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Deep Eutectic Solvents as an Eco-Efficient Alternative for the Sustainable Recovery of Multitarget Alkaloids from *Bocconia frutescens*

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Abstract

Deep eutectic solvents (DESs) have emerged as sustainable alternatives to conventional organic solvents for the extraction of bioactive natural products. This study developed a green extraction strategy for recovering neuroactive alkaloids from *Bocconia frutescens* roots using DES-based systems. Thirteen DES formulations were evaluated and compared with conventional aqueous and ethanolic extraction. UHPLC-DAD and UHPLC-ESI-HRMS analyses identified sanguinarine and chelerythrine as the major benzophenanthridine alkaloids present in the extracts. Among the solvents tested, a citric acid–glucose (1:1) DES exhibited the highest extraction efficiency. Extraction parameters were optimized using a Box–Behnken response surface design, evaluating the effects of plant material-to-solvent ratio, temperature, and effect of water addition. Under optimized conditions, alkaloid recovery increased four-fold compared with the central experimental point, reaching a maximum alkaloids concentration of 400.1 ppm. The optimized alkaloid-rich extract displayed potential neuroprotective activity, inhibiting acetylcholinesterase and butyrylcholinesterase with IC_{50} values of 2.96 and 46.79 µg/mL, respectively. Additionally, the extract strongly inhibited $A\beta_{1–42}$ aggregation, exhibiting an IC_{50} value of 0.70 µg/mL. Finally, to assess the greenness of the analytical method here employed, the green analytical procedure index (GAPI) and the analytical greenness metric approach and software (AGREE version 0.5 BETA) were used, and these metrics show that the ecofriendly character is mainly based on factors such as the low sample size, the use of bio-based solvents and the lack of other hazards associated with handling toxic petroleum-derived solvents. These results demonstrate that DES-assisted extraction is an efficient and environmentally sustainable approach for obtaining alkaloid-enriched extracts from *B. frutescens* and highlight their potential as multitarget agents for Alzheimer's disease management.

Keywords: plants alkaloids; *Bocconia frutescens*; cholinesterase inhibition; amyloid-β aggregation inhibitor; alternative extraction; deep eutectic solvents (DES)

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Academic Editor: Rumyana Simeonova

Received: 4 August 2026
Revised: 27 September 2026
Accepted: 28 September 2026
Published: 13 November 2026

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1. Introduction

The multitarget approach in the search for neuroprotective drugs represents one of the most active fields in neuropharmacology. Given the multifactorial nature of neurodegenerative diseases, such as Alzheimer's disease (AD), bioactive molecules isolated from

plants capable of simultaneously modulating multiple pathological mechanisms, including oxidative stress, protein aggregation, cholinesterase inhibition, and neuroinflammatory processes, represent promising therapeutic alternatives to conventional single-target approaches [1,2]. This strategy offers significant advantages by comprehensively addressing the complex underlying pathophysiology of these disorders [3].

Within the extensive chemical diversity of natural products, plant-derived alkaloids represent one of the most valuable classes of chemical scaffolds with significant polypharmacological potential [4]. Due to the structural diversity of their nitrogen-containing frameworks, these secondary metabolites display notable affinity for multiple neural receptors and enzymatic targets, a property further enhanced by their evolutionary capacity to permeate biological barriers, including the blood–brain barrier (BBB). Prototype alkaloids such as galantamine and huperzine A have historically demonstrated the therapeutic relevance of these compounds in the treatment of central nervous system disorders [5]. More recently, accumulating evidence has indicated that alkaloids belonging to isoquinolines, and β-carbolines families may exert multitarget neuroprotective effects through the dual inhibition of key enzymes, including acetylcholinesterase (AChE) and monoamine oxidases (MAO-A/B) [6]. Concurrently, these compounds exhibit potent reactive oxygen species (ROS) scavenging activity and regulate neuroinflammatory signaling pathways, thereby highlighting their potential as multifunctional therapeutics against neurodegenerative disorders [7].

In the diverse botanical landscape, the Papaveraceae family represents one of the most chemically prolific and extensively studied chemotaxonomic sources of biologically active alkaloids. Characterized by a predominantly cosmopolitan distribution concentrated across the temperate and subtropical zones of the northern hemisphere, this family encompasses approximately 44 genera and nearly 825 accepted species [8]. Amidst this botanical diversity, the genus *Bocconia* stands out within the neotropical regions of Central and South America, with *Bocconia frutescens* L. emerging as a species of special phytochemical interest. Quantitative profiling of extracts derived from this plant has revealed a high concentration of benzylisoquinoline alkaloids, most notably those belonging to the benzo[*c*]phenanthridine subclass [9].

Although plant-derived alkaloids hold immense therapeutic promise, their recovery through conventional extraction techniques such as exhaustive maceration, acid-base extraction, and Soxhlet extractions are severely limited by a heavy reliance on volatile organic and chlorinated solvents like chloroform, dichloromethane, and methanol [10]. Beyond the substantial environmental footprint and acute occupational hazards associated with these volatile, carcinogenic, and neurotoxic vehicles, prolonged thermal exposure frequently triggers the degradation of bioactive scaffolds or the generation of structural artifacts [11]. Crucially, the persistence of trace residual solvents within the final matrix introduces a major confounding variable, as intrinsic in vitro cytotoxicity can obscure the genuine neuroprotective efficacy of the isolated metabolites [12]. Accelerating the transition toward green chemistry by leveraging ecofriendly solvent systems and high efficiency assisted extraction technologies is therefore imperative to ensure process sustainability while safeguarding the chemical purity and diagnostic validity of subsequent biological assays.

Deep eutectic solvents (DES) have emerged as one of the most promising alternatives within green chemistry. These systems, formed by combining a hydrogen bond acceptor and a hydrogen bond donor in specific molar ratios, stand out for their low volatility, high thermal stability, biodegradability, and low toxicity [13]. Beyond their environmental advantages, DES offer exceptional physicochemical versatility, allowing their properties adapted to the nature of the target metabolite. This not only optimizes yield and selectivity in the extraction of complex compounds like alkaloids [14], but is also key to preserving,

and even enhancing, the stability and biological effect of the resulting extracts, positioning them as revolutionary tools in natural products research. The aim of this study was to implement an extraction method for active alkaloids from *Bocconia frutescens* compounds with recognized multitarget activity using deep eutectic solvents in order to maintain or enhance their biological activity.

2. Results and Discussion

2.1. Analysis of Bocconia frutescens *Extracts and Characterization of Major Metabolites*

The family Papaveraceae constitutes a major reservoir of biologically active alkaloids, with its alkaloidal profile driving the primary medicinal properties attributed to these species [15]. Extracts derived from *Bocconia frutescens* (Papaveraceae) have historically demonstrated insecticide, antitussive, anti-inflammatory, antitumor, and antibacterial activities [16]. Given that the secondary metabolites isolated from the *Bocconia* genus belong to the benzophenanthridine, protoberberine, and protopine structural classes, the chemical exploration of this species holds critical relevance for neurodegenerative disease research [17].

Based on these considerations, thirteen deep eutectic solvents (DESs) were evaluated in this study to develop a selective method for alkaloid extraction, alongside conventional extraction procedures employing ethanol and water. The compositions and molar ratios of the evaluated DESs are listed in Table 1.

Table 1. DES constituents used for extraction of *Bocconia frutescens* roots *.

DES	DES Constituents		Molar Ratio
1	ChCl	Sorbitol	(4:1)
2		Fructose	(5:2)
3		Urea	(1:2)
4		Oxalic acid	(1:1)
5		1,2-propanediol	(1:2)
6	Citric Acid	Glucose	(1:5)
7		Glucose	(1:1)
8		Malic Acid	(1:1)
9		L-Proline	(1:1)
10		Saccharose	(1:1)
11		Fructose	(1:1)
12		Sorbitol	(1:1)
13	Oxalic acid	L-Proline	(1.1)

* A total of 100 mg of plant material was extracted with 5 g de DES.

After extraction, the eutectic solvents were recovered by solid-phase extraction (SPE), and the resulting extracts were analyzed by HPLC-DAD, with chromatograms monitored at 330 nm, a wavelength commonly used for alkaloid detection [18,19]. The first set of experiments was run at 20 °C Chromatographic analysis revealed the presence of four detectable compounds. The chromatographic profiles were dominated by two major constituents, which were subsequently characterized by UHPLC-ESI-HRMS. Accurate mass measurements, and MS/MS fragmentation analyses enabled their tentative identification as the benzophenanthridine alkaloids sanguinarine and chelerythrine. The observed fragment ions at m/z 317, 304, 274, 246, 218, and at m/z 333, 304, 290, 274, respectively, were consistent

with fragmentation patterns previously reported for these compounds isolated from the roots of species belonging to the genus *Bocconia* [20–22].

Both compounds have previously been reported in *Bocconia frutescens*, supporting the reliability of the analytical approach and confirming the selectivity of the extraction solvents toward the major bioactive alkaloids of the species. Figure 1 shows a representative chromatographic profile together with the mass spectra of the two major compounds extracted using the different solvents.

