of waste, mainly comprised of husk and shell. The two by-products have several bioactive compounds, mainly phenolic compounds with antioxidant activity. Given the above, this work seeks to establish productive and adequate conditions for the recovery of compounds with antioxidant activity (juglone among them) from such discards to use the extract as a bioherbicide.
Results: Temperature and extraction solvent (type of solvent and use of hydroalcoholic mixtures) are relevant factors on phenolic compounds' (TPC, Folin-Ciocalteu method) recovery and antioxidant activity (AA, DPPH method), observing values from 2 to 17 mg GAE/g shell and 0.5 and 23 mg GAE/g husk, and 3 to 28 mg TE/g shell and 0.2–36 mg TE/g husk for TPC and AA, respectively. Using 50°C and ethanol as extraction solvent, 14,000 µmol TE/100 g of ORAC activity was obtained for both shell and husk. This value increased when a hydroalcoholic mixture was used. Juglone was recovered only from the husk at 166 mg/100 g. The bioherbicidal potential of the extract was evaluated; a phytotoxic effect and a lower plantar density when applying the product to broad-leaf weeds were observed.

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* Corresponding author.
E-mail address: carmensoto@creas.cl (C. Soto-Maldonado).

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Conclusions: In conclusion, the walnut process's residues have a high potential to be used under the circular economy concept in the agri-food sector by obtaining products with high added value.

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1. Introduction

Walnut (*Juglans regia*) is one of the most important dried fruits in the world. Every year over 3.5 million tons are produced and commercialised, about 1.5 million tons without the shell. China, USA and Chile are the highest exporters of this product. In the production process, 3 important residues are generated, corresponding to the green husk, the shell and the industrial wastewater from walnut washing, which are currently undervalued and in general, must be disposed of, carrying the associated economic and environmental costs [1].

Over the next 35 years, agriculture will face an unprecedented confluence of pressures, including a 30% increase in the world's population, intensifying competition for increasingly scarce land, water and energy resources. The export markets will demand good agricultural practices, ensuring the correct development of the food generation processes. Due to the above, the revaluation of waste becomes an alternative that improves the sustainability of production processes. In this regard, the search for alternatives used or added value of both – the husk and the shell – has been studied.

The shell is the outer layer (endocarp). Due to its composition [2,3] it has been used as abrasives; as a filter medium, as source of pyroligneous acids (which have antioxidant and antimicrobial activity); in the production of activated carbon and natural fibres; as an alternative substrate for growing plants; as a source of xylose to produce lactic acid; and as in the treatment of malaria [4,5,6,7,8]. On the other hand, considering that the shells have a high content of lignin and that polyphenolic compounds are precursors of this molecule [6], it is suggested that this residue may be a great source of phenolic compounds, recognized for their multiple properties such as antioxidants, antibacterial, and anti-inflammatory [9,10,11,12].

In the case of walnut green husk (exocarp and mesocarp), it is used as a source of nogalina, a colourant used to varnish furniture. However, some studies [13] identified six compounds: ferulic acid, vanillic acid, Coumaric acid, Syringic acid, myricetin and juglone in five species of *Juglans regia.* Other authors report thirteen compounds between phenolic compounds and naphthoquinones [14,15] in the green husk, including chlorogenic, p-coumaric, ellagic, ferulic, gallic, protocatechuic, sinapic, syringic and vanillic acids, (+)-catechin, myricetin, 1,4-naphthoquinone and juglone. It is important to consider that several studies point juglone as the compound that has the biological potential. Juglone (5-hydroxy-1,4-naphthoquinone) is the compound responsible for walnut allelopathy [16,17]. It has been isolated from different walnut family plants (*Juglandaceae*), including *J. nigra* and *J. regia*, being abundant, especially in walnut leaves, shells, and roots. Plants are seen to be affected by the absorption of juglone through its roots, observing that it is toxic to herbaceous and woody plants [18], in addition to inhibiting a variety of microorganisms, including bacteria, algae and fungi [19,20].

Given the information related to both walnut shells and walnut green husks, there are currently a few studies that seek to evaluate the presence of compounds and their activity, mainly antioxidants for walnut shells [6,21,22,23,24]. In general, the literature shows the presence of different compounds in the walnut green husk, and the use of some extracts or specifically of juglone, due to its antiproliferative of cancer cells, antioxidants, or antimicrobial properties [17,20,25,26].

Regarding the extraction processes, for both wastes, studies have been focused on using a single extraction solvent under specific conditions of time and temperature, without establishing which is the best recovery conditions or using pure solvents over mixtures with water, which, as is recognized, allow in many cases, to increase the recovery yields of phenolic compounds [27].

The extraction process is of great relevance, suggesting that extracts containing juglone can be used as biopesticides. The commercialization of organic pesticides of chemical synthesis allows the increase in the crops' productivity, whose losses (without the use of pesticides) were estimated up to 78% [28]. Unfortunately, these are distributed in the ecosystems, generating a significant exposure, through inhalation and/or consumption of food and liquids, and can induce damage to human health due to their persistence and bioaccumulation, and their persistence in fruits and vegetables can generate a food safety problem. Also, alterations in fat metabolism and porphyria, perinatal death and congenital malformations, and prevalence of cancer, among other problems are observed [29]. These facts create the need to search for different alternatives to obtain natural pesticides that can generate an efficient replacement for harmful substances.

The efficient use of walnut industry wastes as a rich source of bioactive compounds will highlight the importance of walnut production and develop new applications for these by-products that are generated in large quantities. Hence, this work aimed to establish the effect of the type of solvent and extraction temperature on antioxidant phenolic content from shell and walnut green husk and the bioherbicidal potential of extracts with highest juglone concentration.

Given the above, interest arises in evaluating the recovery process of polyphenolic compounds and their antioxidant activity from both discards of the walnut processing industry to achieve added-value products and accomplish a degree of sustainability.

2. Materials and methods

2.1. Materials

Walnut shells were obtained from a walnut packaging company, in the city of Limache (33°'1'00 "S 71°16'00 "W; Chile), Valparaíso region. The green husk was obtained from a walnut plantation in the Libertador General Bernardo O'Higgins Region (33°56'00"S 71°50'00"W; Chile). Both samples were of Chandler variety. The walnut green husk samples were lyophilized, and both raw materials packaged and stored under freezing until use. In the case of green husk, samples were obtained when the husk split (mature walnut harvest). Prior experiences indicate that green husk collected 3 d after harvest contain juglone near to cero.

