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Extraction of phenolic compounds from *Juglans regia* L. leaves using aqueous solutions of eutectic solvents

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ABSTRACT

The leaves of *Juglans regia* L. (walnut tree) are a rich source of bioactive phenolic compounds, particularly flavonoids and phenolic acids, which can be used in cosmetic applications due to their antioxidant properties. Conventionally, these compounds are extracted using volatile organic solvents. This study focuses on a more sustainable approach by designing a heat-assisted extraction process using aqueous solutions of eutectic solvents (ES) composed of cosmetic-compatible ingredients aiming for direct incorporation into cream formulations. In this context, aqueous solutions of the ES betaine + urea and betaine + 1,3-propanediol (PPD) were selected, considering their potential as cosmetic ingredients. The screening process targeted the maximum extraction yield of phenolic compounds (phenolic acids and flavonoids), quantified by HPLC-DAD. The selected variables were water content (25, 50 and 75 % in weight) and the molar proportion of the ES components. For comparison purposes, pure water was used as a reference solvent.

The total phenolic content ranged between 5.5 and 14.6 mg/g of dry plant. Betaine:urea (2:1), betaine:PPD (1:2), betaine, and PPD, all with 50 % water mass percentage, were among those resulting in higher extraction yields and were further selected for bioactivity analysis. The system containing betaine presented the best antioxidant capacity, analysed through ferric reducing power, DPPH radical scavenging, and TBARS assays. The extracts obtained with PPD presented the highest antimicrobial activity against gram-positive and gram-negative bacteria, and yeast. The results show the potential of using binary aqueous mixtures of betaine, or 1,3-propanediol, which are simple ingredients in cosmetics formulations, to efficiently extract phenolic compounds from a natural matrix. Moreover, the bioactivity results, particularly in the antioxidant dimension, support using *J. regia* leaves as a source of natural antioxidants.

1. Introduction

Phenolic compounds (PC) are secondary products from natural metabolic processes from plants and fungi, being the largest group of phytochemicals with more than 8000 structures reported so far [1–3]. Due to their bioactivity, these compounds can be applied in the pharmaceutical, food, and cosmetic industries as a natural alternative to synthetic ingredients. They can be extracted from by-products of agri-food waste and increase the valorisation of those processes, avoiding disposal costs and reducing the environmental impact of waste [4–6]. Among phenolic compounds, flavonoids and phenolic acids are used in cosmetic applications mostly due to their antioxidant activities [1,3,7–9]. Usually, the extraction of phenolic compounds from plant matrices uses organic solvents such as acetone, ethyl acetate, alkyl alcohols and their mixtures that present some environmental and safety concerns due to their volatility, flammability and potential non-biodegradability and toxicity [2,10]. Deep eutectic solvents (DES) present a green alternative to the traditional volatile organic solvents used in extraction processes, as they can be designed to present low volatility, biodegradability, and lower environmental impact [1]. A DES is defined as a mixture of two or more pure compounds for which the eutectic point temperature should be lower than that of an ideal liquid mixture, being liquid at the operating temperature [11]. Several studies have discussed the application of eutectic solvents in the extraction of phenolic

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compounds, evaluating the type of solvent, molar ratio of the components in the ES, solid/liquid ratio, extraction time, temperature, and technique in the optimization of the extraction processes [1,2,12–17]. The most common DES for the extraction of natural compounds are formed by choline chloride or betaine with urea, benzoic acid, ethylene glycol, or glycerol, and other alcohols, amino acids, carboxylic acids, or sugars [2,14,18]. Deep eutectic solvent-based aqueous biphasic systems have also been identified as promising systems for extracting bioactive compounds with different polarities from plant matrices [19]. The back-extraction process can be achieved by adding water as an anti-solvent, facilitating the purification and recovery of the target compounds [19]. Other alternative solvents, such as aqueous mixtures of salts, organic acids and polyalcohols, were applied to extract phenolic compounds [20,21]. Some DES present high viscosity compared with traditional organic solvents due to the extensive hydrogen bonding, van der Waals and electrostatic interactions [2,14], which can hinder their application in extracting target compounds from natural sources. Thus, water is often added to the eutectic mixtures, reducing the viscosity and energy consumption and increasing the mass transfer from the natural matrix to the solvent [14,22]. It is well reported that high water content can lead to weakened interactions within the components of the eutectic solvent and even to the complete disruption of eutectic solvent interactions [23–25], ultimately reducing the extraction capacity of the solvent [26]. However, adding water can also increase the overall polarity of the solvent, with an increase in extraction efficiency depending on the polarity of target analyte compounds [26] and biomass.