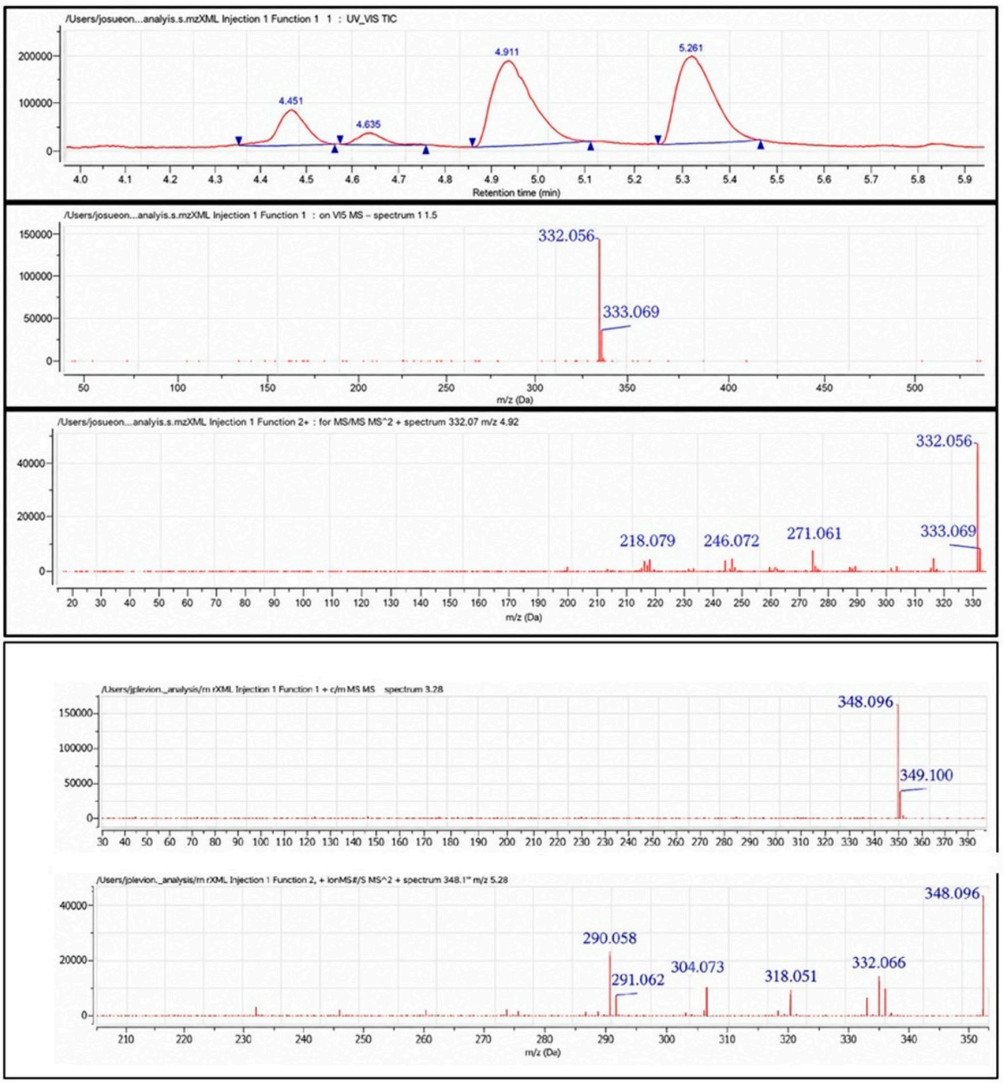


Figure 1. (**a**) UHPLC-DAD chromatographic profile recorded at 330 nm of the extract obtained using the Citric acid-Glu (1:1) DES; (**b**) positive-ion ESI mass spectrum corresponding to the compound eluting at 4.9 min; (**c**) MS/MS fragmentation pattern of the ion at m/z 332; (**d**) positive-ion ESI mass spectrum corresponding to the compound eluting at 5.2 min; and (**e**) MS/MS fragmentation pattern of the ion at m/z 348.

Deep eutectic solvents (DESs) previously reported in the literature for the extraction of alkaloidal metabolites [23,24], together with additional DESs exhibiting acidic properties, were evaluated in this study. Following the assessment of their selectivity toward alkaloids associated with potential neuroprotective activity, the extraction performance of each DES was determined by quantifying the recovery of sanguinarine and chelerythrine. This screening aimed to identify the most effective solvent system for the extraction of bioactive alkaloids from the roots of *Bocconia frutescens*.

Among the first group of choline chloride-based DESs (DES 1–5), a comparatively low extraction efficiency was observed. However, within this group, DES 4, which incorporated an acidic component, exhibited the highest recovery of both alkaloids. This observation suggested that solvent acidity could play a key role in the extraction process, potentially by enhancing alkaloid solubilization and promoting favorable intermolecular interactions between the solvent and the target compounds. Based on this premise, a second group of citric acid-based DESs (DES 6–12) was subsequently evaluated.

A substantial improvement in alkaloid recovery was observed for the citric acid-based DESs, as evidenced by the increased chromatographic peak areas of sanguinarine and chelerythrine. Among the evaluated systems, DES 7 (citric acid–glucose, 1:1 molar ratio) displayed the highest extraction efficiency, indicating a particularly favorable solvent environment for alkaloid solubilization. In contrast, the oxalic acid-based DES exhibited the lowest extraction capacity. These results highlight the importance of DES composition in determining extraction performance and suggest that the extraction efficiency is strongly influenced by the nature of the intermolecular interactions established between the solvent components and the alkaloid molecules.

The superior performance of the citric acid-based systems may be attributed to their enhanced ability to establish extensive hydrogen-bonding networks, which can facilitate interactions with the nitrogen-containing functional groups present in benzophenanthridine alkaloids. In addition, the acidic character of these DESs may contribute to improved solvation of the target compounds, thereby increasing their extraction efficiency. Conversely, the lower performance observed for the oxalic acid-based DES suggests that solvent acidity alone is insufficient to guarantee efficient extraction, and that the balance between acidity, hydrogen-bond donor/acceptor capacity, and the overall physicochemical properties of the eutectic system plays a decisive role. Based on these findings, the most promising DES systems were selected for further evaluation of the effect of extraction temperature on alkaloid recovery.

To investigate the influence of temperature on extraction efficiency, all DESs (DES 1–13) were evaluated at 40 °C. These conditions were selected to ensure the physicochemical stability of the eutectic systems while allowing the assessment of temperature-dependent extraction performance. The maximum temperature was limited to 40 °C to minimize the risk of disrupting the eutectic structure and to avoid potential thermal degradation of both the solvent components and the target alkaloids. The extraction efficiencies of the evaluated solvent systems are presented in Figure 2 and are expressed as the chromatographic peak areas (mUA) of the major alkaloids, sanguinarine and chelerythrine, determined by UHPLC-DAD.

The superior extraction performance observed for the citric acid-based deep eutectic solvents (DESs) can be attributed to their physicochemical characteristics and favorable interactions with alkaloidal compounds. Benzophenanthridine alkaloids, such as sanguinarine and chelerythrine, contain basic nitrogen atoms that are readily protonated under acidic conditions, thereby enhancing their solubility in the extraction medium. Moreover, the results underscore the critical influence of DES composition on extraction efficiency, as the molar ratio of the constituent components directly determines key solvent properties. Variations in the relative proportions of hydrogen-bond donors and acceptors can significantly affect viscosity, polarity, acidity, and hydrogen-bonding capacity, which in turn influence mass-transfer processes and solvent–solute interactions during extraction. Therefore, the differences observed in alkaloid recovery among the evaluated DESs are likely associated with changes in these physicochemical parameters arising from variations in solvent composition [25–27].

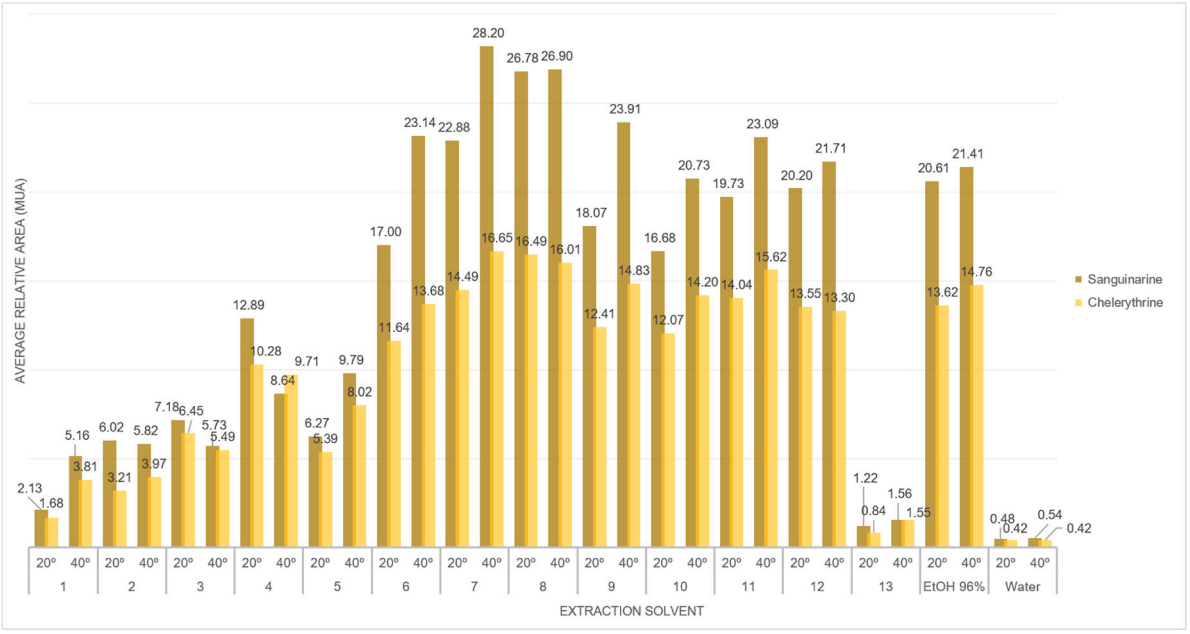


Figure 2. Average relative area of identified compounds in DESs extracts, ethanolic extract, and water extract.