The composition of raw materials in dry basis was of 3.0 ± 0.2 and 18.0 ± 0.5% ash, 9.5 ± 0.4 and 4.2 ± 0.3% of protein, 1.6 ± 0.0 and 2.0 ± 0.1% of lipids, 48.0 ± 1.1 and 21.5 ± 0.6% crude fibre, and 37.9 and 54.3% non-nitrogen extract for shell and green husk,

respectively. The fibre composition shows 14.98 and 6.56% of hemicellulose, 36.98 and 17.35% of cellulose, 30.49 and 11.14% of lignin, and 4.65 and 4.13% of pectin for shell and green husk, respectively.

The extraction solvents (ethanol, methanol, acetone) were of technical grade, while the reagents used in determining the presence of various compounds were of analytical grade (Sigma-Aldrich).

2.2. Extraction process

Both shell and lyophilized walnut green husk were ground using an Ika Basic A11 mill. Extraction was carried out using a solid/solvent ratio of 1/20 g /mL. In a first stage, the process temperature was evaluated (40, 50, 60°C) using commercial ethanol (96%), acetone (99.9%) and methanol (99.8%) as extracting solvents. After that, using the selected temperature, the effect of hydroalcoholic mixtures in the extraction was evaluated. The extracting solvent considers a commercial organic solvent/water ratio of 100/0, 75/25, 50/50, 25/75 and 0/100.

2.3. Determination of total phenolic compounds (TPC) content and antioxidant activity

Total polyphenolic compounds (TPC) and antioxidant activity were determined by the Folin-Ciocalteu method, and DPPH and ORAC methods, respectively, according to Soto-Maldonado et al. [25]. Briefly, for the TPC method, 3.75 mL of water, 0.25 mL two-fold diluted Folin-Ciocalteu reagent, 0.5 mL of phenolic extract and 0.5 mL of 10% sodium carbonate were mixed. The absorbance was measured at 765 nm after 1 h at room temperature. A mixture of water and reagents was used as a blank. Total phenolic content was expressed as mg gallic acid/g raw material (mg GAE/g). For antioxidant capacity determination, DPPH radical scavenging method was applied using 2 mL of 3.6 10^{-5} mol/L of methanolic solution of DPPH (Sigma) and 50 μL. The absorbance at 515 nm was determined at time zero (0) and 16 min using a UV–VIS spectrophotometer, observing the discolouration of the DPPH solution (free radical scavenging). Trolox was used as the standard. The antioxidant capability was expressed as mg Trolox per gram of husk or shell. The ORAC method was applied using 0.2 mL of fluorescein (108 nmol/L in PBS buffer pH 7.4) and 0.02 mL of phenolic extract. The samples were incubated at 37°C for 10 min, and then, 0.075 mL of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) (79.7 mmol/L in PBS buffer pH 7.4) was added. Fluorescence was recorded for 60 min using 485/538 nm excitation/emission wavelengths. Trolox was used as the standard. Results were expressed as μmol TE per 100 g of dry husk or shell.

2.4. Juglone content determination

The juglone content in the walnut green husk extracts was determined by high-performance liquid chromatography (HPLC) using a Photo Diode Array (PDA) according to Soto-Maldonado et al. [25]. The juglone content in the walnut green husk extracts was determined by high-performance liquid chromatography (HPLC) using a Photo Diode Array (PDA) detector in PerkinElmer 200 series equipment. The equipment was controlled using TotalChrome® Chromatography Data System (CDS) software. A Kinetex Evo ® C18 100 A 5 μm (250 mm × 4.6 mm) column was used. The oven temperature was 25°C. A gradient of solvents was used as the mobile phase: solvent A was 2.0% acetic acid in aqueous solution, and solvent B was 0.5% acetic acid in aqueous solution and acetonitrile (1:1 ratio). The gradient used was: 90% A to 40% A (50 min), 45% A to 0% A (10 min), and 0% A to 90% A (5 min). The feed flow was 1.0 mL/min, and the sample volume injected was 20 μL. The total measurement time was 65 min. Juglone detection was done at 280 nm.

2.5. Bioherbicide activity

The potential bioherbicidal effect was studied using a selected extract. For this case, the extract was diluted to 200 mL extract /L solution and was applied twice as a spray using a dose of 80 L/Ha. The dilution was carried out with the purpose of minimizing the effect that ethanol could exert on the plants. On the other hand, the commercial product Nuquat, which has Paraquat in 26.7% as an active compound, was applied using a dose of 4 L/Ha. Also, a control without any treatment was done. In all the cases the wetting was 400 L/Ha. Biomass balance (density of plants), phytotoxicity measurements (8, 15 and 30 d after the first application) and percentage of controlled weeds were measured and calculated. The field trials were carried out in San Felipe, Valparaíso Region (32°44′59.1″ S 70°43′33″ O, Chile). Plant density was 48.1 plant/m^2 and 66.5 plant/m^2 at the beginning of the trial, for broad-leaf and narrow-leaf weed plants, respectively. The data were subjected to Fisher's Test variance according to the experimental design used, and when it gave significant differences, they were compared using the Duncan Test.

Phytotoxicity was evaluated regarding the standards of EWRS (European Weed Research Society), which is related to visual damage of weeds (Table S1).

3. Results and discussion

3.1. Extraction of phenolic compounds from shell and green husk of walnut, and its antioxidant capacity (DPPH method)

Three extraction temperatures and three extracting solvents (methanol, ethanol, acetone), and the effect of the hydroalcoholic mixtures in the process were evaluated.

3.1.1. Walnut shell

Fig. 1 shows the effect of the process temperature on the recovery of phenolic compounds (TPC) and the antioxidant activity. In the first case (TPC), the best results were observed using methanol as extracting solvent; there are no significant differences ($P > 0.1$) in the results obtained at 40°C, regardless of the processing time. In the case of 50°C, it is possible to appreciate that at a time of 6 h there is a greater recovery of phenolic compounds, compared to what is obtained between 0.5 and 3 h of the process ($P < 0.05$). Comparing the best results obtained at 40°C and 50°C, the differences are slight ($P < 0.1$). The best results were observed with ethanol and acetone regarding the antioxidant activity and 50°C. It is interesting to indicate that in the case of TPC, the lowest recovery is obtained when the process occurs at 60°C, this fact suggests the potential degradation of phenolic compounds as an effect of the extraction temperature. On the other hand, the results obtained from TPC do not correlate with the antioxidant activity data; this may be due to the fact that the extraction conditions generate profiles of compounds that have different antioxidant activities.

Then, for evaluation of pure solvent and hydroalcoholic mixtures 50°C is used.

