Synergistic effect of natural deep eutectic solvent and high-intensity ultrasound on obtaining a ready-to-use genipin extract: Crosslinking and anti-neurodegenerative properties

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ABSTRACT

In this paper, genipin, an important natural crosslinker and anti-neurodegenerative compound, was extracted from unripe Genipa americana L., combining high-intensity ultrasound (HIUS) and natural deep eutectic solvents (NADESs). The extraction process conditions were evaluated step-by-step to reach the best genipin recovery. The obtained ready-to-use genipin-NADES extract was examined regarding its crosslinking properties and anti-neurodegenerative capacity. For the conditions tested, the highest genipin recovery was obtained using 40 % water and 60 % betaine:lactic acid NADES in molar ratio 1:3 (n/n) as the solvent, a solvent:feed ratio of 19 (w/w), and HIUS acoustic power of 14 ± 1 W. The HIUS-assisted extraction using NADES as solvent showed to be a promising and efficient green extraction technique to obtain genipin. The ready-to-use genipin-NADES extract presented crosslinking capacity and anticholinergic activity. These results indicate that genipin-NADES extract can be directly applied in hydrogels for drug delivery, films, tissue engineering, and others. Moreover, it can be used in food, supplements, and medicine to enhance their neuroprotective effect.

Introduction

The use of natural deep eutectic solvents (NADESs) has emerged as a new smart strategy to replace toxic, non-biodegradable, and flammable organic solvents, such as methanol, acetone, and formic acid, for the extraction of plant metabolites (Gutiérrez, Alcalde, Atilhan, & Aparicio, 2020). Even compared to generally recognized as safe (GRAS) solvents, such as ethanol or water, NADESs have proven to be more efficient extraction solvents (Benvenutti, del Pilar Sanchez-Camargo, Zielinski, & Ferreira, 2020). They are composed of at least a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), which are natural metabolites such as sugars, alcohols, organic acids, amino acids, etc., linked mainly by hydrogen bonds (Silva et al., 2021). However, other types of interactions can be established between the components. NADESs have gained attention due to their physicochemical qualities (i.e., a liquid state within a wide temperature range, chemical or thermal stability) as well as their low toxicity, high biocompatibility and easy preparation (Gutiérrez et al., 2020). Besides, as a result of HBA:HBD network formation, the solvation of target compounds is favored, which makes NADES ideal as green extraction solvents for the development of sustainable and efficient procedures (Benvenutti, Zielinski & Ferreira, 2019).

At the beginning of NADES utilization, the extract post-treatment, mainly the separation of the target compounds from the NADES, was considered an issue due to its inherent low vapor pressure, which makes it difficult to recover the compounds from the extract solution (Dai, Verpoorte & Choi, 2014). However, since they are generally natural and non-toxic substances NADES may be directly incorporated in pharmaceutical, cosmetic, and food formulations without demanding expensive purification steps, a major advantage over conventional solvents (Pani et al., 2019). Hence, nowadays, some works consider the extracts from NADES as a ready-to-use product (Dai et al., 2020, Pani et al., 2019, Silva et al., 2021).

To achieve an even greener and more efficient process, the NADES is often combined with an emerging extraction technique, such as the high-intensity ultrasound (HIUS). HIUS is an eco-friendly extraction technique due to the low solvent consumption and extraction time, which enhances the extraction yield compared to conventional
4-(amino sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was used as a serum, acetylthiocholine iodide (ATCI), trizma hydrochloride (Tris-)

VI-S was from Electrophorus electricus, whereas BChE was from equine. Acros Organics (Geel, Belgium) (®)

Materials and methods

Material and methods

Chemicals and material

All chemicals were of analytical reagent grade (except those specifically indicated) and used as received. Acetonitrile (ACN) (purity 99.8 %) and formic acid (purity > 99 %) were purchased from VWR Chemicals (Barcelona, Spain). Deionized water was obtained from a Milli-Q Milipore® system A10 (Billerica, MA, USA). ChCl (98 %), Bet (≥ 97 %), lactic acid (LaAc) (≥ 90 %), propylene glycol (ProGly) (99 %), and propionic acid (ProAc) (99 %) were acquired from Tokyo Chemical Industry (Tokyo, Japan), Genipin analytical standard was supplied by Sigma-Aldrich (Madrid, Spain). The 4-(amino359 sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Unripe genipap fruits were donated by “Fazenda Lagoa” (Ponte Alta do Tocantins, TO, Brazil). The entire fruits were partially freeze-dried, ground, and stored at –18 °C until their use.