To further evaluate the influence of the extraction conditions on alkaloid recovery, the effects of extraction method and temperature were statistically assessed using a two-way analysis of variance (ANOVA), considering extraction method and temperature as fixed factors and including their interaction. The analysis demonstrated that both factors significantly influenced the recovery of the two target alkaloids. For sanguinarine, significant effects were observed for extraction method ($F(14, 60) = 6976.56$, $p < 0.0001$) and temperature ($F(1, 60) = 1101.42$, $p < 0.0001$). Moreover, a significant extraction method × temperature interaction was detected ($F(14, 60) = 177.68$, $p < 0.0001$), indicating that the effect of temperature on sanguinarine recovery depended on the extraction system employed.

Similarly, chelerythrine recovery was significantly affected by extraction method ($F(14, 60) = 4431.26$, $p < 0.0001$) and temperature ($F(1, 60) = 580.87$, $p < 0.0001$). A significant extraction method × temperature interaction was also observed ($F(14, 60) = 54.22$, $p < 0.0001$), demonstrating that the response to temperature was dependent on the extraction system.

Considering the significant interaction effects observed for both alkaloids, Tukey's HSD post hoc test was applied to compare the individual extraction method–temperature combinations. The results are expressed as mean ± standard deviation ($n = 3$), and different letters indicate statistically significant differences among extraction conditions according to Tukey's HSD test ($p < 0.05$), as presented in Table S1.

A particularly relevant finding arising from this analysis is that increasing the extraction temperature from 20 to 40 °C did not necessarily improve alkaloid recovery across all DES systems. This behavior is clearly illustrated by the results obtained for sanguinarine. For DES7, increasing the temperature resulted in a marked increase in sanguinarine recovery, from 22.88 ± 0.37 at 20 °C to 28.20 ± 0.45 at 40 °C. In contrast, DES8 showed comparable recoveries at 20 °C (26.78 ± 0.43) and 40 °C (26.90 ± 0.43), with no statistically significant difference according to Tukey's HSD test. An opposite trend was observed for DES4, for which sanguinarine recovery decreased from 12.89 ± 0.20 at 20 °C to 8.64 ± 0.14 at 40 °C.

These contrasting responses are consistent with the highly significant extraction method × temperature interaction identified by the two-way ANOVA and demonstrate that the influence of temperature on alkaloid recovery is strongly dependent on the extraction system employed. Thus, temperature cannot be considered an independent determinant of

extraction efficiency; rather, its effect must be interpreted in the context of the specific DES formulation. These findings provide additional insight into the extraction behavior of the different DES systems and strengthen the interpretation of the experimental results.

2.2. Effect of Extraction Parameter Modifications on the Sanguinarine and Chelerythrine Recovery

Given that the citric acid–glucose (1:1) DES exhibited the highest extraction efficiency in terms of sanguinarine and chelerythrine recovery, its performance was further investigated by evaluating the effect of modifying key extraction parameters on the profile of these major metabolites. The parameters assessed included water content in the eutectic solvent, extraction time, plant material-to-solvent ratio, and extraction temperature. This study aimed to identify the appropriate experimental ranges and the most influential variables for subsequent optimization of the extraction method. As established in Section 2.1, increasing the extraction temperature enhances alkaloid recovery; therefore, it is essential to include this parameter as an experimental variable to determine the optimal extraction conditions through the experimental design approach described in the following sections.

2.2.1. Influence of Water Addition to the DES on the Sanguinarine and Chelerythrine Recovery

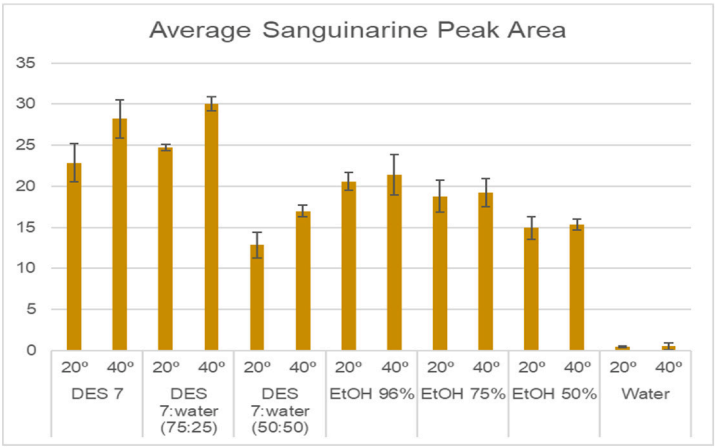
The extraction efficiency of the DES 7 was evaluated for the recovery of sanguinarine and chelerythrine after the addition of 25% and 50% (w/w) water to the solvent system. Extractions were performed in triplicate for each solvent under investigation at two temperatures, 20 °C and 40 °C. The performance of the DES formulations was compared with that of conventional ethanol–water mixtures containing equivalent proportions of water, as well as with water used as the sole extraction solvent. Figure 3 summarizes the chromatographic peak areas of the two major alkaloids obtained in extraction system at both temperatures.

The incorporation of water into deep eutectic solvents (DESs) is a widely employed strategy to enhance extraction efficiency by reducing solvent viscosity and improving mass transfer within the plant matrix. Dai et al. demonstrated that moderate water addition significantly decreases the viscosity of DESs while preserving their supramolecular structure [28], thereby facilitating solute diffusion and enhancing extraction performance. However, excessive water content progressively weakens the hydrogen-bonding network responsible for maintaining the eutectic structure.

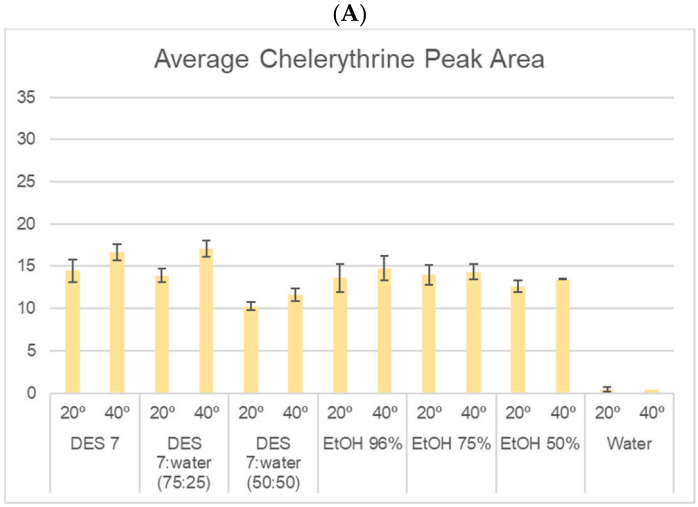
Statistical analysis by ANOVA demonstrated that both the extraction solvent and temperature significantly influenced the recovery of sanguinarine and chelerythrine. The extraction solvent was the most influential factor, exerting a highly significant effect on sanguinarine ($F = 212.64$, $p < 0.0001$) and chelerythrine ($F = 161.36$, $p < 0.0001$) extraction. Temperature also significantly affected the recovery of sanguinarine ($F = 25.88$, $p < 0.0001$) and chelerythrine ($F = 16.69$, $p = 0.0003$). A significant solvent × temperature interaction was observed for sanguinarine ($F = 3.97$, $p = 0.0053$), indicating that the effect of temperature depended on the extraction medium employed. In contrast, no significant interaction was detected for chelerythrine ($F = 1.66$, $p = 0.167$), suggesting a more consistent response of this alkaloid to temperature across the evaluated solvents. The results are expressed as mean ± standard deviation ($n = 3$), and different letters indicate statistically significant differences among extraction conditions according to Tukey's HSD test ($p < 0.05$), as presented in Table S2.

These results allowed us to conclude that the formulation containing DES-water 75:25 provided the greatest recovery of sanguinarine, while both the neat DES and the DES-water 75:25 yielded the highest concentrations of chelerythrine. These findings were further supported by Tukey's post hoc analysis, which confirmed that DES 7 systems formed the highest statistical groups for alkaloid recovery. For sanguinarine, the DES 7-water 75:25

at 40 °C produced the highest extraction yield and was statistically comparable to the neat DES 7 at the same temperature, with both treatments significantly outperforming the hydroalcoholic mixtures and water. Similarly, for chelerythrine, the DES 7-water 75:25 and neat DES 7 at 40 °C constituted the highest statistical group, whereas aqueous extracts resulted in significantly lower recoveries. In all cases, water was the least effective extraction medium, consistently yielding the lowest concentrations of both alkaloids. The DES 7-water 75:25 at 40 °C showed the highest mean recovery for both alkaloids. However, these values were not significantly different from those obtained with the corresponding undiluted DES at 40 °C, as both treatments shared the same statistical significance group (a) according to Tukey's HSD test ($p < 0.05$), (see Table S2).



(**A**)



(**B**)

Figure 3. Average Peak areas of the major alkaloids (mUA) (**A**) Sanguinarine, (**B**) Chelerythrine, obtained using citric acid–glucose (1:1) deep eutectic solvents containing different water contents, compared with conventional extraction solvents.