As can be seen in Fig. 2a, when using pure solvents, the best results are obtained with methanol and water with similar values ($P > 0.1$). In the case of ethanol and acetone, the results are much lower ($P < 0.01$). Because dielectric constants of water, methanol, ethanol, and acetone are 82, 33, 24 and 21 (at 25°C), respectively, the results obtained in this work suggest that the phenolic compounds extractable from the walnut shell are polar and that the hydroalcoholic extraction mixtures generate a synergistic effect

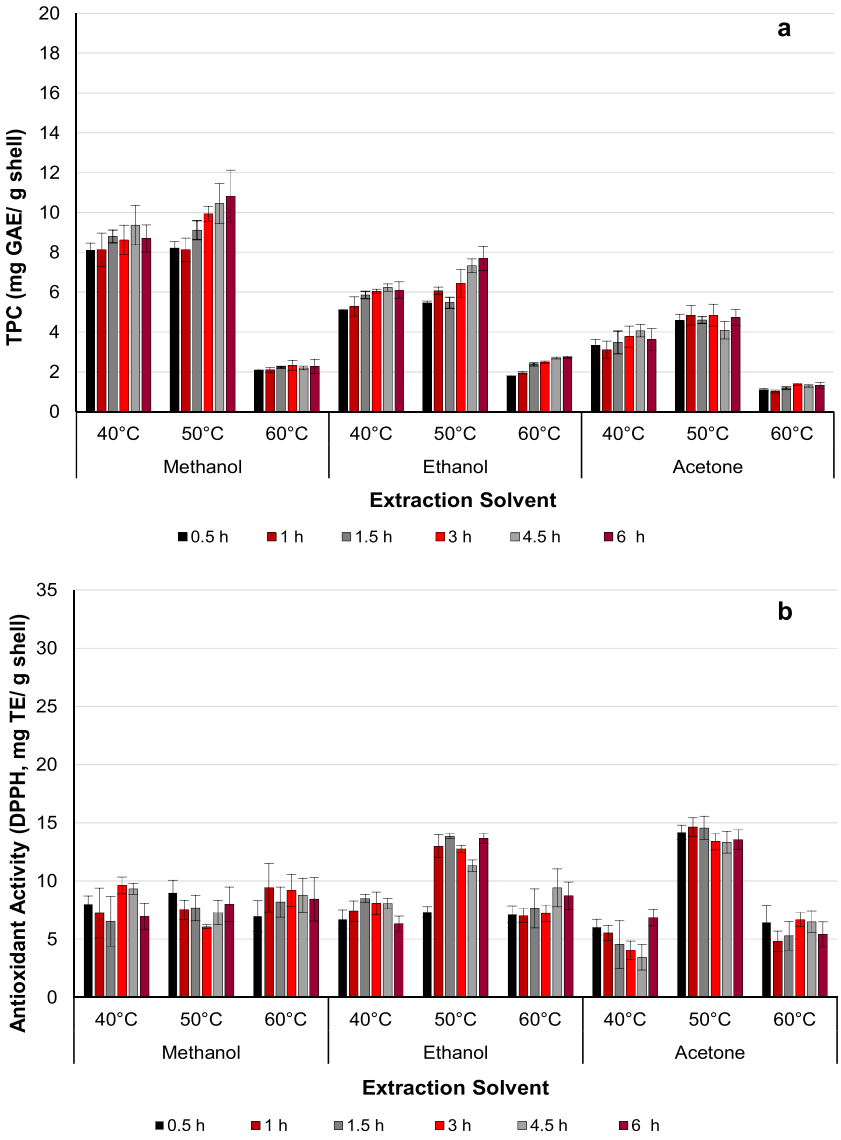

Fig. 1. Effect of temperature and solvent of extraction in (a) TPC and (b) antioxidant recovery from walnut shell. Mean ± SD (n = 3).

in the recovery of phenolic compounds. Various authors have determined the presence of TPC in the walnut shell. In some cases, the analysis was performed directly on the raw material, while in others extracts or infusions were made in which the amount of TPC was quantified. Yang et al. [30] observe similar results for water, methanol and ethanol with a mean value of 25.93 ± 1.89 mg GAE/g extract and values of 179.48 and 200.40 mg GAE/g extract when using n-butanol and ethyl acetate, respectively, while Wang et al. [31] report 14.81 mg GAE/g extract when ethanol was used. Depending on the solvent used, the origin and variety of the raw material and the extraction method, the content of TPC in the walnut shell ranges from less than 1 mg/g shell to 32.76 mg GAE/g shell [6,21,23,24,31,32,33]. When hydroalcoholic mixtures are used, an increase in the recovery of phenolic compounds is observed. For example, in the case of methanol and 6 h of process, an increase from 10.81 ± 1.299 to 16.53 ± 0.88 mg GAE/g shell ($P < 0.01$) was observed when a 50/50 methanol/water ratio was used; similarly, it happens for mixtures with ethanol and acetone where the increase was from 7.69 ± 0.60 to 17.06 ± 0.31 mg GAE/g Shell ($P < 0.01$), and 4.72 ± 0.40 to 16.38 mg GAE/g Shell, respectively ($P < 0.05$). Similarly, it is possible to observe an increase in process time, reaching the highest values between 4.5 and 6 h. This increase in the recovery of TPC from walnut shells, by increasing the presence of water in the extraction solvent, validates the fact that the phenolic compounds present in this raw material are potentially polar. In this regard, the significant increase observed in the case of acetone–water mixtures is interesting. This is consistent with that reported by other authors with various raw materials such as apple [34] and berries [35], for example, who indicate that the best results of TPC and antioxidant capacity recovery are obtained with aqueous mixtures.

Fig. 2b shows similar results of antioxidant activity when hydroalcoholic mixtures are used. Besides, slight differences generated by the extraction time are appreciated. Concerning the different organic solvents used, when using methanol mixed with water, the results do not exceed 21.2 ± 1.65 mg TE/g shell, while, in the case of using acetone mixed with water, values up to 28.74 ± 2.1 6 mg TE/g shell are achieved. Hydroalcoholic mixtures generate higher results in both, TPC and AA determination, but with behaviours depending on the organic solvent used. Regarding the effect of pure solvents, the results suggest that the compounds that possess antioxidant activity are non-polar compounds. However, it is also possible to observe that water incorporation produces an increase in antioxidant activity recovered, probably because the