Synthesis of NADES

NADESes were prepared by mixing different HBDs and HBAs with a molar ratio (HBA:HBD) (n/n) of 1:2. Combinations of Bet and ChCl as HBAs with ProAc, ProGly, and LaAc as HBDs were synthesized. For those syntheses, NADES components were placed in a glass vial and stirred at 80 °C for 30 min, observing the formation of the homogeneous liquids (Adeyemi, Sulaiman, Almazroui, Al-Hammadi & AlNashel, 2020). The solvents were stored in a vacuum desicator at room temperature. After NADESes preparation, they were combined with different amounts of water to prepare the extraction solvents.

NADES HIUS-assisted genipin extraction

The samples for the HIUS-assisted extraction were prepared using a solvent:genipin ratio to reach a final mass of 30 ± 1 g, the solvent/feed ratio (S/F) (w/w) initially used was 14; also, the water proportion to form the solvent with NADES was initially 50 %. Genipin extraction was carried out according to Neves, Strieder, Silva, and Meireles (2020), with some modifications. At the beginning of the study, a 12.6 mm diameter ultrasonic probe 450 Digital Sonifier (Branson, Danbury, CT, United States) at 19 kHz and a nominal power of 315 W. The processing time was 3 min for all experiments. During the HIUS-assisted extraction process, the sample temperature increase was controlled using an ice bath. After selecting the most suitable NADES, different HBA:HBD ratios (1:2, 1:3 and 1:4) (n/n) were tested. Then, the S/F (29, 19, 14, 9) (w/w); and the %H₂O in the solvent formulation (30, 40, 50, 60 %) were evaluated. Afterward, different HIUS nominal power was tested: 0, 180, 270, 315 and 405 W, referring to the amplitudes (%): 0, 40, 60, 70, and 90, respectively. The acoustic powers supplied by the ultrasound probe in that nominal powers were determined by calorimetric assays as described by Mason, Lorimer, Bates and Zhao (1994). Using 90 % of amplitude, the temperature profile was measured to calculate the acoustic power; the process was run without an ice bath and the processing time was 9 min. The acoustic power, specific energy, and ultrasound intensity were calculated according to Eq. (1), 2, and 3. For each nominal power used, we had the acoustic powers of 14 ± 2, 22.1 ± 0.2, 27 ± 1, and 34 ± 1 W. Finally, the extracts were separated from the biomass by a paper filtration. An aliquot of 200 μL was collected for each run, diluted 1:5 in water, and filtered through a 0.45 μm filter to quantify the extraction rate. The extracts were stored at –18 °C until performing the analyses.

Acoustic power (W) = \( mC_p \frac{dT}{dt} \) (1)

Specific energy (kJ/kg) = \( \frac{\text{Acoustic power} \times \text{processing time}}{\text{sample mass}} \) (2)

Ultrasound intensity (W/cm²) = \( \frac{\text{Acoustic power} \times 4}{\pi D^2} \) (3)

where \( m \) is the sample mass (g); \( C_p \) is the specific heat of water (J/g °C), \( \frac{dT}{dt} \) is the change in temperature throughout the time range (°C/s), and \( D \) is the diameter of the probe in cm.

Experiments were carried out in triplicate. For each step, the same fruit was used to avoid the effect of different raw materials.

HPLC-DAD method

Genipin extraction rate was determined using an HPLC-DAD Agilent
1100 series system (Agilent Technologies, Santa Clara, CA, United States). Genipin was separated using a column Poroshell 120, C18 (4.6 × 100 mm, 2.7 μm) (Agilent Technologies, Santa Clara, CA, United States). Separation was carried out according to Nathia-Neves, Nogueira, Varadanega, and Meireles (2018), using a mobile phase composed of water (A) and ACN (B), both acidified with 0.1 % formic acid and applying the following gradient: 0 min, 99 % A; 9 min, 75 % A; 10 min, 99 % A and 13 min, 99 % A. Injection volume was 10 μL. The column temperature was set at 35 °C and the flow rate at 1.5 mL/min. Genipin was detected at 420 nm and identified by comparing its retention time and UV–vis spectra to the reference standard (St-Gp). The extraction rate (mg/g of genipap mass) was calculated according to Eq. (4).

\[ \text{Genipin extraction rate} = \frac{\text{genipinnmass (mg)}}{\text{genipap mass (g)}} \]  

(4)

For the confirmation analyses, the St-Gp, genipin was extracted with water (Gp-H2O), genipin extracted with NADES (Gp-NADES), selected NADES, and St-Gp with NADES (St-Gp + NADES) were evaluated using an MS system from Bruker Daltonik (Bremen, Germany) with electrospray ionization (ESI) (negative mode) and an ion trap as the analyzer. The extracts were diluted in water to reach the genipin concentration of 0.5 μg/ml. St-Gp, NADES, and (St-Gp + NADES) were prepared based on the same concentration. ESI nebulizer gas flow was set at 20 psi, spray voltage at 2000 V, and source temperature at 250 °C. The analytical column used was a C18 (2.1 × 100 mm, 1.9 μm) (Thermo Fisher Scientific, Vilnius, Lithuania) at 35 °C. Water (solvent A) and ACN (solvent B), both with 0.1 % formic acid (v/v), were used as mobile phases. Initial composition was established at 99 % A and 0.1 % B of C, and this percentage was reduced up to 75 % A at 7 min, maintaining this composition until 8 min. Then, it was increased up to 99 %, until 14 min. The sample flow rate was set at 0.25 mL/min, and the sample injection volume was 10 μL. The area for each m/z was normalized to the total summed area.