Based on the findings obtained in this study and the experimental procedure employed, the DES 7–water ratio was selected within the range of 50:50 to 80:20 for further optimization using a Box–Behnken experimental design. This interval was chosen because it provided distinct extraction yields, allowing a comprehensive evaluation of the effect of water addition on alkaloid recovery. Furthermore, assessing this factor in combination with the other experimental variables enabled the identification of the optimal extraction conditions and a better understanding of the interactions governing the extraction process.

2.2.2. Effect of Extraction Time on Sanguinarine and Chelerythrine Recovery

The influence of extraction time on the performance of the selected deep eutectic solvent (DES) was investigated by evaluating the recovery of the major alkaloids. Extractions were performed in triplicate at five extraction times ranging from 30 to 150 min. Conventional solvents were not considered at this stage, as DES 7 had previously exhibited the highest extraction efficiency, surpassing both water and ethanol. Consequently, the effect of extraction time was assessed exclusively using this solvent system to identify the conditions that maximize alkaloid recovery.

The experiments were conducted at 20 °C using a DES 7–water ratio of 75:25. This composition was selected because the addition of water reduces the viscosity of the DES, thereby improving mass transfer and facilitating solvent penetration into the plant matrix while maintaining the intermolecular interactions responsible for alkaloid solubilization. Figure 4 shows the mean chromatographic peak areas of sanguinarine and chelerythrine obtained at the extraction times evaluated. These results provide insight into the time required to achieve maximum recovery under the selected conditions.

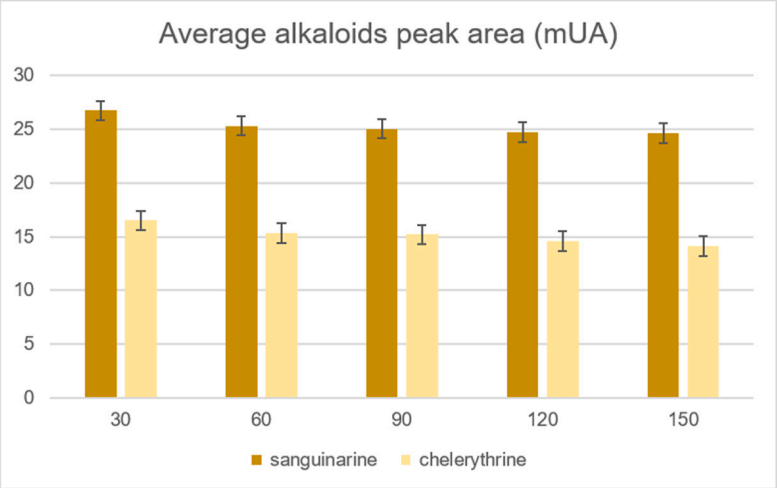


Figure 4. Effect of time extraction on amount of major *Bocconia frutescens* alkaloids recovery.

The influence of extraction time on the recovery of sanguinarine and chelerythrine was evaluated over the range of 30–150 min. As shown in Figure 4, the average peak area of both alkaloids decreased progressively with increasing extraction time. Sanguinarine peak area decreased from 26.75 ± 0.90 mUA at 30 min to 24.62 ± 0.92 mUA at 150 min, whereas chelerythrine decreased from 16.50 ± 0.90 mUA to 14.15 ± 0.92 mUA over the same period. One-way ANOVA indicated that extraction time did not significantly affect the recovery of sanguinarine ($F = 2.69$, $p = 0.093$) or chelerythrine ($F = 2.93$, $p = 0.077$). Consistently, Tukey's HSD post hoc test showed no significant differences among the five extraction times evaluated ($p > 0.05$) (See Table S3). Therefore, extending the extraction period beyond 30 min did not result in a statistically significant improvement in alkaloid recovery. Considering that 30 min provided the highest mean recovery for both target alkaloids while requiring the shortest extraction period, this condition was selected for subsequent experiments, thereby improving the overall time efficiency of the extraction process.

These results suggest that extraction equilibrium was reached within the first 30 min and that extending the extraction period does not improve alkaloid recovery. Similar behavior has been reported for the extraction of benzophenanthridine alkaloids from *Macleaya cordata*, where maximum extraction yields were achieved at relatively short extraction times and no significant improvements were observed thereafter [29]. The authors reported

that microwave-assisted extraction enabled efficient recovery of sanguinarine and chelerythrine within only a few minutes, demonstrating the rapid mass-transfer kinetics of these compounds. Likewise, studies focused on the separation and purification of *M. cordata* alkaloids have shown that once solvent–matrix equilibrium is achieved, prolonged processing times do not enhance recovery and may even promote degradation or loss of target compounds [30]. Therefore, the present results indicate that an extraction time of 30 min is sufficient to maximize the recovery of sanguinarine and chelerythrine using selected DES, while longer extraction times offer no analytical advantage.

2.2.3. Effect of Vegetal Material-Solvent Ratio on Chelerythrine Recovery

The effect of the plant material-to-solvent ratio on extraction efficiency was evaluated using chelerythrine recovery as a marker compound. Quantification of the alkaloid was performed using an analytical standard of chelerythrine chloride (PhytoLab®). Seven different plant material: DES (DES7-water 75:25) ratios were assayed, while the extraction temperature was maintained at 40 °C. This temperature was selected based on previous experiments, in which the highest recovery levels of the major alkaloids identified in *Bocconia frutescens* root were obtained. Figure 5 presents the amount of chelerythrine extracted under each solid-solvent ratio evaluated, expressed as concentration (ppm).

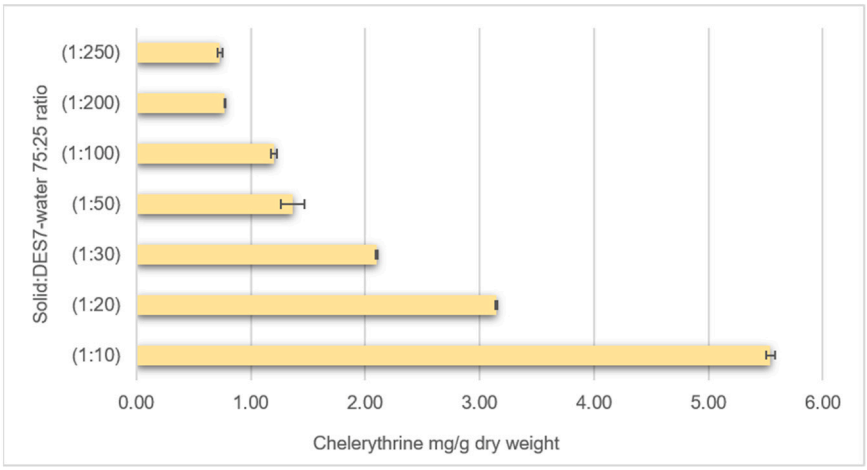


Figure 5. Chelerythrine recovery at different plant material-to-solvent ratios.

The solid-to-solvent ratio significantly affected chelerythrine recovery (one-way ANOVA, $F(6, 14) = 4493.41$, $p < 0.0001$). The highest chelerythrine content was observed at a solid-to-solvent ratio of 1:10 (5.543 ± 0.042 mg/g DW), which was significantly higher than those obtained at all other ratios evaluated according to Tukey's HSD test ($p < 0.05$). Chelerythrine content progressively decreased as the solvent proportion increased, reaching 0.773 ± 0.006 and 0.730 ± 0.020 mg/g DW at ratios of 1:200 and 1:250, respectively. No significant difference was observed between these latter two conditions (Tukey's HSD, $p = 0.8875$) (See Table S4). These results indicate that increasing the solvent-to-solid ratio beyond the evaluated lower ratios did not improve chelerythrine recovery under the experimental conditions.

The observed decrease in chelerythrine recovery may be partly attributed to the dilution effect associated with increasing solvent volume. As the amount of DES increases, the analyte concentration in the resulting extract may decrease even when the total amount transferred from the plant matrix remains relatively unchanged. In addition, changes in the solid-to-liquid ratio can modify the partition equilibrium between the plant matrix and the extraction medium, thereby influencing the concentration gradient and the driving force

for mass transfer. These combined effects may account for the differences in chelerythrine recovery observed across the evaluated solid-to-liquid ratios.

Previous studies have identified the solid-to-liquid ratio as one of the most influential parameters in DES-based extraction systems [31], demonstrated that changes in solvent loading significantly affected the alkaloid recovery from lotus leaves, highlighting the importance of optimizing this parameter to maximize extraction performance. Likewise Dai et al. reported that the physicochemical properties of DES [28], particularly viscosity and hydrogen-bonding interactions, strongly influence solute diffusion and extraction efficiency. Therefore, optimization of the plant material-to-solvent ratio is essential to achieve efficient mass transfer while minimizing solvent consumption. The results obtained in the present study indicate that a 1:10 ratio provides the most favorable conditions for obtaining chelerythrine-enriched extracts from *Bocconia frutescens* under the experimental conditions evaluated.

2.3. Analytical Performance of Chelerythrine Quantification

The external calibration curve for chelerythrine showed excellent linearity over the investigated concentration range of 0.2–1000 ppm, with the regression equation $y = 0.174245x + 0.283888$ and a coefficient of determination of $R^2 = 0.999967$. Instrumental repeatability was satisfactory across the calibration range, with RSD values for the three independent injections ranging from 1.20 to 1.94%. The estimated instrumental LOD and LOQ were 0.156 and 0.472 ppm, respectively, indicating adequate instrumental sensitivity for chelerythrine determination under the chromatographic conditions employed.