Juglans regia L. leaves extract is rich in phenolic compounds, presenting relevant antioxidant, antitumor, antiproliferative and anti-inflammatory bioactivities [22,27,28], which can be explored for cosmeceutical applications. Previous studies have reported the use of aqueous DES based on choline chloride and carboxylic acids [29], hydroethanolic solutions [27,28,30], glycerol and alkanediols [22] to extract phenolic compounds from *J. regia* leaves. On the other hand, cosmetics-compatible components, such as urea and betaine, have been previously used to extract bioactive natural compounds aiming for direct incorporation in the final product [31,32]. These compounds are widely applied in cosmetic formulations as moisturizer agents for maintaining hydration and skin barrier integrity [33–35]. Betaine is also used as a cleansing and viscosity-increasing agent [35]. Therefore, considering the potential direct use of the extract for cosmeceutical formulations, urea, betaine, and 1,3-propanediol were chosen as components of the extraction eutectic solvent as an alternative to the use of conventional volatile organic solvents that are usually removed by evaporation at the end of the extraction.

This study aims to design a more efficient and sustainable extraction process of the bioactive phenolic compounds from the leaves of walnut trees (*J. regia*), evaluating several aqueous solutions of eutectic solvents and their constituents. Besides the innovative application character of the selected compounds to form the eutectic mixtures, the effect of changing their ratio, water content, or even the study of the aqueous solutions of the individual compounds are evaluated, as these are parameters of primary importance but have been less explored or overlooked in previous extraction studies. Finally, the bioactivity in different dimensions (antimicrobial, antioxidant, anti-tyrosinase and photostability) of the *J. regia* leaves extracts with higher content in phenolic compounds will be evaluated. This information is essential to define the functionality of the liquid extracts obtained in this work, as previous studies were mainly focused on the bioactivity of the dry residue extracted with conventional volatile organic solvents and/or water, as already discussed in the literature [27,28].

2. Materials and methods

2.1. Materials

The source and mass purity of the compounds used in this work are listed in Table 1. The solutes and the organic solvents were stored at room temperature. All the solids were kept in desiccators to avoid water contamination. The water used was of ultrapure quality (resistivity of 18.2 MΩ·cm, free particles ≥ 0.22 μm and total organic carbon < 5 μg·dm^{-3}).

Table 1
Purity (%) and source of chemical compounds used in this work.

Compound	Mass purity (%)[a]	CAS number	Source
1,3-Propanediol	≥ 99.5	57-55-6	Sigma-Aldrich
Urea	≥ 99.5	57-13-6	ITW Reagents
Betaine	≥ 98.0	107-43-7	Alfa Aesar
Chlorogenic acid	≥ 95.0	327-97-9	Sigma-Aldrich
p-Coumaric acid	≥ 98.0	501-98-4	Sigma-Aldrich
Quercetin 3-*O*-glucoside	≥ 99	482-35-9	Sigma-Aldrich

[a] The mass purities were provided by the manufacturer.

Juglans regia L. dried leaves were purchased from Soria Natural, S.A., Spain. According to the distributor, the leaves were collected in Soria (Spain) and naturally dried in a room with controlled humidity. The samples were reduced to fine powder and stored protected from light in a desiccator to avoid water contamination.

2.2. Extraction method

2.2.1. Preparation of the solvents

First, the solubility of urea and betaine in water was measured between 25 °C and 55 °C to assist in preparing the solvents. The methodology and results of the solubility experiments can be found in Section S1 and Table S1.1 of Supporting Information. Considering the solubility results at 25 °C, aqueous solutions of betaine (Bet) and urea were prepared with water mass fraction (w_{water}) of 0.50 and 0.75.

The pure organic solvent 1,3-propanediol (PPD) was also studied and as it is completely miscible with water, its aqueous solutions were prepared with w_{water} of 0.25, 0.50 and 0.75. Aqueous mixtures of the eutectic solvents, betaine + urea and 1,3-propanediol + betaine in the molar proportions of 1:1, 1:2 and 2:1, were prepared, with w_{water} of 0.25, 0.50 and 0.75. All the systems, apart from Bet:Urea (2:1) with $w_{water} = 0.25$, resulted in a homogeneous liquid solution at room temperature. Hence, Bet:Urea (2:1) with $w_{water} = 0.25$ was not used as extraction solvent.

All mixtures were weighted in an electronic balance (Denver Instruments, precision of ±0.1 mg). The solutions were prepared by stirring at room temperature until a homogenous mixture was obtained, and its composition is listed in Table S2.1.

2.2.2. Heat-assisted extraction

The optimum extraction conditions, namely solid/liquid ratio, temperature, and extraction time were previously evaluated [30], aiming at the maximum extraction yield of phenolic compounds. The extractions were carried out considering the solid–liquid ratio of 30 g of plant per litre of solvent. Hence, 300 mg of finely ground plants were weighed in an analytical balance. Then, 10 mL of solvent was added to the flask at the beginning of the extraction. The flasks were placed in a thermostatic bath at 50 °C for 2 h with continuous magnetic stirring. Samples were centrifuged using an Eppendorf Centrifuge 5810 R at 6000 rpm for 15 min at room temperature. After the centrifugation, the sample was filtered in a qualitative filter paper Prat Dumas (surface weight 85 g/m^2, pore 10–12 μm, thickness 200 μm). The samples were diluted in pure water and filtered using Branchia nylon syringe filters (pore 0.22 μm, 13 mm of diameter), followed by quantification using HPLC analysis, as described in the next section.