Reverse phase ultra-high performance liquid chromatography-quadrupole-time of flight mass spectrometry (RP/UHPLC-Q-TOF MS/MS) analysis

NADES-genipin extract (Gp-NADES) samples obtained under the best condition tested were dissolved 1:10 in water to a final concentration of 1 mg/mL, and 2 μL (in triplicates) were injected into a UHPLC (model 1290) coupled to a quadrupole Q-TOF (6540 series) from Agilent Technologies (Germany). The conditions used for compound separation and detection were the same as previously described (Dauber et al., 2022). LC-MS raw data files were converted to ABF format for data processing, and data processing was performed using MS-DIAL (v. 4.80) (Tsugawa et al., 2015). Peak area calculation was performed by combining data for different detected molecular species ([M−H], [M + CI], [M + FA-H] adducts). Total compound contribution (%) was calculated as compound area/Total Ion Current area * 100, and the selected NADES.

Effect of the presence of NADES on genipin properties

NADES-genipin extract (Gp-NADES) obtained under the best condition tested was analyzed to evaluate the influence of the NADES on genipin anti-neurodegenerative effect (based on its anticholinergic activity) and genipin crosslinking capacity. Gp-NADES, Gp-H2O, and St-Gp solution were examined and compared. Before the anticholinergic activity and crosslinking assays, the pH of the extracts was adjusted at 8 and 6 with NaOH 10 M, respectively (the amount added did not affect the concentration of the extracts).

Anticholinergic activity

The anticholinergic capacity of the genipin extracted by NADES was tested by in-vitro assays based on the AChE and BChE inhibitory capacity performed based on Ellman’s method by fluorescent enzyme kinetics. The samples tested were the St-Gp, Gp-H2O, and Gp-NADES. The method was carried out according to Sánchez-Martínez, Bueno, Alvarez-Rivera, Tudela, Ibáñez & Cifuentes (2021). One hundred μL of the sample at the concentrations in the range of 200 μg-2000 μg/mL, 100 μL of buffer (150 mM Tris-HCl pH = 8) and 25 μL of 1.6 U/mL AChE or BChE in buffer were filled in a microplate of 96-well and then, incubated for 10 min. Afterward, 25 μL of ABD-F (125 μM) in buffer and 50 μL of ATC at a concentration of the K\text{a} (Michelais–Menten constant) value in H2O were added. The K\text{a} constant is the substrate concentration at which the reaction rate is half of the maximum velocity rate. The fluorescence was logged at λ\text{excitation} = 389 nm and λ\text{emission} = 513 nm every 10 min at 37 °C. This kinetic measurements seeks to obtain the V\text{mean} value, which resembles the enzymatic mean velocity achieved during kinetic measurement. Galantamine hydrobromide in EtOH/H2O (1:1, v/v) was used as the reference inhibitor for AChE and BChE enzymes (Sánchez-Martínez et al., 2021). The K\text{m} Value was measured by adding 100 μL of ATC at different concentrations (0.4 – 4 mM) in H2O, 50 μL of H2O, and 100 μL of buffer and starting the reaction by adding 25 μL of ABD-F (125 μM) in buffer and 25 μL of 1.6 U/mL AChE or BChE in the buffer. V\text{mean} and K\text{m} were calculated by Gen5\text{TM} version 2.0 Data Analysis software from BioTek Instruments, Winooski, VT, USA. The 96-well was placed in a spectrophotometer reader Synergy HT (BioTek Instruments, Winooski, VT, USA).

Crosslinking capacity

The crosslinking reaction between genipin and primary amines from amino acids, proteins or polymers produces a blue pigment (Neves, Valdés, Silva, Meireles, Ibáñez, & Cifuentes, 2022). The effect of NADES on the genipin crosslinking capacity was evaluated by determining the absorption modification of the solutions at 590 nm (wavelength corresponding to the blue). For this, lysine (lys), St-Gp; Gp-H2O, and Gp-NADES were prepared at pH 6 and diluted to reach the same concentration (80 μg/mL). All extracts with and without the amino acid (used as amine supplier) were put in a 96-well microplate. Immediately, the microplate was read at 590 nm in a spectrophotometer Synergy HT (BioTek Instruments, Winooski, VT, USA) for the time 0 and every 2 min until 30 min. The results were expressed in Δ\text{absorbance} calculated according to Eq (5).

\[ \Delta \text{absorbance} = A_t - A_0 \]  

(5)

where, \( A_t \) is the absorbance at time t, and \( A_0 \) correspond to the initial absorbance (time 0).