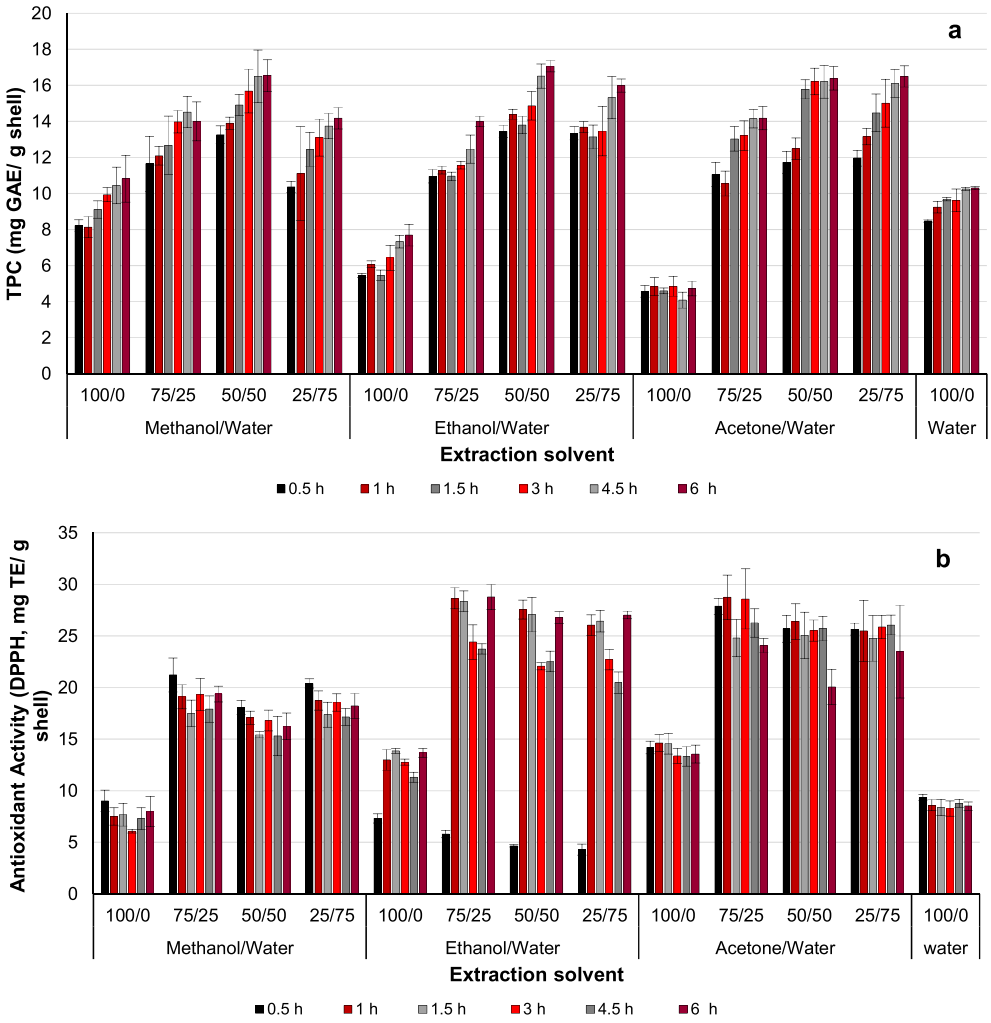

Fig. 2. Effect of solvent extraction on TPC (a) and AA (b) recovery from walnut shell. Extraction conditions: 50°C.Mean ± SD (n = 3).

extraction of polar antioxidant compounds generate a synergistic effect with non-polar compounds.

The use of hydroalcoholic mixtures for the recovery of phenolic compounds is an alternative that has been evaluated by other authors and different raw materials. Such is the case indicated by Laroze et al. [36] using an ethanol/water mixture to recover phenolic compounds with antioxidant activity from raspberry pomace, where the best results of TPC and antioxidant activity (DPPH, ABTS and FRAP), were obtained with a 50/50 or 25/75 mix; validating the results obtained in this work regarding the effect of the use of hydroalcoholic mixtures. Similarly, Cerda et al. [37] report an increase of up to 10 times in the recovery of phenolic compounds and antioxidant activity of an extract obtained from thyme leaves when a methanol/water mixture is used instead of pure solvents. In the same way, Sun et al. [38] show that the use of hydroalcoholic mixtures (ethanol/water) generates a positive effect on the recovery of phenolic compounds, antioxidant activity and even the profile of compounds recovered from propolis, with respect to pure solvents.

On the other hand, for TPC determination the best results are obtained with polar solvents. An explanation of this behaviour is that although the method for determining TPC used is the "gold standard", there are various compounds that can interfere to the extent due to their reducing characteristics. It is also important to mention that the DPPH method is an easy-to-implement measurement method that allows generating comparisons with other authors but has the characteristic of detecting antioxidant activity generated only by the SET mechanism, that is, it stabilizes free radicals through electron donation.

The antioxidant capacity of walnut shells has been very few studied. Only 4 studies report antioxidant activity of peel extracts with the DPPH method, expressing their results as, % inhibition (or free radical scavenging) or as the concentration of the extract that allows reaching 50% inhibition (IC50). Yang et al. [23] report IC50 values between 81 and 263 μg extract/mL depending on the extraction solvent, and a DPPH radical scavenging capacity between 60 and 80% when the extract concentration was 500 μg /mL; similarly, Moghaddam et al. [32] report high variability in the results, with IC50 values between 16 and 7090 μg extract / mL for water and dichloromethane, respectively; while Akbari et al. [21] show 7.19% inhibition and an IC50 of 410 μg/mL using a methanolic extraction. These results cannot be compared with those obtained in this work, which are expressed as Trolox equivalent per g of raw material, considering that it seeks to establish conditions to recover antioxidant compounds, rather than obtaining an extract rich in them.

3.1.2. Walnut green husk

A very marked effect of process temperature is observed when the extraction is done for walnut green husk. The behaviour depends on the organic solvent used in the extraction, and the type of analysis developed. In the case of TPC recovery, Fig. 3a shows