2.3. Chromatographic analysis

The quantification of the phenolic compounds was described thoroughly elsewhere [30]. Briefly, the solutions containing the extracts

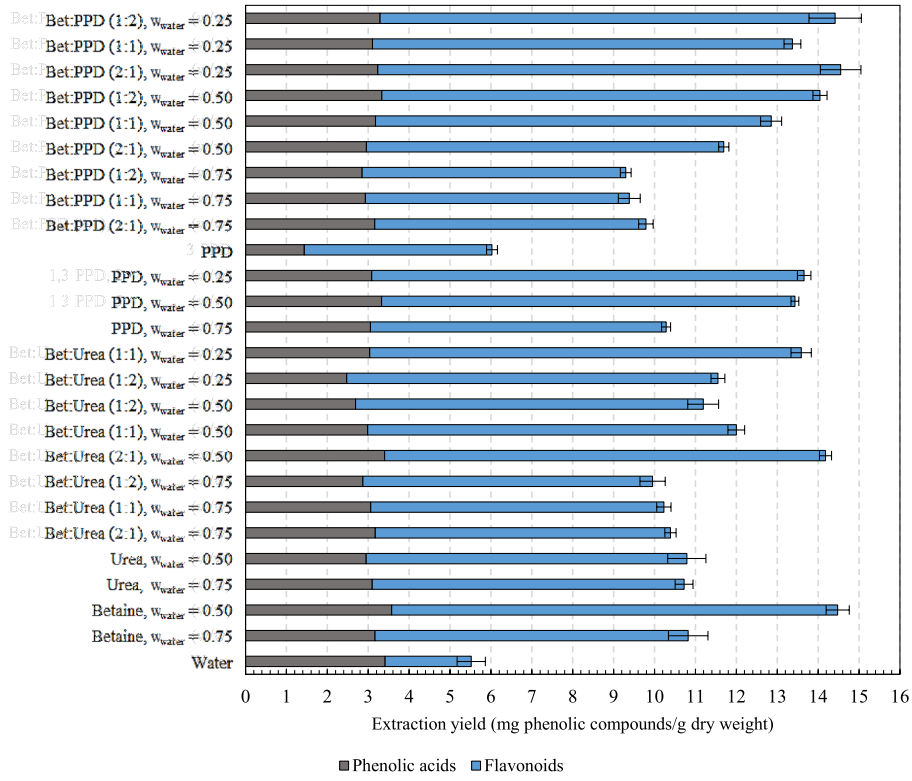

Fig. 1. Extraction yield of the main phenolic acids and flavonoids, and their sum (total phenolic compounds) present in the extracts of *Juglans regia* L. leaves (mean ± SD) obtained with several solvents composed by water, betaine, urea and 1,3-propanediol.

were diluted with water and filtered through 0.22 μm disposable nylon syringe filter and analysed by HPLC-DAD-ESI/MSn. Double detection was carried with a diode array detector (DAD) operating at 280 and 370 nm. A Xcalibur® data system (Thermo Scientific, San Jose, CA, USA) was used in data acquisition. The quantification of the main phenolic acids (chlorogenic acid and *p*-coumaric acid), and the flavonoids (quercetin 3-*O*-glucoside and quercetin-*O*-pentoside) was carried out. The identification of the compounds present in this natural matrix by comparison with their UV spectra and retention times was obtained in previous works [27,30].

2.4. Bioactivity assays

2.4.1. Antioxidant activity

The antioxidant activity of the solutions containing the extracts was evaluated by three assays: ferric reducing power (RP), DPPH radical scavenging and thiobarbituric acid reactive substances (TBARS).

The ferric reducing power assay used potassium ferricyanide (1 % *w/v*, 0.25 mL) and ferric chloride (0.1 % *w/v*, 0.8 mL). The absorbance was measured at 690 nm in a microplate reader (model ELx800, BioTek) using the equation,

$$RP(\%) = [(A_b - A_S)/A_b] \times 100 \quad (1)$$

where A_S is the absorbance of the sample solution and A_b is the absorbance of the blank (pure solvent).

The DPPH assay was performed using a methanolic solution of DPPH radical of 6 × 10^{-5} mol/L. The radical scavenging activity (RSA) was calculated using the equation,

$$RSA(\%) = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100 \quad (2)$$

where A_S and A_{DPPH} are the absorbances of the sample and the DPPH solution, respectively. The evaluation was determined by UV–Vis spectroscopy (model ELx800, BioTek).