Statistical analysis

Statistical analysis was performed using Minitab 18® software. The results were expressed as mean values ± standard deviation (SD) of three replicates. Analysis of variance (ANOVA) using Tukey’s test
examined the differences between the mean values. The difference was considered statistically significant when the p-value was below 0.05.

Results and discussion

NADES selection

NADES components selection

Screening of six NADESs was carried out to select the most suitable for extracting genipin from unripe *Genipa americana* L. All NADESs were based on ChCl or Bet in combination with ProAc, ProGly or LaAc at a 1:2 M ratio. The choice of these six solvents was based on the literature (Ivanović, Alanoń, Arraz-Roman, Segura-Carretero, 2018; Yu et al., 2021). According to the previous studies, terpenoids with similar chemical characteristics as genipin were extracted using these combinations. One of the main issues of using NADESs in extraction processes is their high viscosity, leading to low mass transfer and low target compound diffusion to the extraction medium (Zainal-Abidin, Hayyan, Hayyan & Jayakumar, 2017). In fact, even with organic acid-based NADESs, which present relatively high fluidity at room temperature, viscosity is significantly higher than that of the commonly used organic solvents (Sánchez, González, Salgado, Parajo & Domínguez, 2019). In this sense, the combination of NADES with water can reduce viscosity and, consequently, decrease surface tension and increase molecular diffusion. Thus, all experiments were conducted using 50% of water in NADES as standard conditions in an initial selection of the NADES.

Fig. 1a) shows the effect of the NADES combination on genipin extraction efficiency. ChCl:ProGly was the less favorable to recover genipin, followed by ChCl:LaAc, Bet:ProGly and ChCl:ProAc, which showed intermediate values of genipin recovery. The highest genipin extraction efficiency was obtained using Bet:ProAc, and Bet:LaAc. The Bet:LaAc was the one presenting the smallest standard deviation. Therefore, it was selected for further studies. It should be noted that organic acid-based NADESs (e.g., ChCl:LaAc, Bet:LaAc, and Bet:ProAc) showed a good capacity for extraction genipin.

Moreover, a control experiment was also performed using water as a solvent. However, the analysis of the genipin content by HPLC-DAD of this sample was not consistent, and high variability was observed in samples stored for 1–5 days (data not shown). This effect may be a consequence of different reactions occurring between genipin and other compounds extracted by water, such as amines from amino acids and sugars. Renhe, Stringheta, Silva and Oliveira (2009) have previously observed this effect, demonstrating an unstable blue color formation in *Genipa americana* L. extracted with ethanol and water. However, this effect was not observed by using NADES, highlighting the importance of these types of solvents as an alternative for maintaining genipin in its stable form. This effect might be explained by the hydrogen bond capability of NADES and their electrostatic interactions with target components, which might increase its stability. Dai et al. (2014) reported the influence of NADES on the stability of carthamin due to the molecular hydrogen bond interactions between the solvent and the bioactive compound. Additionally, NADES can enhance the selectivity of compound recovery (Zainal-Abidin et al., 2017).

NADES molar ratio selection

After selecting the HBA and HBD for the NADES preparation, it is important to verify its proportion that influences NADES’s chemical stability and physicochemical properties. Viscosity plays a crucial role in the extraction performance (see Table S1 – Supplementary material). Lower viscosities improve the diffusivity of the solvent through the matrix, thus, favoring the interactions between the target compound and the NADESs, consequently improving the extraction process. Table S1 shows the viscosity of the three different Bet:LaAc molar ratios (1:2; 1:3, and 1:4) evaluated in this work (at 25 °C). Results showed dynamic viscosities of 966 ± 4 mPa-s; 465 ± 1, and 311.5 ± 0.4 mPa-s, respectively. Therefore, by decreasing the proportion of LaAc, an increase in viscosity was observed, which can hinder the effectiveness of HIUS extraction. Those values agree with other previously published studies that reported similar data (Gutiérrez, Alcalde, Atilhan & Aparicio, 2020; Sánchez et al., 2019). Moreover, the solvent viscosity directly affects the efficiency of the HIUS-assisted extraction.