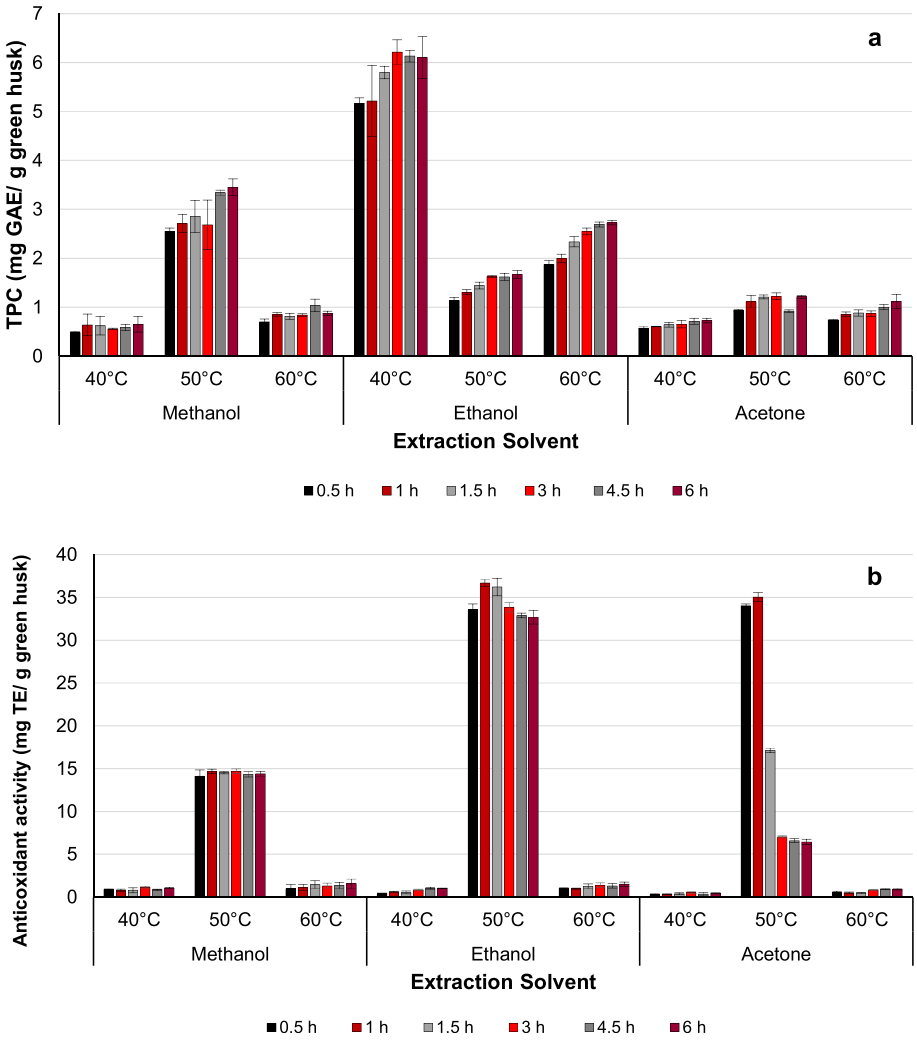

Fig. 3. Effect of temperature and solvent extraction in TPC and antioxidant recovery from walnut green husk. Mean ± SD (n = 3).

that using methanol and acetone the best results are obtained at 50°C, but with values much lower than those observed with ethanol at 40°C (reaching up to 6.1 mg GAE/g green husk). However, when what is sought is to establish the capacity to recover antioxidant compounds, it is possible to observe that the best result is obtained at 50°C for any of the solvents used. This behaviour is probably due to an increase in the compounds' solubility measured by the process temperature, up to a condition where the thermal inactivation (or degradation of these) of phenolic compounds with antioxidant capacity occurs [27]. It is important to mention that walnut green husk samples correspond to those obtained from mature samples, at the time of splitting and harvesting the walnut, so its decomposition can be accelerated. Therefore, the operational conditions of extraction can affect the recovery of the compounds as mentioned above. Samples of the same origin [25] but immature and obtained with ethanol or methanol at 40°C present a TPC content over 20 mg GAE/g green husk, which is higher than that observed in this work. However, the authors do not evaluate the temperature effect on TPC recovery. On the other hand, this work seeks to use the walnut green husk that is generated as a discard in the ripening stage.

Several authors have reported the extraction of phenolic compounds from the walnut green husk, in most cases, the extraction is carried out with solvents under simple maceration or using Soxhlet equipment. In contrast, in others they are helped with ultrasound, microwaves or even supercritical extraction, obtaining mixed results in the recovery of phenolic compounds, antioxidant activity, and the composition of the resulting product [4]. To our knowledge, there is only one study regarding the effect of temperature in the recovery process of phenolic compounds from the walnut green husk. This evaluation was done by Tabaraki and Rastgoo [39] using ethanol as solvent, observing that the optimal temperature for TPC recovery was 48°C, which is in agreement with the results reported in our work. Additionally, Han et al. [33] evaluated the effect of temperature and process time, on tannins' recovery from walnut green husk, using acetone as solvent, and using the Folin-Ciocalteu method for its quantification, which allows estimating a recovery behaviour of total phenolic compounds based on these variables. As previously indicated, the Folin-Ciocalteu method is the gold standard for this type of analysis, although it is not specific as it can quantify different molecules capable of generating an oxide reduction reaction. The results of Han et al. [33] show that the best results are achieved with 40 and 50% acetone, a temperature of 50°C and a processing time of 3 h, managing to recover about 26 mg tannins/g sample. In this work, the improvement was to evaluate the hydroalcoholic solvent effect on TPC and antioxidant activity at 50°C, which was selected.

Among the solvents evaluated by the different authors, methanol is one of the most used [21,22,25,40,41,42] observing results between 15.2 and 108.11 mg GAE/g extract or 17 to 115.29 mg