The TBARS assay was performed in brain porcine tissue in the presence of $FeSO_4$ using tris-HCl buffer solution (20 mM), ascorbic acid (0.1 mM; 0.1 mL), trichloroacetic acid (28 %, *w/v*, 0.5 mL), and thiobarbituric acid (TBA, 2 %, *w/v*, 0.38 mL). The lipid peroxidation inhibition *LPI* (%) was calculated using the expression,

$$LPI(\%) = [(A_C - A_S)/A_C] \times 100\% \quad (3)$$

where A_C and A_S are the absorbance of the control and the sample solutions, respectively. For all antioxidant assays, the concentrations responsible for 50 % of antioxidant activity (EC_{50}) were obtained by the gradient dilution method. Each solution containing the extract obtained with the solid/liquid ratio of 30 g/L was dissolved in the extraction solvent and submitted to successive dilutions.

2.4.2. Anti-tyrosinase activity

The tyrosinase inhibitory activity was determined as previously described [36]. Each solution containing the extract obtained with the solid/liquid ratio of 30 g/L was dissolved in the extraction solvent and submitted to successive dilutions. Kojic acid and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were utilized as the positive control and substrate, respectively. Shortly, 1 mL of 2.5 mL L-DOPA solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8), followed by adding 0.1 mL of the sample solution. The mixture was incubated at 30 °C for 10 min followed by the addition of 0.1 mL of the tyrosinase enzyme solution (142 Units/mL). The absorbance was measured at 475 nm using a UV/visible spectrophotometer (UV-1600PC, VWR, Pennsylvania, United States). The tyrosinase inhibition *TI(%)* was calculated using the following equation:

$$TI(\%) = [(A_b - A_s)/A_b] \times 100 \quad (4)$$

The EC_{50} values can be calculated using a gradient dilution method with the calibration curve of tyrosinase inhibition percentage versus extract concentration.

2.4.3. Antimicrobial activity

The methodology used for the antimicrobial activity assay is described in detail elsewhere [27]. The extracts were tested against *Escherichia coli*, *Pseudomonas aeruginosa*, MRSA and *Candida albicans* by the microdilution method in 96 well microtiter plates.

The extracts were diluted in a gradient using the same solvent as the extraction, ranging from 15 mg of plant/mL of solvent to 3.75 mg of plant/mL of solvent, based on the initial concentration of 30 g of plant/L of solvent. The minimum inhibitory concentrations (MICs) and minimum antimicrobial concentrations (MACs) of the extracts were determined. Ampicillin (10 mg/mL), imipenem (1 mg/mL) and vancomycin (1 mg/mL) were used as positive controls.

2.4.4. Photostability

The extracts were diluted in buffer solutions containing citric acid and sodium citrate with a pH range of 3 to 6, reaching a final concentration of 5 mg of plant/mL of solvent. The tested pH range is within a healthy skin pH [37]. The extracts were irradiated for 90 min using a UV irradiation chamber (UV-consulting Peschl, Castellón, Spain) with a 45 $mJ{\cdot}cm^{-2}$ flux of UVA and UVB radiation. Afterwards, samples were analysed for total phenol content using the Folin-Ciocalteu method [38]. The antioxidant activity was measured using the DPPH method.

2.5. Statistical analysis

All the extractions were performed in duplicate, and all assays of bioactivity of the extracts were performed at least in triplicate. The statistical analysis was made using SPSS software (Version 25; IBM Corp., New York, NY, USA). One-way ANOVA was performed to determine the difference between the results, considering $p < 0.05$. The post hoc comparison was performed using Tukey's HSD test.

3. Results and discussion

3.1. Phenolic compounds extraction yield

The total yield of each extraction was evaluated by quantifying the four most abundant phenolic compounds present in *J. regia* leaves: quercetin 3-*O*-glucoside, quercetin-*O*-pentoside, chlorogenic acid and *p*-coumaric acid [27,28]. The yield of extraction of phenolic acids is the sum of chlorogenic acid and *p*-coumaric acid amounts, and for flavonoids is the sum of quercetin 3-*O*-glucoside and quercetin-*O*-pentoside, quantified in the HPLC-DAD analysis. The phenolic profiles exemplified in Figures S2.1 to S2.3 (Supporting Information) agree with those previously reported in the literature for the extracts obtained with water, ethanol, and their mixtures [27,28,39]. Fig. 1 presents the extraction yield of the main phenolic compounds for pure water, pure 1,3-propanediol (PPD) and the aqueous solutions studied in this work. The numerical results are also reported in Table S2.1. In the Supporting Information, the graphical representation of the extraction yield of flavonoids and phenolic acids and their statistical analysis can be found in Figures S2.4 and S2.5.

Depending on the solvents, the extraction yields of phenolic acids varied in the range 1.4–3.6 mg/g of dry plant and that of flavonoids varied between 2.1 and 11.3 mg/g of dry plant. The total phenolic content varied between 5.5 and 14.6 mg/g of dry plant.