These analytical parameters support the linearity, sensitivity, and injection repeatability of the HPLC-DAD measurements used for chelerythrine quantification and subsequent extraction optimization. However, recovery experiments were not performed; consequently, the accuracy of the complete analytical procedure was not independently established, and the method should not be regarded as fully validated. This limitation should be considered when interpreting the absolute quantitative values. Nevertheless, the analytical performance established for the calibration and instrumental measurements supports the use of chelerythrine content as a comparative response variable for evaluating extraction conditions within the experimental framework of this study. The analytical validation data is presented in Table S6.

2.4. Optimization of Deep Eutectic Solvent-Based Extraction Conditions for Alkaloid Recovery from Bocconia frutescens

Optimization of the extraction conditions is an important step toward maximizing the recovery of sanguinarine and chelerythrine from *Bocconia frutescens*. These benzophenanthridine alkaloids exhibit a broad spectrum of biological activities that support their application as potential neuroprotective agents. Recent evidence indicates that sanguinarine possesses neuroprotective, anti-inflammatory, and antioxidant properties through the modulation of multiple cellular signaling pathways involved in the pathogenesis of neurodegenerative disorders [32]. In particular, the regulation of oxidative stress and neuroinflammatory processes has attracted considerable attention because of their central role in the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Consequently, the development of efficient extraction strategies is relevant for obtaining extracts enriched in these bioactive compounds and facilitating their subsequent biological evaluation.

In this context, response surface methodology (RSM) is widely used for the optimization of extraction processes involving natural products [33]. Among RSM approaches, the Box–Behnken design (BBD) allows linear, quadratic, and interaction effects among experimental factors to be evaluated using a relatively small number of experimental runs

compared with full factorial designs [34,35]. In the present study, BBD was used to investigate the effects of plant material concentration (A), extraction temperature (B), and DES content in the DES–water mixture (C) on chelerythrine recovery. This approach enabled the construction of a quadratic response surface model and the exploration of the experimental domain to identify conditions associated with maximum chelerythrine recovery.

The chelerythrine content obtained across the 15 experimental runs ranged from 0.725 to 40.010 mg/g DW, as shown in Table 2.

Table 2. Box–Behnken experimental design matrix and experimental chelerythrine content.

Nº.	A (mg/mL)	B (°C)	C (DES:Water)	Chelerythrine Content, D (mg/g DW)
1	35	20	80:20	1.474
2	10	40	65:35	25.520
3	35	30	65:35	2.694
4	10	30	50:50	40.010
5	35	20	50:50	2.811
6	35	30	65:35	2.646
7	10	20	65:35	32.020
8	60	40	65:35	1.362
9	35	40	50:50	5.937
10	10	30	80:20	35.190
11	35	40	80:20	2.786
12	35	30	65:35	2.600
13	60	20	65:35	0.725
14	60	30	50:50	0.977
15	60	30	80:20	0.803

The experimental data were fitted to the following quadratic equation expressed in terms of the actual factor values:

$$D = 96.82169 - 2.641982A + 1.048794B - 1.524935C + 0.0226023A^2 + 0.00713667AB + 0.00309778AC - 0.0186643B^2 - 0.00302381BC + 0.0109862C^2$$

where D is the chelerythrine content (mg/g DW), A is the plant material concentration (mg/mL), B is the extraction temperature (°C), and C is the DES content in the DES–water mixture (%).

The overall quadratic model was statistically significant ($F = 29.88$, $p = 0.000801$) and explained 98.17% of the observed response variability ($R^2 = 0.9817$). The adjusted and predicted coefficients of determination were 0.9489 and 0.7079, respectively. The RMSE was 3.28 mg/g DW, and the PRESS value was 858.37. Among the individual model terms, only the linear effect of plant material concentration (A) and its quadratic term (A^2) were statistically significant ($p < 0.001$). Temperature (B), DES content (C), the remaining quadratic terms, and the interaction terms were not statistically significant at $p < 0.05$. These results indicate that plant material concentration was the predominant factor affecting chelerythrine recovery within the investigated experimental domain.

Despite the statistical significance of the overall regression and its high R^2, the lack of fit was significant ($F = 8094.07$, $p = 0.000124$). The pure error estimated from the three replicated center points was very small (SS = 0.00442), which resulted in a particularly stringent lack-of-fit test. Nevertheless, the significant lack of fit indicates that the quadratic

equation did not capture all systematic variation within the experimental domain. In addition, the difference between the adjusted R^2 (0.9489) and predicted R^2 (0.7079) indicates lower predictive performance than would be inferred from R^2 alone. Accordingly, the model should be interpreted cautiously and used primarily to describe the overall response surface and identify a potentially favorable experimental region rather than as a fully validated predictive model.

Predicted conditions and experimental comparison

Within the evaluated experimental domain, numerical optimization of the quadratic model predicted a maximum chelerythrine content of approximately 38.01 mg/g DW at 10 mg/mL plant material concentration, 26.0 °C, and a 50:50 DES: water ratio. The closest experimental condition included in the BBD was 10 mg/mL, 30 °C, and a 50:50 DES: water ratio. Under these conditions, the experimental chelerythrine content was 40.010 mg/g DW, whereas the model predicted 37.70 mg/g DW. The absolute difference between the experimental and predicted responses was 2.31 mg/g DW, corresponding to a relative deviation of approximately 5.8% with respect to the experimental value.

Although the experimental and predicted values were relatively close under these conditions, this experimental run was included in fitting the quadratic model and therefore cannot be considered an independent model-validation experiment. Furthermore, no additional experiment was performed at the exact model-predicted conditions (10 mg/mL, 26.0 °C, and 50:50 DES:water). Consequently, the calculated optimum should be regarded as a model-based exploratory prediction, and the absence of independent experimental validation represents a limitation of the present study.

The response surface indicated that the region associated with the highest chelerythrine recovery was characterized primarily by a low plant material concentration (10 mg/mL), consistent with the significant linear and quadratic effects observed for factor A. Although the predicted optimum also occurred at a 50:50 DES:water ratio and a moderate temperature, the individual effects of DES content and temperature were not statistically significant within the investigated ranges. Therefore, specific mechanistic effects of these variables cannot be established from the BBD alone. Nevertheless, the high chelerythrine recovery experimentally observed under these conditions, together with the preceding solvent-screening results, supports the potential of the citric acid–glucose DES–water system for the recovery of benzophenanthridine alkaloids from *Bocconia frutescens*.

The application of DESs to alkaloid extraction within the Papaveraceae family remains scarcely explored. To the best of our knowledge, no previous independent studies have reported the use of DESs for alkaloid extraction from species of the genus *Bocconia*. The most directly comparable study was reported by Strzemski et al. [36], who evaluated volatile natural deep eutectic solvents based on menthol, camphor, and thymol for the extraction of isoquinoline alkaloids from the roots and aerial parts of *Chelidonium majus*. Appropriate menthol–camphor and menthol–thymol formulations increased the recovery of protopine, chelidonine, berberine, chelerythrine, and coptisine by 16%, 35%, 76%, 12%, and 180%, respectively, compared with acidified methanol, whereas sanguinarine recovery was comparable between the extraction systems. These results support the suitability of eutectic systems for recovering isoquinoline alkaloids from members of the Papaveraceae family.

More recently, a hydrophobic NADES-based liquid–liquid microextraction procedure was applied to the analytical determination of opium alkaloids in beverages prepared from *Papaver somniferum* seeds [37]. However, because that procedure involved analytical microextraction from a liquid food matrix rather than direct extraction from solid plant material, its extraction performance is not quantitatively comparable with that of the present method.

Although these reports provide relevant contextual benchmarks, a strict quantitative comparison among studies is limited by differences in plant species and tissues, endogenous alkaloid concentrations, sample pretreatment, DES composition, solid-to-liquid ratio, extraction time and temperature, analytical methodology, and the units used to report extraction yield. Therefore, the present results do not establish the universal superiority of DES-assisted extraction over previously published methods. Rather, they demonstrate that, under the controlled conditions evaluated in this study, the optimized citric acid–glucose DES system provided greater recovery of sanguinarine and chelerythrine than the aqueous and alcoholic extraction systems tested using the same batch of *B. frutescens* roots and the same analytical procedure.

2.5. Green Analytical Chemistry Metrics

For evaluating the greenness of the analytical method here employed and to assess the possible adverse effects on the human safety and health, as well as for the environment, we have used the green analytical procedure index (GAPI) as a green semi-quantitative metric, and the analytical greenness metric approach and software AGREE which is derived from the 12 principles of green analytical chemistry (GAC).