Fig. 1b) presents the effect of Bet:LaAc acid molar ratio (n/n) (1:2, 1:3, and 1:4) on genipin extraction rate; as can be seen, the LaAc concentration affected the potential of the NADES as a solvent (p-value = 0.003) influencing viscosity and NADESs formation. When solvent viscosity increases, acoustic cavitation bubbles within the fluid occur morecreepily and thus, the tensile strength of the liquid increases. Hence, these results suggest the higher suitability of molar ratio 1:4 to perform the extraction due to its low viscosity. However, other aspects such as the better formation and interaction between each NADES components should also be considered. Hence, the 1:2 ratio impairs the extraction due to the high viscosity, and the 1:4 proportion was not suitable to generate an efficient NADES as a solvent; therefore, 1:3 ratio was finally selected (see Fig. 1b) to conduct the subsequent steps was 1:3 (Bet: LaAc).

Selected NADES Bet:LaAc (1:3) was characterized by FT-IR to verify the formation of the hydrogen bonds between both components and, consequently, the correct preparation of the new solvent. Hence, Figure S1 (Supplementary material) shows the FT-IR spectra of Bet (a), LaAc (b) and Bet:LaAc (1:3) NADES (c). The bands at 3359 and 3285 cm⁻¹ in Fig. S1(a) are associated with the asymmetric and symmetric stretching of N=H bonds in the Bet structure, while the bands at 1695 and 1616 cm⁻¹ are characteristic of the asymmetric and symmetric stretching of carboxylate group present in its structure (Li et al., 2020). Fig. S1b) shows a band at 3401 cm⁻¹ associated with the stretching of the hydroxylated group of the LaAc structure. The interaction of both molecules is established by the formation of a hydrogen bond between the carboxylate group in Bet and the hydroxylated group of LaAc as a consequence of the strong electronegativity of the O' of (COO⁻) group.
and the positive charge of the H- in the OH– group. Such interaction modifies the stretching vibration of carbonyl and hydroxyl groups bringing about a shift of the wavelength, as can also be seen for both specific bands in Fig. S1c (Li et al., 2020).

Evaluation of main factors involved in NADES HIUS-assisted genipin extraction

In order to improve genipin recovery, the main parameters involved in NADES HIUS-assisted extraction were tested, as follows: solvent/feed ratio (S/F) (w/w) (29, 19, 14, and 9), proportion of water in the extraction solvent % (w/w) (30, 40, 50, and 60), and HIUS acoustic power (control, 14 ± 1, 22.1 ± 0.2, 27 ± 1, and 34 ± 1 W).

Solvent/feed (S/F) ratio selection

Results of genipin recovery are presented in Fig. 2a). The best S/F was 19 (1.5 g of genipap mass and 28.5 g of solvent) (p-value < 0.001), using the initial parameters of (water in the extraction solvent % (w/w) 50; HIUS amplitude (%) 70). The equilibrium between the solvent and extractable material is important to achieve an adequate recovery of the target compound. When the amount of solvent is small, the NADES can only interact with a part of the target compound. However, when the extractable material exceeds a certain value, NADES cannot interact with the extractable material to recover the target compound. Indeed, the selected proportion S/F of 19 allows a suitable interaction between the target compounds with the solvent, maximizing the extraction rate (Liu, Li, Fu, Zhang, Wang & Wang, 2019).

Study of water proportion on the extraction solvent

Water proportion on the solvent formulation by NADES can affect the efficacy of the final solvent (p-value = 0.001), as can be seen in Fig. 2b). It is important to use the minimal quantity of NADES, which reach a higher extraction efficiency to reduce the process costs. Water addition increases polarity, reduces surface tension and reduces the viscosity of NADES. However, excessive addition of water can impair the hydrogen bond between the HBA and HBD, reducing NADES stability and hindering the interactions between the compound and NADES (Sánchez et al., 2019). Experiments were developed fixing the S/F at 19 and using the HIUS amplitude at 70 %. The minimal amount of water that reached the best extraction conditions was 40 %. Using 30 % of water, the extraction was less efficient, probably due to the higher viscosity. From 50 % of water onwards, the genipin recovery process becomes less efficient. Thus 40 % was selected for further studies.

HIUS acoustic power

After fixing the best extraction conditions regarding the NADES and solvent formulation and solvent/feed proportion, we evaluated the best HIUS power. The higher the power used in the HIUS process, the greater the acoustic cavitation. Acoustic cavitation offered by HIUS resulted in higher performance on genipin extraction from unripe Genipa americana L., besides its diffusion into the Bet-based NADES. The effects of this phenomenon include heat, free radical formation, and high shear rates, in which the last one promotes a vegetable cell rupture, facilitating the access by the solvent to recover the target compound. As mentioned in the literature, the increasing temperature and turbulence favored genipin recovery and diffusion in the NADES solvent (Strieder et al., 2021). Although these different stresses: thermal, chemical and mechanical, respectively, can increase the extraction rate, they can slightly promote the degradation of the target compound. In fact, some authors directly apply the maximum nominal power to extract the target compound (Neves, Strieder, Vardanega, Silva & Meireles, 2020). However, by studying the minimal nominal power that can be used to recover as much as possible of the target compounds, it is possible to reduce the extraction cost and energy consumption. This complies with the