Taking water as the reference solvent, while a high extraction yield was obtained for the phenolic acids (3.4 mg/g dry plant), for the flavonoids, one of the lowest extraction capacities was achieved (1.8 mg/g dry plant), resulting in the lowest total phenolics content (TPC=5.5 mg/g of dry plant). As discussed previously [29,30], pure water presents high extraction yields for the more polar phenolic acids, but low for the flavonoids present in the extract. These results are related to the very low solubility of quercetin in water [40].

The other pure compound that is liquid at room temperature, 1,3-propanediol (PPD), showed a similar total phenolics extraction yield compared to water (6.0 mg/g dry plant), with higher amounts of flavonoids. Still, in this case, its poor performance is most likely due to the high viscosity of the solvent. Adding water as a co-solvent had a positive impact, and by preparing a solution with $w_{water} = 0.25$ or 0.50, the extraction yields significantly increased to 13.7–13.4 mg/ g dry plant, respectively, which belongs to the set of solvents for which the maximum extraction yield was obtained. It should be noted that by further increasing the amount of water to $w_{water} = 0.75$ the extraction yield decreased.

When considering the aqueous solutions of either urea or betaine, only the aqueous solution of betaine ($w_{water} = 0.50$) resulted in the maximum extraction yield (TPC=14.5 mg/ g dry plant) that also decreased when adding water ($w_{water} = 0.75$).

Considering the families of solvents composed of betaine + urea or PPD+urea, similar extraction yields were obtained for the phenolic acids, with ranges 2.5–3.4 mg/g of dry plant and 2.9–3.3 mg/g of dry plant, respectively. The water amount in those aqueous mixtures does not seem to interfere in the extraction yield of phenolic acids. Furthermore, the aqueous mixtures of the pure components of the ES presented extraction efficiency like that of the eutectic solvent mixtures.

In comparison with phenolic acids, flavonoids presented a wider range of extraction yields, ranging in betaine + urea from 7.1 to 10.8 mg/g of dry plant and in betaine + 1,3-propanediol solvents from 6.4 to 11.3 mg/g of dry plant, showing that the range of the extraction yield is close for both families of solvents. Once again, using aqueous mixtures of ES does not significantly enhance the extraction of flavonoids compared with the extraction yield obtained using the aqueous mixtures of the pure compounds. In fact, betaine with 50 % mass of water presented one of the highest extraction yields of flavonoids.

In summary, considering the total phenolics content, the set of solvents that resulted in the same maximum extraction yield were the aqueous solutions of:

- PPD with $w_{water} = 0.25$ or 0.50;
- Betaine with $w_{water} = 0.50$;
- Betaine:Urea (2:1) with $w_{water} = 0.50$;
- Betaine:Urea (1:1) with $w_{water} = 0.25$;
- Betaine:PPD (1:2) with $w_{water} = 0.25$ or 0.50;
- Betaine:PPD (1:1) with $w_{water} = 0.25$ or 0.50;
- Betaine:PPD (2:1) with $w_{water} = 0.25$.

As can be seen, none of the aqueous solutions containing $w_{water} = 0.75$ resulted in the maximum extraction yield. That was also the case of the aqueous solutions containing higher amounts of urea, either in their aqueous solutions or in the eutectic systems (Betaine:urea 1:2, $w_{water} = 0.25$, 0.50 or 0.75).

Two other studies have also addressed the extraction of phenolic compounds from the leaves of walnut trees using aqueous solutions of eutectic solvents based on choline chloride and carboxylic acids [29] and aqueous solutions of several alkanediols or glycerol that are often applied as hydrogen bond donors to form ES [22]. A direct comparison of the extraction yield values is not straightforward as the biomass source is different due to diverse exogenous factors such as geographical location, seasonal differences, type of cultivar, and soil. The extraction conditions (time, temperature and water content) varied, but a solid–liquid ratio of 30 g/L was set in both works.

Vieira et al. (2018) [29] optimized the heat-assisted extraction using aqueous solutions of eutectic systems based on choline chloride and carboxylic acids. A preliminary solvent screening at fixed extraction conditions (60 min, 50 °C and 20 % *w/w* of water) allowed the selection of choline chloride mixtures with butyric acid (extraction yield of 33.7 mg/g of dry plant) or phenylpropionic acid (extraction yield of 34.3 mg/g of dry plant) at a mole ratio 1:2. The extraction capacity of the solvents was compared with water, ethanol, and a mixture of ethanol and water (50:50 % *v/v*), resulting in extraction yields of 8.8, 10.3, and 24.9 mg/g of dry plant, respectively, highlighting the better extraction capacity of choline chloride-based solvents in comparison with more traditional solvents [29]. Thus, the extraction yield increased 4 times compared to