GAPI, proposed by Płotka-Wasylka [38], allows to evaluate the greenness of different stages of the analytical assay employing the GAPI pictogram. This pictogram is composed of five pentagrams scaled with colors indicating the different levels of impact (green for low, yellow for medium and red for high). The first three pentagrams of the GAPI pictogram are associated with sample preparation. According to our method, the first part of the first pentagram (number 1) is marked with a red color corresponding to an off-line sample collection, the second part marked with green is associated with sample preservation (not needed), the third part related with sample transportation is marked with yellow (required), and as sample storage is not needed, the number 4 is marked in green (Figure 6a). The second pentagram (number 5) is red because an extraction procedure is needed for sample processing, and the circle placed inside means that the analytical assay could be used to both qualitative and quantitative analyses. The third pentagram (numbers 6 to 8) is associated with different stages of sample processing and is marked as red for number 6 (macroextraction, 100 mg of sample), red for number 7 (MeOH is used in the extraction stage) and yellow for number 8 (SPE is an additional sample processing). The fourth pentagram is in yellow and illustrates that the volume of solvents is in the range of 10–100 mL (a total of 46.8 mL were used, where 10 mL are from the DES:H_2O mixture, 20 mL are from SPE and 16.8 mL are from LCMS analysis) and the health and safety hazards of the employed solvents have highest NFPA scores of 3 (health hazard NFPA = 1 methanol, 2 citric acid, 3 formic acid; safety hazard NFPA = 2 methanol, 1 citric acid, 2 formic acid). Finally, the fifth pentagram associates energy consumption by the instruments (number 12), occupational hazards (number 13), wastes produced (number 14), and their treatment (number 15). The energy consumption is marked with red (>1.5 kWh per sample for HPLC-DAD and LCMS), the occupational hazard is marked with green (hermetization of analytical process), the volume of waste is >10 mL (cc. 16.8 mL LCMS) and it is not treated which gives red marks for numbers 14 and 15.

On the other hand, AGREE metric, proposed by Pena-Pereira et al. [39], is calculated through the assessment of the 12 GAC principles and is scored on a 0 to 1 scale. The pictogram of AGREE represents the 12 principles of GAC as 12 segments around the central circle which reveals both the color (green to red) and the final score of the greenness evaluation results. In our work, the pictogram representing the result of the assessment is presented in Figure 6b.

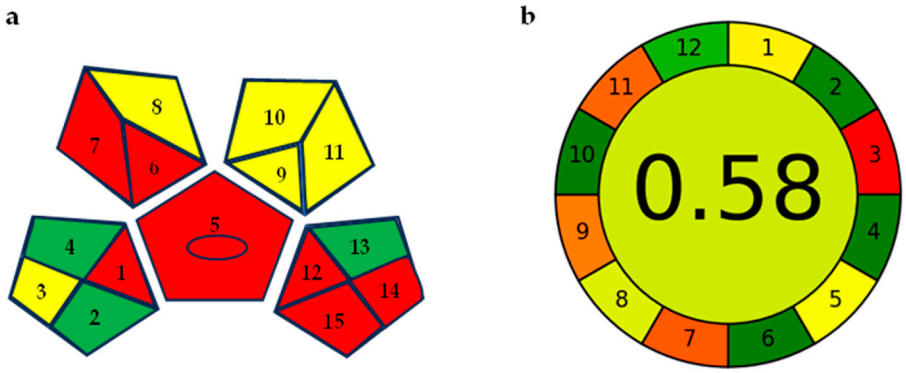


Figure 6. Pictograms of green analytical chemistry metrics. (**a**) GAPI, (**b**) AGREE.

As shown in Figure 6b the segments 2 (minimal sample size), 4 (number of steps involved in the sample preparation), 6 (derivatization), 10 (reagents from renewable sources) and 12 (safety of operators) are in line with the GAC principles: 100 mg of sample is used, few steps are required for sample preparation (extraction, SPE and solvent evaporation), no derivatization is needed, renewables are used (citric acid, glucose, formic acid and methanol) and MeOH is used in the SPE procedure. Next, segments 1 (sampling procedure) and 5 (automatization and sample preparation) show some drawbacks (off-line analysis, the method is not automatized and miniaturized), while segment 8 (multi-analyte) represents a benefit (four analytes are determined in a single run). Finally, the less favorable segments are 3 (measurements in situ), 7 (generation of analytical waste), 9 (use of energy) and 11 (use of toxic reagents). These results are obtained because the measurement is off-line, about 47 mL of waste is generated (extraction 10 mL, SPE 20 mL and LCMS 16.8 mL), about 1.15 kWh are consumed for a single LCMS analysis, and a total of 9.93 mL of toxic solvents are used in SPE and LCMS (MeOH and formic acid).

Although the AGREE calculation excludes information from the pre-extraction procedures (the health and safety hazards for compounds and solvents, energy consumption, and waste generated) and does not consider the greenness of sample preparation, these parameters are included in the GAPI approach which make these two metrics complementary. As the identification and quantification of chelerythrine from extracts of roots of *B. frutescens* has not been reported before, a comparison with other methods is not possible. However, from the results obtained with these two metrics, and considering that a direct analysis of chelerythrine in the plant material is implausible, we can conclude that the reduced energy and material inputs required for sample preparation, the low sample size and low amount of solvent required in the extraction, the possibility to apply bio-based solvents, and the lack of other hazards associated with the use of oil-derived solvents such as hexane, DCM and $CHCl_3$ widely used for the obtention of extracts from of *B. frutescens* [15] are the key factors that highlight the green character of the method described here.

2.6. Potential Neuroprotective Activity of the Bocconia frutescens Alkaloid Extract

The alkaloid-rich extract obtained under the optimal extraction conditions established through the Box–Behnken experimental design described in Section 2.3 (10 mg/mL, 33 °C, and DES–water ratio of 50:50), was evaluated against biological targets associated with Alzheimer's disease, including cholinesterase activity and amyloid-β peptide aggregation. These assays were conducted to assess the potential neuroprotective activity of the extract recovered using the selected deep eutectic solvent (DES) and to determine its potential as a source of multitarget bioactive compounds relevant to the prevention and management of neurodegenerative disorders.

Direct bioautography assays against both cholinesterases revealed inhibitory activity for the *Bocconia frutescens* root extracts. Enzyme inhibition was evidenced by the presence of clear zones on the TLC plates, see Figure S1. Notably, the extract obtained with the DES produced more intense inhibition zones than the ethanolic extract, indicating a higher concentration of cholinesterase-inhibitory constituents recovered under the optimized DES extraction conditions.

Considering the promising results obtained in the bioautography assays, the cholinesterase inhibitory activity of the DES extract was further evaluated at 1000 ppm. The extract demonstrated potent inhibitory effects against both acetylcholinesterase and butyrylcholinesterase, with inhibition percentages greater than 98% in both cases. Notably, these values exceeded those observed for physostigmine, which was used as the positive control in the assay. The corresponding IC_{50} values were 2.96 µg/mL for AChE and 46.79 µg/mL for BChE, indicating a markedly higher inhibitory potency toward acetylcholinesterase. The dose–response curves of the ethanolic extract and DES extract are shown in Figure 7. The results clearly indicate that the DES extract produced a greater inhibitory effect than the ethanolic extract at the tested concentrations, confirming the enhanced recovery of bioactive cholinesterase-inhibitory constituents achieved through the optimized DES extraction process.

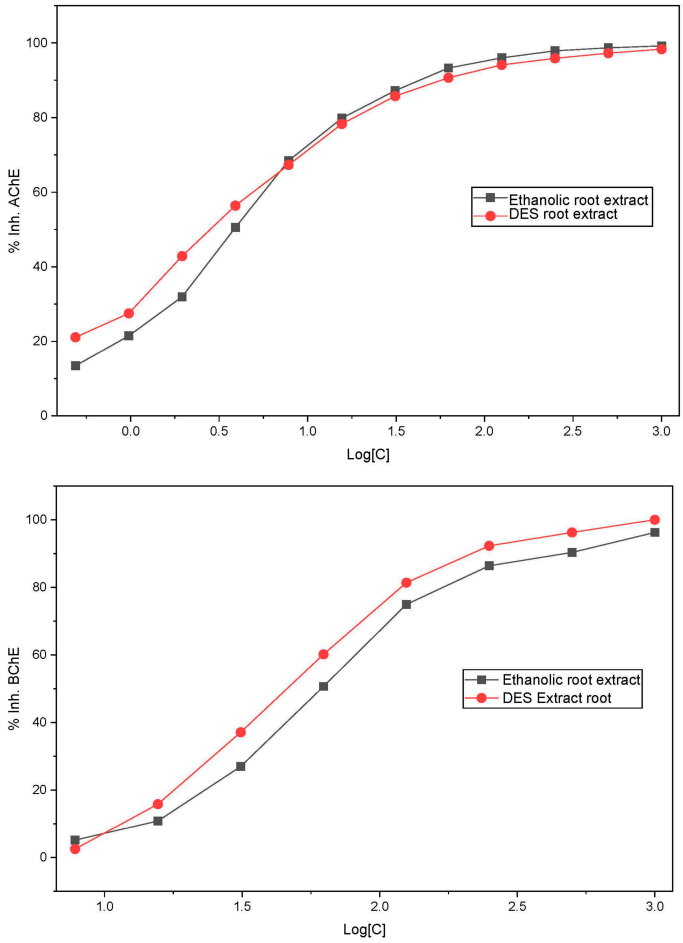


Figure 7. The dose–response curves of the ethanolic extract and the citric acid–glucose (1:1) DES extract.

Given its pronounced anticholinesterase activity, the alkaloid-rich extract was further evaluated for its ability to inhibit amyloid-β ($A\beta_{1–42}$) peptide aggregation. Complete inhibition (100%) was observed over a broad concentration range, from 500 to approximately

30 µg/mL. Figure 8 shows the fluorescence kinetics of $A\beta_{1–42}$ aggregation in the presence of the *Bocconia frutescens* root extract. Compared with the control, a marked reduction in fluorescence intensity was observed throughout the incubation period, indicating a strong inhibitory effect on amyloid fibril formation.