Fig. 2. Extraction yields are based on the genipin extraction rate (mg/g of genipap) obtained by solid–liquid extraction assisted by HIUS. Experiments were carried out in triplicate using 1:3 Bet:LaAc, a) with different solvent/feed (w/w) ratio (S/F) (9, 14, 19, 29) and fixing the % of water at 50 % and the acoustic power at 27 W; b) with different water concentration in the solvent (30, 40, 50, 60 %) and applying 27 W, as acoustic power and 19 as S/F; c) applying different acoustic power on HIUS extraction and using 40 % of water and 19 as S/F. Control sample: without HIUS process.
sustainability premises and avoids the target compound degradation due to the acoustic cavitation effect. Fig. 2c) shows the effect of applied acoustic power on genipin recovery efficiency when previously selected parameters were fixed. A solid–liquid extraction without HIUS assistance was performed to observe the effect of the HIUS process on genipin extraction. The HIUS-assisted extraction process influenced the recovery of genipin by the solvent (p-value < 0.001). However, even an acoustic power of 14 W was enough to present better extraction yields, and it could be possible due to the high efficiency of NADES as a genipin extraction solvent. In addition, the higher acoustic power applied probably promoted higher free radical formation, which furthers a slight chemical degradation of the genipin since the genipin is thermal stable (Strieder et al., 2021). The result is very favorable from an energy point of view, as the lower power was already sufficient to promote acoustic cavitation and increase extraction efficiency. Higher energy in the HIUS process did not increase the genipin extraction rate, probably because with 14 W, the maximum recovery of the compound had already been reached.

**Evaluation of solvent and matrix effect on genipin determination**

The matrix effect is caused by the influence of matrix composition on the target analyte’s detection signal, which can be enhanced or suppressed (Meerpohl et al., 2018). Hence, genipin quantification can be impaired by this phenomenon. In this sense, Fig. S2 (Supplementary material) shows the calibration curves for genipin in the concentration range (0–500 µg/mL) in water solvent and in *Genipa americana* L. extract obtained using water as extraction solvent, respectively. For the last one, the initial amount of genipin was quantified and excluded from the results.

As shown in Fig. S2, the matrix did not cause any effect on the genipin detection by HPLC-DAD because the calibration curve of St-Gp in water and in the genipap extract presented a similar slope.

In the same way, the NADES effect on genipin signal was also evaluated by comparing the peak areas obtained for genipin extract with and without NADES. As shown in Table S2 (Supplementary material), similarity in the range 80–100 % was obtained in all experiments, indicating that there is no significant influence of the NADESes on genipin signal that could preclude the correct determination of this compound in NADES extracts.

**Validation of the HIUS-assisted genipin extraction with NADES**

Genipin calibration curve (y = (13.81 ± 0.336)x + 95.36 ± 130.91) was obtained in the range 1–1000 µg/mL, reaching a determination coefficient of 0.9993. The repeatability of the method was evaluated inter-day and intraday. The relative standard deviation for intraday experiments was 8 % and 11 % for inter-day (n = 5). Also, the identification of the genipin was confirmed by HPLC-(ESI)-MS (Fig. 3). As can be observed, the St-Gp peak appears at 11 min and is associated with two m/z: 224.7 and 206.7 and (the green and red one, respectively), in which the first one represents the molecular ion (genipin exact mass: 226.084) and the second one is a genipin fragment related to the loss of an H₂O molecule from molecular ion (Chen et al., 2014). We could observe the same peaks at the same retention time (Rt) for the extracts Gp-NADES, Gp-H₂O₂, and St-Gp + NADES. These results confirm the effective extraction of genipin by using both H₂O and NADES solvents. For NADES extracts, three main peaks are present at a retention time of 14 min. Those peaks are associated with the m/z 304.8, 232.7, and 160.7: blue, pink, and light blue chromatograms, respectively (Fig. 2). Also, the samples in which NADES is present (Gp-NADES and St-Gp-NADES) show those peaks which should be related to the NADES signal.

**Identified compounds in NADES-genipin extract**

Table 1 summarizes the main compounds identified by RP/UHPLC-Q-TOF MS/MS in NADES-genipin extract, ordered based on their retention time. The most abundant compound was malic acid, followed by citric acid, mannitol and genipin. Other interesting compounds were also detected, such as geniposide and genipin 1-gentiobioside. These results partially agree with previous studies where genipin, geniposide and/or geniposide acid have been identified in methanolic and ethanolic extracts of *Genipa americana* L. fruits (Neri-Numa et al., 2020; Nathia-Neves et al., 2018; Bentes & Mercadante, 2014). However, in none of these works, the presence of malic acid was observed, which might be a consequence of the higher capacity of NADES to recover this compound.