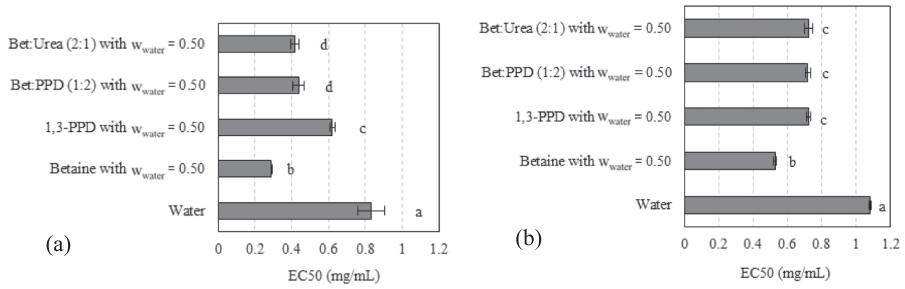

Fig. 2. Antioxidant results for the (a) DPPH and (b) ferric reducing power of the different liquid extracts. Each value is the mean of three replicate determinations ± standard deviation. Results in mg of plant per ml of extract. Means with different letters are significantly different ($p < 0.05$). Trolox EC_{50} values: 30 μg/mL (reducing power), 43 μg/mL (DPPH scavenging activity).

the pure water result. Further optimization of the extraction conditions (time, temperature and water content) resulted in a higher extraction yield (37.9 mg/g of dry plant) using aqueous solutions of choline chloride:butyric acid (1:2 mol ratio). The optimized extraction conditions were 180 min, 30 °C and 53 % *w/w* of water. The effect of changing the mole ratio of the ES components or the final mixtures' bioactivity was not evaluated.

Finally, Vieira et al. (2020) [22] compared the heat-assisted extraction using aqueous solutions of glycerol and several alkanediols (1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,3-butanediol, 1,2-pentanediol, 1,5-pentanediol, and 1,2-hexanediol). The anti-inflammatory activity and cytotoxicity of extracts and pure solvents were also evaluated. The extraction conditions were set at 120 min, 50 °C and 20 % *w/w* of water. The extraction capacity of the solvents was compared with water and a mixture of ethanol and water (80:20 % *w/w*), resulting in extraction yields of 14.1 and 27.8 mg/g of dry plant, respectively. The solvents showing the best-combined results were 1,2 and 1,3-propanediol, also increasing 2 times the pure water result, with extraction yields of 27 mg/g of dry plant.

In this work, water was the reference solvent with an extraction yield of 5.5 mg/g of dry plant, at similar extraction conditions (120 min and 50 °C). The best solvents (betaine:urea (2:1), betaine:PPD (1:2), betaine, and PPD, all with 50 % water mass percentage) allowed the extraction yield to increase 3 times. While the magnitude of the extraction capacity is not as high as using choline chloride-based solvents, the extracts from this work have the enormous advantage of potentially being used directly as ingredients in the cosmetic and pharmaceutical areas while reducing the complexity of the solvent systems. In fact, aqueous solutions of only one of the ES constituents originate extracts with similar extraction efficiency.

For the following bioactivity analysis, the extracts obtained with the aqueous solutions ($w_{water} = 0.50$) of betaine, PPD, Betaine:Urea (2:1) and Betaine:PPD (1:2) were selected, considering the maximum extraction yield and the highest amount of water, since water is a low cost and non-toxic solvent that can be directly incorporated in cosmetic formulations.

3.2. Bioactive properties of *J. regia L.* extracts

3.2.1. Antioxidant activity

The objective of the antioxidant assays is to determine the ability of the extracts to protect the biological molecules of the free radicals produced in the oxidative processes [41,42]. The antioxidant activity of the liquid extracts was assessed by three in vitro assays. The results of the DPPH and ferric reducing power assays are presented in Fig. 2.

For all the extracts in the solvents tested, the EC_{50} values are lower than the results obtained by the reference solvent (water), meaning they have a higher antioxidant activity. For the DPPH assay, the best result was found in the aqueous extract of betaine, followed by betaine:urea (2:1) and betaine:PPD (1:2), all containing $w_{water} = 0.50$. It is important to mention that no antioxidant activity was observed in the pure solvents that were also tested. For the reducing power results, the aqueous solution of betaine also presented the best result. The results of the TBARS assay are presented in Fig. 3.

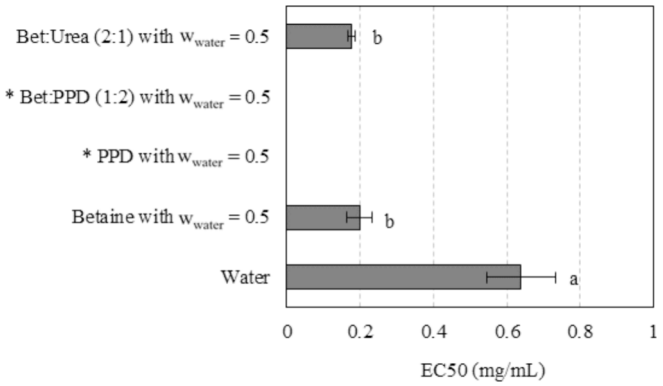

Fig. 3. Antioxidant results for the TBARS assay applied to the different liquid extracts. Each value is the mean of three replicate determinations ± standard deviation. Results in mg of plant per ml of extract. Means with different letters are significantly different ($p < 0.05$). Solvents marked with an asterisk were not able to measure. Trolox EC_{50} value: 5.8 ± 0.6 μg/mL.