Figure 8 illustrates the time-dependent evolution of Thioflavin T fluorescence during $A\beta_{1–42}$ peptide aggregation in the presence of different concentrations of the alkaloid-rich extract obtained from *Bocconia frutescens*. The negative control (methanol, A08) exhibited the highest fluorescence increase throughout the assay, reaching approximately 35 RFU after 4 h of incubation. This profile is indicative of the progressive aggregation of the peptide and the continuous formation of β-sheet-rich structures characteristic of amyloid fibrils.

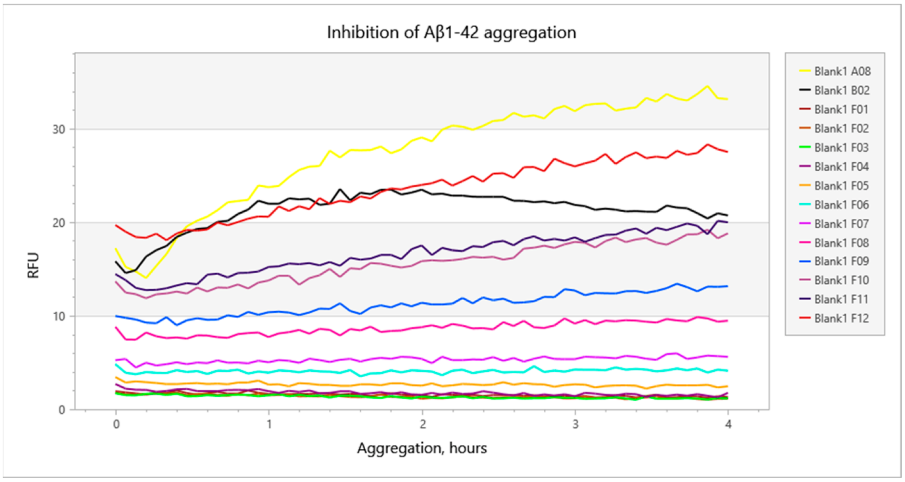


Figure 8. The fluorescence kinetics of $A\beta_{1–42}$ aggregation in the presence of the *Bocconia frutescens* root DES extract. A08 sample: methanol control, B02 sample: tannic acid, F01 sample: 500 µg/mL, F02 sample: 250 µg/mL, F03 sample:125 µg/mL, F04 sample: 62.5 µg/mL, F05 sample: 32 µg/mL, F06 sample: 16 µg/mL, F07 sample: 8 µg/mL, F08 sample: 4 µg/mL, F09 sample: 2 µg/mL, F10 sample: 1 µg/mL, F11sample: 0.5 µg/mL, F12 sample: 0.25 µg/mL.

In contrast, the positive control (tannic acid, B02) produced a marked reduction in fluorescence relative to the negative control, reaching values close to 20 RFU at the end of the experiment. This result confirms the ability of tannic acid to interfere with $A\beta_{1–42}$ fibrillization and validates the sensitivity of the assay for detecting aggregation inhibitors.

The *B. frutescens* extract displayed concentration-dependent effects on $A\beta_{1–42}$ aggregation. Several concentrations yielded fluorescence profiles substantially lower than those observed for tannic acid, remaining nearly constant throughout the 4 h incubation period and maintaining fluorescence values close to the baseline (<5 RFU). Such behavior suggests an almost complete suppression of amyloid aggregate formation, indicating a strong ability of the extract to interfere with both the nucleation and elongation stages of fibril assembly.

Other concentrations produced intermediate fluorescence levels, characterized by gradual increases over time that nevertheless remained significantly lower than those of the negative control. These findings indicate partial inhibition of the aggregation process, which may be associated with a lower abundance of active constituents or a diminished contribution of synergistic interactions occurring at higher extract concentrations.

Notably, several extract concentrations exhibited inhibitory activity superior to that of tannic acid, as evidenced by considerably lower fluorescence signals throughout the assay. This observation suggests that the alkaloids present in *B. frutescens*, particularly sanguinarine and chelerythrine, may act as effective modulators of amyloid aggregation.

Their activity may involve interactions with early oligomeric species and/or hydrophobic regions that play a critical role in fibril nucleation and growth.

Overall, these results demonstrate that the alkaloid-rich extract obtained under the optimized extraction conditions possesses a remarkable ability to inhibit $A\beta_{1–42}$ aggregation, surpassing the activity of tannic acid at several concentrations. These findings further support the multitarget potential neuroprotective activity of *B. frutescens* and complement the observed acetylcholinesterase and butyrylcholinesterase inhibitory activities, suggesting that the extract constituents may simultaneously modulate multiple pathological pathways associated with Alzheimer's disease.

The present study highlights the potential neuroprotective activity of an alkaloid-enriched extract obtained from *Bocconia frutescens* using an optimized deep eutectic solvent (DES)-based extraction process. The extract exhibited pronounced inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), together with a marked concentration-dependent reduction in amyloid-β ($A\beta_{1–42}$)-associated ThT fluorescence. These two responses are relevant to key pathological processes implicated in the onset and progression of Alzheimer's disease (AD). Collectively, the findings support the initial hypothesis that alkaloid-rich extracts from *B. frutescens* represent a promising source of compounds with multitarget neuroprotective potential. Furthermore, the results underscore the importance of the extraction methodology in maximizing the recovery of bioactive metabolites and influencing the biological performance of the resulting extract.

Among the most noteworthy findings was the potent anticholinesterase activity displayed by the extract, particularly against AChE, for which an IC_{50} value of 2.96 µg/mL was determined. Cholinesterase inhibition remains one of the most widely employed therapeutic strategies for the symptomatic management of AD, as it increases synaptic acetylcholine levels and partially compensates for the progressive cholinergic dysfunction associated with disease progression [40]. Although both AChE and BChE contribute to acetylcholine hydrolysis, AChE constitutes the primary therapeutic target during the early stages of AD, whereas the contribution of BChE becomes increasingly relevant in the advanced stages of the disease [41]. Therefore, the ability of the extract to inhibit both enzymes suggests a broad spectrum of biological activity with potential relevance across different stages of neurodegeneration.

The observed anticholinesterase activity may be attributed, at least in part, to the abundance of the benzophenanthridine alkaloids sanguinarine and chelerythrine, which were identified as major constituents of the extract. These compounds have attracted considerable attention owing to their diverse biological properties, including antioxidant, anti-inflammatory, and neuroprotective activities. Previous studies have demonstrated that sanguinarine modulates signaling pathways involved in oxidative stress, apoptosis, and neuroinflammation, all of which are closely associated with the pathogenesis of neurodegenerative disorders [42]. In addition, computational and biochemical studies have suggested that benzophenanthridine alkaloids can interact with the catalytic sites of cholinesterases, thereby contributing to their inhibitory activity [43].

The *B. frutescens* root extract produced a marked concentration-dependent decrease in ThT fluorescence, resulting in a calculated IC_{50} of 0.70 µg/mL under the experimental conditions evaluated. Importantly, concentration-matched extract blanks containing the extract and ThT, but no $A\beta_{1–42}$ peptide, were included in the assay. The fluorescence signal from each extract blank was subtracted from that of the corresponding peptide-containing sample before calculating the aggregation-inhibition percentage, thereby minimizing the contribution of extract-associated background fluorescence to the measured signal.

Nevertheless, given the complex chemical composition of the plant extract, additional matrix-related effects on the ThT fluorescence signal cannot be completely excluded. Plant

constituents may affect ThT-based measurements through fluorescence quenching, inner-filter effects, absorption at the excitation or emission wavelengths, or direct interactions with the ThT probe. Although the use of concentration-matched extract blanks corrects for extract-associated background fluorescence, it does not necessarily eliminate all potential sources of interference. Therefore, the pronounced decrease in ThT fluorescence and the resulting IC_{50} should be interpreted as indicating potential in vitro activity against $A\beta_{1–42}$ aggregation rather than as definitive evidence of direct inhibition of amyloid fibril formation [44]. Similar antiamyloidogenic properties have been reported for other isoquinoline alkaloids, including berberine, which has shown the potential to inhibit amyloid fibril formation and attenuate Aβ-induced neurotoxicity [45]. These reports provide relevant context for the response observed with the alkaloid-rich *B. frutescens* extract; however, complementary fluorescence-independent methods and studies with isolated constituents are required to confirm the antiaggregation activity and identify the compounds responsible for the observed response.

The relevance of these findings lies in the potential of the extract to modulate multiple targets associated with AD. Increasing evidence suggests that single-target therapeutic approaches may have limited efficacy against multifactorial disorders such as AD. Consequently, the development of multitarget agents capable of acting on complementary pathological mechanisms has emerged as an important strategy in the search for compounds with potential neuroprotective activity [46]. In the present study, the *B. frutescens* root extract showed marked inhibitory activity against AChE and BChE, together with potential in vitro activity against $A\beta_{1–42}$ aggregation, as indicated by the concentration-dependent reduction in ThT fluorescence. Taking together, these findings suggest that alkaloid-rich extracts from *B. frutescens* may represent a promising source of naturally occurring compounds with multitarget potential relevant to neuroprotection. However, further studies using complementary experimental approaches are required to confirm the antiaggregating effect and establish the biological relevance of these activities.