**Effect of the presence of NADES on genipin properties**

As mentioned, it is known that when NADES is used as an extraction solvent, its separation from the target compound is complex due to its low volatility (Zai et al., 2014). As some authors have considered the extract with NADES as a ready-to-use extract, it is important to test if the target compound will present the same properties in the presence of NADES. For this reason, we tested the effect of the NADES on two important genipin properties: its anticholinergic activity and its cross-linking capacity.

**Anticholinergic activity**

It has been reported that genipin presents AChE and BChE inhibition capacity (Huang et al., 2019). Based on that, Gp-NADES and Gp-H₂O extracts and St-Gp were tested to evaluate the effect in AChE and BChE inhibition capacity. For enzyme activation, it is necessary a buffer solution at pH 8. However, NADES extracts present a pH of 3.0 ± 0.2 due to the presence of lactic acid in their composition. For this reason, 10 M NaOH was added to the samples until reaching the desired pH of 8. AChE and BChE enzymatic activities were expressed as % of inhibition using 66 µg/mL of genipin in each extract form. As shown in Table 2, extracts obtained with NADES present higher AChE and BChE inhibition capacity than the genipin standard at the same concentration.

In the same way, these values were also higher than the ones obtained for Gp-H₂O. These results demonstrated that not only the selected NADES did not impair the genipin’s AChE and BChE activity but enhanced it. This effect can be explained as a result of the use of natural compounds as NADES constituents, which are expected to promote a synergic bioactive effect. Additionally, the higher AChE inhibitory capacity found in NADES extract could be due to the extraction of other metabolites, such as malic acid (identified by RP/UHPLC-Q-TOF MS/MS, Table 1). This compound has also been observed in high amounts in wild edible *Silene* species (Zengin et al., 2018), where it has been related to AChE and BChE inhibitory capacity. Apart from malic acid, other genipin-related compounds, such as geniposide and genipin 1-gentiobioside, have been related to neuroprotective and antioxidant capacities (Liu, Li, Höltscher & Li, 2015; Huang et al., 2019; Neri-Numa et al., 2020; Zhang et al., 2021). These iridoid molecules act by reducing amyloid plaques, inhibiting τ phosphorylation, and preventing memory impairment and loss of synapses (Zhang et al., 2021). Despite Neri-Numa and co-authors (2020) observed in the ethanol extract that genipin was the iridoid that most contributed to the antioxidant and antiproliferative activities of *Genipa americana* L. extract. At the same time, it has to be noted that a recently published article has shown that geniposide, genipin and genipin 1-gentiobioside, among other compounds from *Gardenia jasminoides*, present hepatotoxicity in vitro (Liu et al., 2015), however, further in vivo experiments are needed to confirm those results.

**Crosslinking capacity**

In the presence of oxygen, genipin can spontaneously react with primary amines of amino acids, peptides, proteins, or other polymers which contain amine groups through a crosslinking process used for some technological applications (Neri-Numa et al., 2020). This reaction produces a blue pigment with a maximum absorbance at 590 nm (Neves, Valdés, Silva, Meireles, Ibañez, Cifuentes, 2022). Fig. 4 presents the
Fig. 3. HPLC-(ESI)-MS chromatograms (0.5 µg/mL of evaluated samples) a) St-Gp; b) genipin extracted by water (Gp-H₂O); c) genipin extracted by NADES (Gp-NADES); d) pure Bet:LaAc NADES; e) the mixture of standard genipin and Bet:LaAc NADES (St-Gp + NADES). Small boxes represent the m/z spectrum in each case.
Table 1
Tentatively identified genipin compounds and total compound contribution (%) obtained from NADES-genipin extract and analyzed by RP/UHPLC-ESI (-) Q-TOF-MS/MS.