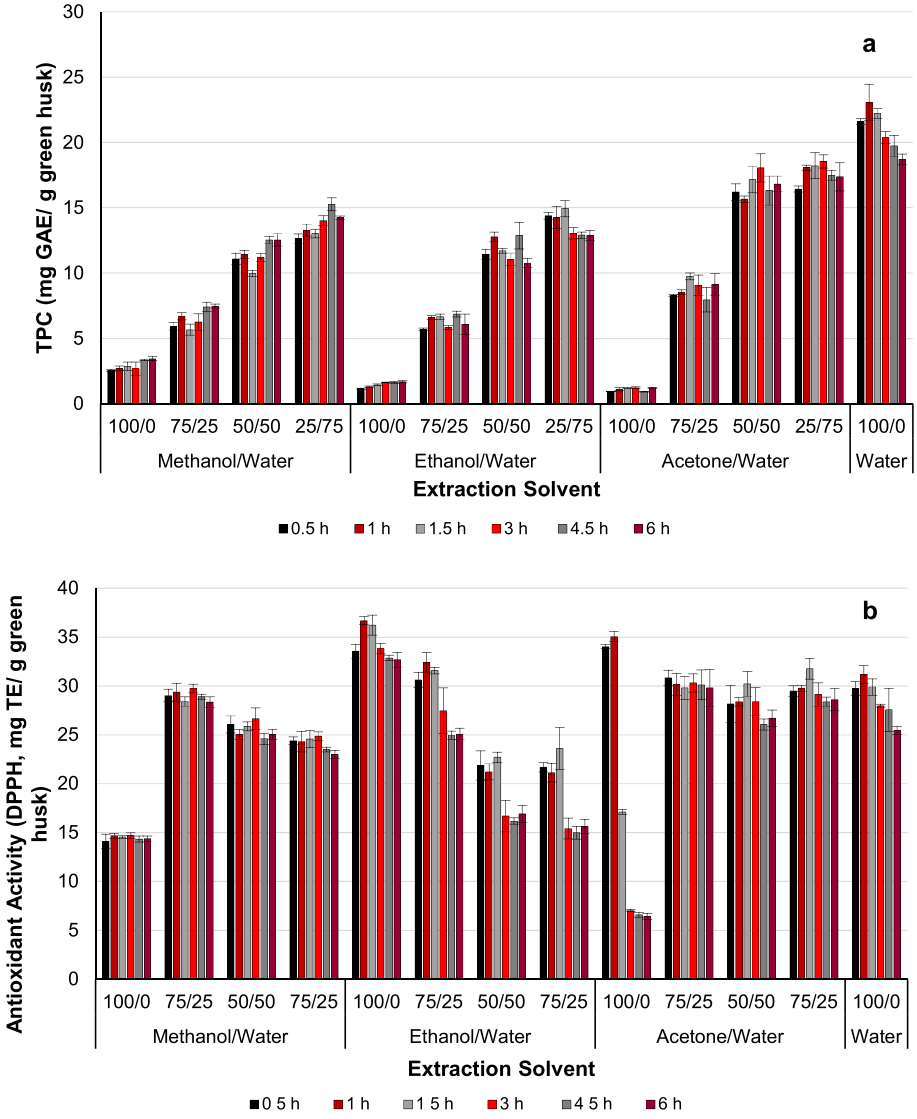

Fig. 4. Effect of solvent extraction on TPC (a) and AA (b) recovery from walnut green husk. Extraction conditions: 50°CMean ± SD (n = 3).

GAE/g green husk, measured by the Folin-Ciocalteu method, or ranges from 3.15 to 15.26 mg/g dry matter of phenolic compounds identified by HPLC [14]. Their variations depend on variety or moment (maturity of the walnut) of obtaining the sample, among other factors. It is important to note that, in most studies, either the variety is not known, or they correspond to cultivars such as Franquette, Vina, Valcon, Valrex, Valnuit, among others. Particularly in this work, the Chandler cultivar was used, which is one of the most cultivated in America, for which 115.3 mg GAE/g green husk is reported when the extraction is carried out with methanol, 60°C using reflux in Soxhlet equipment [42], a value that is much higher than that observed in this work but obtained under different process conditions (Fig. 4a). Other solvents used are ethanol, acetone, and mainly water. In this regard, authors such as Tabaraki and Rastgoo [39] report that the best TPC results in a process using ultrasonic aided extraction, are obtained with methanol, followed by ethanol, selecting the latter for the safety of the solvent. In the case reported by Soto-Madrid et al. [43], when using a mixture of ethanol and water and ultrasound-assisted extraction, they obtained between 31 and 106 mg GAE/g green husk, values that exceed what was observed in this work. Oliveira et al. [19] used boiling water as extracting solvent, obtaining extracts with 32.61 to 74.08 mg GAE/g extract. Yields ranging from 31.63 to 33.69% depending on the variety used, managing to recover between 10.5 and 23.43 mg GAE/g green husk, which agrees with what is presented in this work using simpler technology, are able to be scaled up for less investment.

Authors such as Wenzel et al. [44] use supercritical CO_2 with ethanol at different levels as a co-extractor, observing a variation in the recovery of phenolic compounds between 0.97 and 4.06 mg GAE/g green husk when using the raw material dry. A higher recovery (29.21 mg GAE/g husk) was observed by Romano et al. [45], when the supercritical extraction was carried out at 300 bar, 50°C and a flow of 10 mL/min, using 20% of ethanol as a co-extractor. This type of process has the advantage that supercritical fluids have a high diffusion coefficient and a lower viscosity than liquids, and an absence of surface tension, which promotes a faster extraction of the compounds, both due to their better solubility, as well as easier penetration of the fluid into the pores of the heterogeneous matrix. In addition, the selectivity during the extraction can be manipulated due to the variation in different operating conditions, temperature, and pressure, affecting the solubility of various components in the supercritical fluid, as well as by the incorporation of the co-extractor. Additionally, the previous

results can be improved, as indicated by Wenzel et al. [44], when the raw material is wet, increasing the values from 4.06 to 9.47 mg GAE/g green husk, demonstrating the effect and importance of using hydroalcoholic mixtures even in this type of process.

As shown in Fig. 4, when using hydroalcoholic mixtures, instead of pure organic solvents, there is an increase in TPC recovery and antioxidant activity. For TPC, the best results are obtained by increasing the presence of water in the extraction mixture. As can be seen in Fig. 4, using only water generates the highest recovery of phenolic compounds from the green husk, reaching values of 23 mg GAE/g green husk, while when using organic solvents, only ranges of values between 2.5 and 18 mg GAE/g green husk are reached, the latter case being the result obtained with acetone/water 25/75. The results of this work agree with those reported by Fernández-Agulló et al. [46], who studied the effect of the type of solvent on obtaining phenolic compounds from walnut husk, using methanol, ethanol, water and 50% methanol, and 50% ethanol. The authors observed that hydroalcoholic mixtures allow obtaining extracts rich in phenolic compounds (mean value of 82.98 mg GAE/g extract) over what happens with pure solvents. Also, the recovery of phenolic compounds ranges from 7.4 to 17.8 mg GAE/g green husk when the extraction is carried out with methanol and water, respectively. However, it is necessary to consider two things, no remarkable differences are observed between the results obtained with 50% ethanol and water (mean value of 17.43 mg GAE/g green husk) and, the extraction with water vs the extraction with organic solvents (pure and hydroalcoholic mixtures) are made with different solid/solvent ratios (solid/liquid ratio of 1/50 for water extraction, and a solid/liquid ratio of 1/16,67 for extractions with organic solvents-pure or mixed with water), potentially having a saturation of the extraction solvent in the case of using organic solvents or their mixtures with water, given the lower surface ratio between liquid and solid. This fact is relevant since it has been established that there are several factors that can affect the recovery of phenolic compounds in a solvent extraction process, the solid/liquid ratio being one of them. Authors such as Rajha et al. [47] show that by increasing the level of liquid used for extraction from by-products of the wine industry, the recovery of phenolic compounds decreases; while Elboughdiri [48] shows that there is no significant effect of this variable in extraction from olive leaves; and Wong et al. [49] report that an increase in the extraction solvent generates an increase in the recovery of phenolic compounds and antioxidant activity from *Dukung Anak*, but without being proportional.

Regarding antioxidant activity, the behaviour differs from that observed with TPC. In this case, in general, the best results are seen with the hydroalcoholic mixture that has more organic solvent; even in the case of ethanol, the best results are seen when there is no water in the extraction mixture. This effect may be due primarily to two facts: the compounds with the highest antioxidant activity correspond to non-polar compounds, or that interference is generated in the determination of TPC by other soluble polar compounds. It is interesting to note that Tabaraki and Rastgoo [39] point out that the antioxidant activity of walnut green husk extracts – determined by the DPPH method, decreases according to the following solvents methanol > ethanol > water > acetone. The authors establish the inhibition of the radical DPPH using pure solvents, and with an extraction process that considers 45°C, 30 min, and a solid/liquid ratio of 1/20. In our case, under similar process conditions, and with pure solvents, the best result is seen with acetone > ethanol > water > methanol, showing that the most non-polar compounds are those with the highest antioxidant activity. These differences may also be due to the raw material's variety and origin, not reported by the author.