Regarding the TBARS assay, the extracts from Betaine:Urea (2:1) and Betaine with $w_{water} = 0.50$ presented similar antioxidant activity, with pure water presenting the lowest activity of the solvents tested. For the solvents containing 1,3-propanediol (Betaine:PPD (1:2) and PPD, with $w_{water} = 0.50$), it was not possible to measure the antioxidant activity of their extracts by this method since a colour alteration was observed when adding the reactant, probably due to a reaction between some components of the solvents and the reactants.

Among all the samples tested, the extracts from using aqueous solutions of betaine with $w_{water} = 0.50$ consistently showed the best antioxidant results across all three in vitro assays, with significantly lower EC_{50} values in both DPPH and ferric reducing power and similar results to the extracts of Betaine:Urea (2:1) in the TBARS assay.

3.2.2. Anti-tyrosinase activity

Tyrosinase is multi-copper enzyme essential in melanogenesis and enzymatic browning, also having a role in the neurodegeneration related to Parkinson's disease [28,43]. Anti-tyrosinase compounds can be incorporated in cream formulations to avoid the overproduction of melanin [28]. In this work, the anti-tyrosinase activity of the *J. regia L.* leaves extracts were evaluated using L-DOPA as substrate. Interestingly, only the extract obtained using pure water showed anti-tyrosinase activity, with 26 % of tyrosinase inhibition at the maximum concentration tested (5 mg of plant/mL of solvent). The remaining liquid extracts presented no activity at the concentrations tested.

Table 2
Minimum inhibitory concentration (MIC) and minimum antimicrobial concentration (MAC)[a] of *Juglans regia* extracts tested against *Escherichia coli*, *Pseudomonas aeruginosa*, MRSA and *Candida albicans*.

	Aqueous solutions with $w_{water} = 0.50$										Positive controls					
	Water		Betaine		PPD		Bet:PPD (1:2)		Bet:Urea (1:2)		Ketaconazole		Imipenem		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	>15	>15	15	15	7.5	15	7.5	15	15	15	n.t.	n.t.	<0.0078	<0.0078	n.t.	n.t.
P. aeruginosa	>15	>15	7.5	15	3.75	7.5	3.75	15	7.5	15	n.t.	n.t.	<0.0078	<0.0078	n.t.	n.t.
MRSA	>15	>15	15	15	3.75	7.5	3.75	7.5	15	15	n.t.	n.t.	n.t.	n.t.	0.25	0.5
C. albicans	>15	>15	15	15	3.75	7.5	3.75	15	15	15	0.007	0.15	n.t.	n.t.	n.t.	n.t.

[a] Concentration equivalent to mg of plant/mL of solvent used in the extraction step; n.t.: not tested.

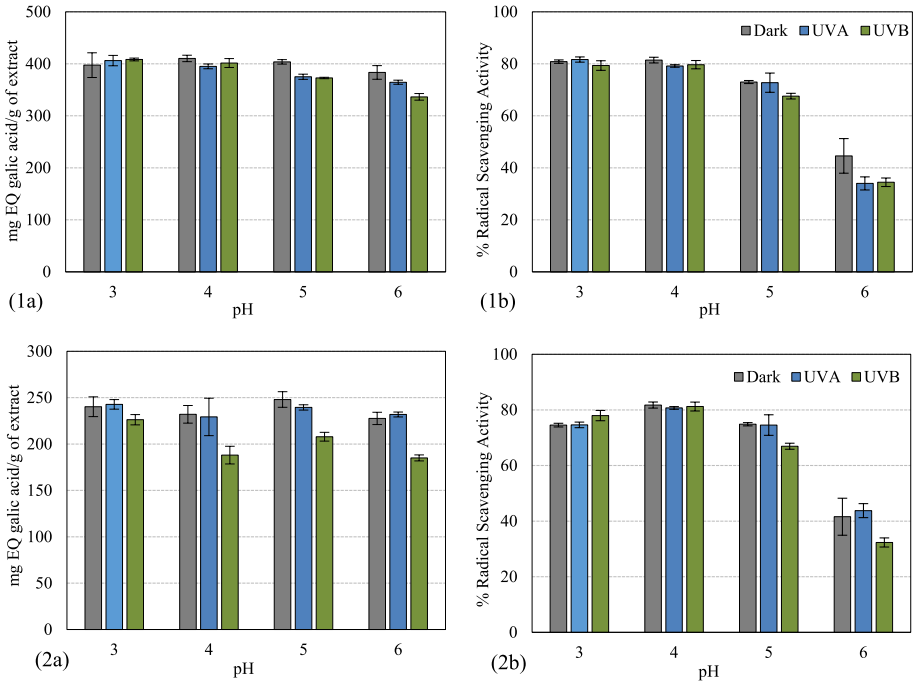

Fig. 4. The photostability profile of *J. regia* extract using PPD, with $w_{water} = 0.50$ (1) and pure water (2). Analysis: (a) total phenolic compound by Folin-Ciocalteu method and (b) antioxidant activity by the DPPH method. Each value is the mean of at least three replicate determinations ± standard deviation.