| Tentatively identified compound | Retention time (min) | Formula | Total compound contribution (%) |
|--------------------------------|----------------------|---------|---------------------------------|
| α-Mannitol                     | 0.551                | C₉H₁₄O₅ | 13 ± 1                          |
| α-Arabinonic acid              | 0.555                | C₉H₁₄O₅ | 1.63 ± 0.09                    |
| α-Gluconic acid                | 0.556                | C₉H₁₄O₅ | 4.4 ± 0.4                       |
| Threonic acid                  | 0.566                | C₉H₁₄O₅ | 2.8 ± 0.1                       |
| α-Malic acid                   | 0.688                | C₉H₁₄O₅ | 35.4 ± 0.4                      |
| Citric acid                    | 0.933                | C₆H₈O₇  | 17.6 ± 0.8                      |
| 2-Furoic acid                  | 0.936                | C₇H₆O₂  | 1.0 ± 0.2                       |
| α-Pyroglutamic acid            | 1.051                | C₉H₁₄NO₅| 4.1 ± 0.3                       |
| Cinnamic acid                  | 1.225                | C₉H₁₄O₂  | 0.60 ± 0.04                     |
| Guanosine                      | 1.894                | C₇H₆N₅O₅| 0.11 ± 0.01                     |
| 3,4-Dihydroxybenzaldehyde      | 3.079                | C₆H₆O₅  | 0.64 ± 0.06                     |
| Genipin 1-gentiobioside         | 3.837                | C₉H₁₄O₁₅| 0.15 ± 0.01                     |
| Genipin                        | 4.478                | C₉H₁₄O₁₀| 3.90 ± 0.12                     |
| Genipin                        | 4.862                | C₉H₁₄O₁₀| 11 ± 1                          |
| Salicylic acid                 | 5.652                | C₆H₈O₃  | 0.21 ± 0.08                     |
| Phellopterin                   | 6.375                | C₇H₁₄O₁₂| 0.16 ± 0.04                     |
| (9Z)-5,8,11,14-Eicosatetraenoic acid | 8.596 | C₁₄H₂₅O₃ | 2.8 ± 0.1                       |
| 9-hydroxy-10E,12Z- octadecadienoic acid | 9.769 | C₁₄H₂₅O₃ | 0.30 ± 0.09                     |

Table 2
AChE and BChE inhibition (%) using the genipin (66 μg/mL) in standard form (St-Gp), extracted by H₂O (Gp-H₂O), and by NADES (Gp-NADES).

| Genipin (66 μg/mL) | % Inhibition | AChE | BChE |
|--------------------|--------------|------|------|
| St-Gp              | 47.2 ± 0.22  | 61 ± 1|      |
| Gp-H₂O             | 61 ± 2³     | 59 ± 3|      |
| Gp-NADES           | 73 ± 10⁴    | 63 ± 2|      |

Fig. 4. ΔAbsorbance at 590 nm of the genipin in standard form (St-Gp) and as extract with water and NADES as a solvent (Gp-H₂O) and (Gp-NADES), respectively. And, St-Gp, Gp-H₂O, and Gp-NADES added with lysine (St-Gp + Lys), (Gp-H₂O + Lys), and (Gp-NADES + Lys), respectively.

change in absorbance at 590 nm of the St-Gp, Gp-H₂O, and Gp-NADES; the increase is a measure of the genipin’s crosslinking capacity.

The absorbance did not increase in the samples without lysine (St-Gp, Gp-H₂O, Gp-NADES). However, when lysine was added, an increase in the absorbance at 590 nm was observed in all samples (St-Gp + Lys, Gp-H₂O + Lys, Gp-NADES + Lys). This increase corresponds with the formation of blue color due to the crosslinking reaction with genipin. This result proves that NADES does not impair the crosslinking capacity of genipin and, therefore, can be maintained as a ready-to-use extract.

Conclusions
An extraction solvent composed of Bet-based NADES with lactic acid (1:3) and 40 % of water (w/w) was the most promising solvent for genipin recovery. Also, the best S/F ratio was 19. This study also pointed out the HIUS as an efficient technique for NADES-based extraction of genipin, even using an acoustic power of 14 W. It means that even an acoustic power of 14 W was enough to present better extraction yields due to the high efficiency of NADES as a genipin extraction solvent. Therefore, the obtained results justify using NADES HIUS-assisted extraction as an alternative environment-friendly, acceptable cost and very efficient technique for genipin recovery.

Additionally, the obtained extract is ready-to-use because NADES did not impair the genipin crosslinking capacity or its anticholinergic activity. Additionally, the NADES as a solvent could enhance the neuroprotective potential mainly due to the capacity to extract other bioactive compounds from GeNie americana L. The results obtained in this work demonstrated the ready-to-use Gp-NADES extract potential in food, supplements, medicines, and cosmetic applications.

CRediT authorship contribution statement
Maria Isabel Landim Neves: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization. Bárbara Socas-Rodríguez: Conceptualization, Methodology, Data curation, Writing – original draft, Visualization. Alberto Valdes: Formal analysis, Writing – original draft, Writing – review & editing. Eric Keven Silva: Writing – review & editing. Alejandro Cifuentes: Conceptualization, Resources, Supervision, Funding acquisition. Angela A. Meireles: Writing – review & editing, Supervision. Elena Ibáñez: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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 data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfoch.2022.100489.

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