Because environmentally non-toxic food-grade organic solvents like water and ethanol are recommended by the US Food and Drug Administration for extraction purposes [39], in this work, the use of ethanol and acetone (both solvents are GRAS) is considered.

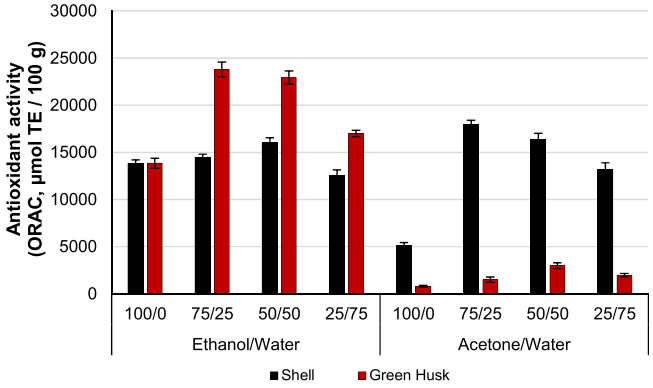

Fig. 5. Effect of solvent extraction on AA recovery from walnut green husk, determined by ORAC method. Extraction conditions: 50°C, time of extraction 6 hours. Mean ± SD (n = 3).

3.2. Determination of antioxidant capacity by ORAC method

When evaluating the antioxidant activity using the ORAC method (Fig. 5), it is possible to see that better results are obtained with ethanol/water mixtures and acetone/water mixtures for green husk and shell, respectively. Additionally, and as has been observed for TPC and the antioxidant activity measured with the DPPH method, the best results are seen with hydroalcoholic mix-

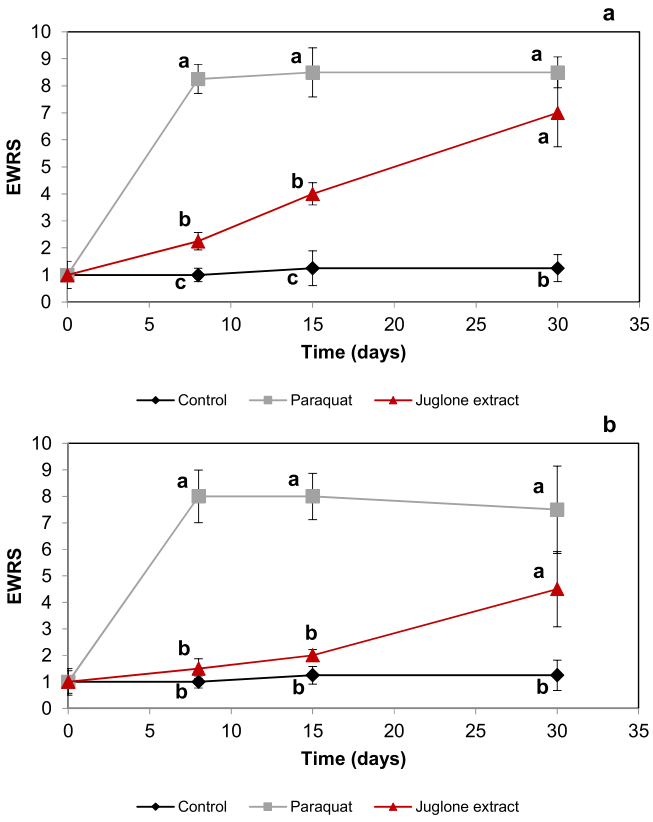

Fig. 6. Damage evolution in broad-leaf weeds (a) and narrow-leaf weeds (b). Phytotoxicity of Paraquat and Juglone extract from green husk walnuts. Each point represents the average among 4 samples (n = 4). Each sample an area of 1 m × 1 m surface. Letters a, b, and c represent the statistically significant difference among treatments, taking into account the Fisher test with 95% confidence.

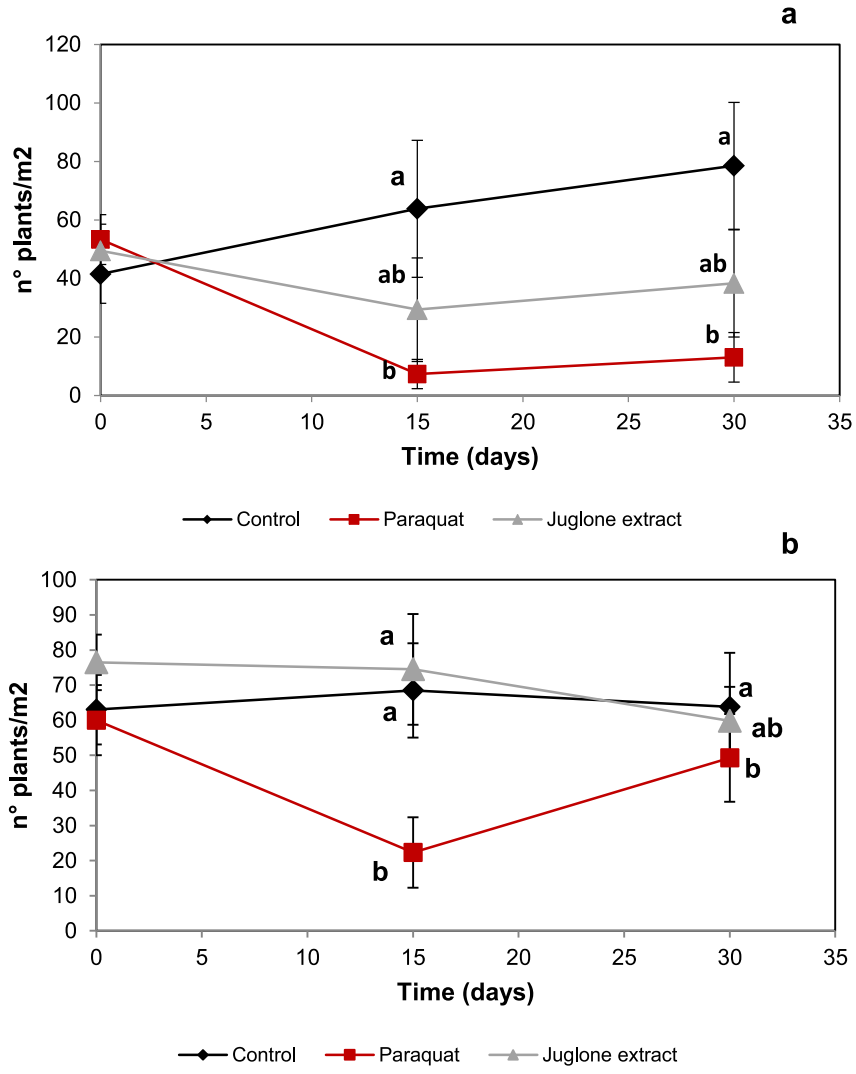

Fig. 7. Data of broad-leaf (a) weed, and narrow-leaf weed (b) density for two treatments and control. Each point represents the average among 4 samples ($n = 4$). Each sample an area of 1 m × 1 m surface. Letters a and b represent the statistically significant difference among treatments, considering the Fisher test with 95% confidence.