3.2.3. Antimicrobial activity

The antimicrobial activity of the *J. regia* L. extracts here obtained were tested against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), Gram-positive bacteria (MRSA) and yeast (*Candida albicans*). *E.coli* is an important species related to healthcare-associated infections [41], whereas *P. aeruginosa* is a Gram-negative bacillus associated with characteristic cutaneous infections [44]. Methicillin-resistant *Staphylococcus aureus* (MRSA) can cause skin and soft tissue infections, being associated with high morbidity and mortality [45]. *Candida albicans* is the most pathogenic Candida species, the most common fungal disease-causing agent in humans [41]. The results are presented in Table 2 as the minimum inhibitory concentration (MIC) and minimum antimicrobial concentration (MAC). It is important to mention that the antimicrobial activities of the pure solvents were also evaluated, showing no contribution to the observed antimicrobial effect at the tested concentrations range.

The tested extracts obtained with PPD presented the highest antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and yeast, with the corresponding lowest MIC and MAC values. The highest MIC and MAC values are found for *Escherichia coli* for all extracts, showing a possible resistance of this strain against these extracts. The aqueous extract was the only one without antimicrobial effects in the tested concentration range. In previous literature studies [27,28,46–49], dry extracts of *J. regia* leaves obtained using different conventional volatile organic solvents and water also displayed antimicrobial activity against *Propionibacterium acnes*, *Bacillus cereus*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Actinomyces viscosus*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Staphylococcus epidermidis*. The reported results show that these extracts can be further explored as part of cosmetic formulations due to their antimicrobial effect against common skin pathogens [46–49].

3.2.4. Photostability

Phenolic compounds, such as flavonoids and phenolic acids, can also be explored as potential cosmetic and sunscreen additives because they can absorb a broad spectrum of ultraviolet rays, protecting the human skin from UVA and UVB radiations [50]. However, these compounds were revealed to be photo-unstable in long-term exposure to ultraviolet radiation and high temperatures [28,51]. In this work, the photostability of the aqueous solutions containing the extracts was tested against UVA and UVB radiation at different pH conditions (between 3 and 6), considering the human skin pH range [52]. The photostability profile for the extracts in water and the aqueous solution of PPD (with $w_{water} = 0.50$) is presented in Fig. 4. The photostability profile and statistical analysis can be found for the remaining solvents in Figure S3.1 of Supporting Information.

As can be seen, for pH 5 and specially pH 6, a decrease in the total phenolic content can be observed, which is more relevant in the

antioxidant activity. As this also happened for the control sample (placed in the dark), the radiation effect did not cause this decrease. Regarding the UVA/UVB exposure, generally, only minor changes can be observed in the antioxidant activity and the total phenolic content compared with the control, showing that the extracts presented excellent photostability for all solvents tested. Thus, the phenolic compounds in *J. regia* leaves can be further exploited as a natural additive in cream, cosmetic and sunscreen formulations.

4. Conclusions

This work explored the *Juglans regia* L. leaves as a promising source of bioactive phenolic compounds with potential application in cosmeceutical formulations. The bioactivity evaluation of the extracts shows that the solvent used in the extraction greatly impacts both the extraction yield and the bioactive properties of the final extracts.

The extraction using pure water resulted in the lowest total yield considering the total phenolic compounds content. However, several aqueous mixtures of eutectic solvents with 25 % or 50 % water (in weight) showed an improved solvent performance for phenolic acids and flavonoids, more than doubling the extraction yield of pure water. Additionally, aqueous solutions of the individual components (betaine and PPD) demonstrated similar extraction capacity to the aqueous eutectic mixtures. This indicates that less complex solutions (binary mixtures) should always be tested in parallel to using aqueous solutions of eutectic mixtures (ternary mixtures).

The aqueous extract showed the highest anti-tyrosinase activity, while the extracts obtained with 1,3-propanediol presented the highest antimicrobial activity against gram-positive bacteria, gram-negative bacteria, and yeast. For the antioxidant activity, the extracts obtained with aqueous solutions of betaine ($w_{water} = 0.50$) were the most effective. The tested extracts generally showed good photostability, particularly at pH values of 3 and 4.

Furthermore, extracts obtained with different solvents also presented different bioactive properties, even those with a similar phenolic profile. A more detailed study of the extracted phytochemicals, other than phenolic compounds, should be carried out to account for those differences.

Declaration of competing 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2024.129214.

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