tures. It is important to consider that to our knowledge, there are no other authors who have evaluated the antioxidant activity of these residues with the ORAC method. It is interesting to consider the fact that DPPH and ORAC methods are useful for the determination of antioxidant activity of both, hydrophilic and hydrophobic compounds. DPPH technique is related to an electron transfer mechanism in antioxidant capacity, but the ORAC method is related to the hydrogen atom transfer mechanism [50]. The latter, has the advantage of evaluating the antioxidant capacity of substances in samples with and without retardation phases in their antioxidant capacities. This is especially beneficial when measuring complex samples with various slow-acting and fast-acting antioxidants, as well as ingredients with combined effects that cannot be pre-calculated. This explains the potential differences between the behaviours observed with the extraction methods and the antioxidant activity, suggesting differences in the profile of phenolic compounds of the different extracts.

Although hydroalcoholic mixtures promote the recovery of a greater antioxidant capacity from both shell and husk, it is necessary to know if these samples contain the compound of interest for this work, which is juglone. As indicated, juglone is soluble in organic solvents and poorly soluble in water (0.052 g/L according to Weidenhamer et al. [51]); therefore, of the solvents evaluated, ethanol is the one that allows the best levels of recovery of antioxidant activity, and it is the solvent with which the bioherbicide effect was evaluated.

3.3. Determination of juglone content in extracts

Regarding the determination of juglone presence in the samples, using HPLC-DAD analysis, the following is observed, those samples extracted with hydroalcoholic mixtures do not present this compound. Also, the presence of juglone is only observed in samples of walnut husk and not in the walnut shell evaluated in this work. In this regard, it is also important to point out that studies carried out by the authors (data not shown) indicate that juglone content varies notably in the green husk with the maturity of the fruit (89 to 1404 mg/100 d.w.) [14,52]. At the moment of the husk split (full maturity of the fruit, and when the harvest is carried out), the juglone content decreases, as well as it is reported by other authors [14,52], being imperceptible after a few days of the splitting if the raw material is not stabilized (freeze or dehydrated), probably due to the degradation and/or oxidation of this compound [53], potentially giving rise to other molecules that

may also have bioactive capacity, including other compounds with allelopathic potential [54]. The samples evaluated in this work show an average juglone concentration of 166.3 ± 15.2 mg/100 g dry husk when the extraction is carried out with ethanol. This value is within the expected, considering the variability in the information regarding the presence of juglone in different parts of the walnut. For example, walnut kernels (edible fraction of the walnut) show juglone concentrations between 2 and 2400 mg/100 g fresh weight [55,56,57]. In the case of leaves, some authors do not detect the presence of juglone [58,59]. Specifically, in the walnut husk case, the same behaviour can be observed, that is, wide ranges in the quantification of juglone, according to the variety of walnut and time of collection of the husk (stages of development). Samples of walnut husk without juglone [59], as with contents ranging from 20.56 to 305.6 mg/100 g fresh [13,25,52,60], or values from 288 to 1404 mg/100 g of dry husk.

The samples obtained in this study, with ethanol as an organic solvent, and containing juglone, are used to evaluate the bioherbicidal effect.

3.4. Determination of bioherbicidal capacity

The allelopathic behaviour of walnut is recognized, indicating that it is due to juglone in its different parts [18,61]. In this regard, studying the bioherbicidal potential of an extract that contains it, as an alternative to chemical products, is seen as an alternative potential for sustainable development in agro-industry.

The potential of juglone ethanolic extracted from walnut green husks as bioherbicidal (phytotoxicity and selectivity) in comparison with a commercial chemical herbicide (Nuquat) over broad-leaf and narrow-leaf weeds regarding the scale of EWRS, was evaluated. Fig. 6a, shows that commercial herbicide presents a more significant damage in broad-leaf weeds than juglone extract; however, after 30 d, juglone extract increases the damage over broad-leaf weeds. These results allow to work in the potential future optimization of the extract formulation and/or juglone dose. In the case of narrow-leaf weeds, Fig. 6b shows that commercial herbicide presents a grater damage at the beginning (7 d). Even after 30 d, juglone extract just reached a damage of 4.5, which means about 12.5% of plant damage regarding the EWRC score (Table S1). Hence, the data of Fig. 6, show specific selectivity of juglone extracts over broad-leaf weeds.

The biomass balance (density of plants) is presented for broad-leaf weeds and narrow-leaf weeds for all treatments in Fig. 7. As shown in Fig. 7a, for broad-leaf weeds, the plant density decreases with the use of the product containing the extract of the walnut husk, 15 d after the first application, and then increases slightly. This behaviour is also presented when a commercial product is applied. For control, the broad-leaf weeds' density increases with time and is higher than those obtained with any of the treatments. Whereas, in the case of narrow-leaf weeds, there is a strong decrease in density when the commercial product is used 15 d after the first application, the density increases again after 30 d, reaching levels close to the initial ones. When the product obtained from the walnut husk is applied, a slight decrease (20%) in density is observed after 30 d of treatment.

4. Conclusions

The residues of the walnut process, such as the husk and the shell, show a high content of fibre as part of their main components, thus suggesting, in addition, a high presence of phenolic compounds with antioxidant activity, which can be recovered efficiently using ethanol as an organic solvent, managing to establish that the raw materials have an antioxidant activity even higher than the walnut kernel and even some super fruits. This fact confirms that these residues are an important source of biological compounds of commercial interest.

On the other hand, it is possible to conclude that the extraction process is fundamental, noting that the most significant effect occurs with the extraction solvent's composition, which even determines whether a compound of interest as juglone is present in the extract.

Finally, concerning the use of the extract as a bioherbicide, it is possible to conclude that there is greater effectiveness on broad-leaf, especially what is related to reducing the number of plants in the application sector. In this way, it is possible to validate their use, as a first step for the recovery of bioactive compounds with both economic and agro-industrial relevance from the walnut industry.

The study of the characteristics of the raw materials, and the extraction process allows establishing the most suitable conditions to be able to generate a first step in the strategy of comprehensive revaluation of the waste produced in this industry, with a focus on the development of circular economy in the agri-food industry, as a source of compounds of high biological and commercial value.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

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