Beyond the intrinsic bioactivity of the extracted alkaloids, the extraction methodology appears to have played an important role in determining the biological performance of the final extract. The citric acid–glucose DES optimized through response surface methodology yielded substantially higher recoveries of sanguinarine and chelerythrine than conventional aqueous and alcoholic extraction systems. These findings are consistent with previous reports highlighting the extraction performance of DESs, which arises from their tunable physicochemical properties, including polarity, viscosity, acidity, and hydrogen-bonding capacity [47,48].

In particular, the acidic nature of the DES likely promoted the protonation of nitrogen-containing alkaloids, thereby increasing their solubility and facilitating their transfer from the plant matrix into the extraction medium. Furthermore, the extensive hydrogen-bonding network characteristic of DESs may enhance solute–solvent interactions and mass-transfer processes, ultimately improving extraction efficiency. Similar observations have been reported for the extraction of alkaloids and other nitrogenous secondary metabolites, for which DES-based systems improved extraction yields and produced extracts enriched in biologically active constituents [49].

The optimization results further support this interpretation. The Box–Behnken experimental design demonstrated that the plant material-to-solvent ratio, extraction temperature, and DES content influenced alkaloid recovery. Under the optimized conditions, the alkaloid concentration was approximately fourfold higher than that obtained at the central point of the experimental design. This substantial improvement highlights the importance of systematically optimizing extraction parameters when the objective is to maximize the recovery of pharmacologically relevant metabolites. It also suggests that the pronounced potential neuroprotective activity observed in this study may depend not only on the in-

trinsic phytochemical composition of *B. frutescens* but also on the capacity of the extraction process to enhance the recovery of bioactive constituents.

From a technological and environmental perspective, the extraction strategy developed in this study offers potential advantages over conventional methods. Conventional alkaloid extraction commonly requires volatile organic solvents, prolonged extraction times, and considerable energy inputs. DESs may provide advantages such as low volatility, tunable physicochemical properties, and the possibility of using components with comparatively favorable safety and biodegradability profiles [50]. Nevertheless, because the complete analytical workflow included SPE processing and methanol elution and was not evaluated using a quantitative greenness metric, the present results do not establish the overall environmental superiority of the method. Therefore, the DES-based procedure should be regarded as a potentially more sustainable extraction alternative whose overall environmental performance requires confirmation through a comprehensive greenness assessment.

The biological activity of the DES-derived extract should be interpreted in conjunction with both its alkaloid content and the physicochemical selectivity of the extraction medium. The enhanced activity may arise from two complementary factors. First, the polarity, acidity, and hydrogen-bonding capacity of the citric acid–glucose DES may favor the extraction of benzophenanthridine alkaloids and other chemically compatible constituents compared with conventional extraction systems, potentially modifying the qualitative profile and relative proportions of compounds in the resulting extract. Second, the response may reflect the greater recovery and, consequently, the higher concentration of the principal bioactive alkaloids, particularly sanguinarine and chelerythrine. Differences in their relative abundance, the presence of other co-extracted constituents, and additive or synergistic interactions may also contribute to the overall biological response.

Because a complete matched dataset comprising the individual concentrations of sanguinarine and chelerythrine and the corresponding biological activity was not available for every conventional extract, the activity could not be reliably expressed per unit of principal alkaloid. Consequently, the relative contributions of solvent-dependent phytochemical selectivity and alkaloid concentration cannot be conclusively distinguished in the present study. The findings demonstrate that the optimized DES procedure produces an alkaloid-rich, biologically active extract, but they do not establish that its greater activity results exclusively from selective phytochemical enrichment or from a concentration-dependent effect. Future studies should compare extracts at equivalent sanguinarine and chelerythrine concentrations and evaluate the isolated alkaloids, both individually and in combination, to distinguish concentration-dependent effects from compositional and potential synergistic effects.

The promising biological activities observed in the present study open new avenues for further investigation of the neuroprotective potential of *B. frutescens*. Future studies should focus on isolating and characterizing the compounds responsible for the observed effects and evaluating potential synergistic interactions among the alkaloids present in the extract. In addition, molecular-docking studies, enzyme-kinetic analyses, and cell-based models could provide deeper insight into the mechanisms underlying the observed bioactivities. Validation in animal models will also be essential to assess bioavailability, blood–brain barrier permeability, safety, and therapeutic efficacy. Such studies will help establish the scientific basis for the potential development of phytopharmaceuticals or bioactive ingredients derived from *B. frutescens* for the prevention or management of neurodegenerative disorders, particularly AD.

Overall, the results demonstrate that DES-assisted extraction constitutes an effective strategy for recovering benzophenanthridine alkaloids from *B. frutescens*. The pronounced cholinesterase-inhibitory activity and the potential in vitro activity against $A\beta_{1–42}$ aggre-

gation, together with the extraction advantages associated with DES technology, support further investigation of this species as a source of bioactive compounds with multitarget neuroprotective potential.

3. Materials and Methods

3.1. Preparation of Bocconia frutescens Extracts

3.1.1. Ethanolic Extraction

Ground root (500 mg) was placed in an Erlenmeyer flask, and 10 mL of 96% *w*/*v* ethanol was added. The mixture was subjected to ultrasound-assisted maceration for 15 min. This procedure was repeated for 11 cycles to ensure exhaustive extraction, maintaining the temperature at 25 °C. The efficiency of each extraction cycle was monitored by thin-layer chromatography (TLC). Finally, the solvent was removed under reduced pressure.

3.1.2. Aqueous Extraction

Ground root (500 mg) was placed in an Erlenmeyer flask, and 10 mL of Milli-Q water (Millipore, Burlington, MA, USA) was added. The mixture was subjected to ultrasound-assisted maceration for 15 min. This procedure was repeated for 19 consecutive cycles to ensure exhaustive extraction. The efficiency of each cycle was evaluated by TLC. Finally, the resulting solution was lyophilized to obtain the dry aqueous extract and determine the extraction yield.

3.1.3. Deep Eutectic Solvent Extraction

- **DESs preparation**

Each DES component was accurately weighed and quantitatively transferred into the same beaker. The mixture was heated under constant stirring until a homogeneous, viscous, and translucent liquid was formed. The preparation system was insulated from air currents and thermoregulated using a heating plate coupled to a thermosensor immersed in the DES mixture. All DES preparations were carried out at either 40 °C and 20 °C.

- **Extraction procedure**

A total of 100 mg of plant material was extracted using different volumes of the selected DES. The extraction conditions were modified according to the objectives of each experiment and are described in detail in Section 2. Each extraction experiment was performed in triplicate ($n = 3$), and the results are expressed as mean ± standard deviation (SD). The resulting mixtures were filtered and subjected to solid-phase extraction (SPE) using Strata SDB-L (styrene-divinylbenzene) cartridges. Sequential elution was performed using 10 mL of water, a 7 mL of methanol–water solution (v/v), and 3 mL of pure methanol to maximize analyte recovery. Elution efficiency was monitored by TLC.

3.2. Alkaloids Content Quantification by HPLC-DAD and LC-MS Analyses

The analysis of DES extracts was performed using a Thermos Scientific Dionex Ultimate 3000 chromatographic system (Waltham, MA, USA) equipped with a Dionex Ultimate 3000 diode array detector (DAD), Dionex Ultimate 3000 RS quaternary pump, in-line degasser, and automatic injector. Data acquisition and processing were carried out using Chromeleon Client software (version 6.80 SR15). Chromatographic separation was performed using a Poroshell 120 EC-C18 column (2.7 µm, 150 mm × 4.6 mm i.d., Agilent, Santa Clara, CA, USA) previously conditioned with 95% mobile phase A and 5% mobile phase B. The mobile phases consisted of (A) 1.0% formic acid in water and (B) methanol. The flow rate was set at 0.8 mL/min. The gradient elution program was as follows: 5% B at 0 min, increasing to 15% B at 5 min, 30% B at 8 min, and 50% B at 15 min; subsequently, the proportion of B

increased to 95% at 20 min, decreased to 5% at 21 min, and was maintained for 4 min to allow column reconditioning before the next run. The column temperature was maintained at 35 °C.

LC–MS analyses were performed using a Bruker Impact II UHR-QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source operated in positive-ion mode. Chromatographic separation was achieved using a Poroshell 120 EC-C18 column (150 mm × 4.6 mm i.d., 2.7 µm particle size), previously conditioned with 95% mobile phase A and 5% mobile phase B. The mobile phases consisted of 1% formic acid in water (A) and methanol (B), and the flow rate was set at 0.8 mL/min. The gradient elution program was as follows: 5% B at 0 min, increasing to 15% B at 5 min, 30% B at 8 min, and 50% B at 15 min. The proportion of B was subsequently increased to 95% at 20 min, decreased to 5% at 21 min, and maintained at this composition for 4 min to allow column re-equilibration before the next injection. The column temperature was maintained at 35 °C. High-resolution mass spectra were acquired over an m/z range of 80–1200. The capillary temperature and spray voltage were set at 350 °C and 5.0 kV, respectively. All solvents and additives were of LC–MS grade.

Alkaloids were identified through the combined analysis of their UV–VIS absorption spectra, retention behavior, MS/MS fragmentation profiles, and literature data. Among the identified compounds, sanguinarine and chelerythrine were selected as analytical marker compounds due to the selectivity exhibited by the extraction procedure toward these alkaloids, although additional compounds were also identified.

Sample preparation consisted of diluting 100 µL of DES extract to a final volume of 1.0 mL with 1% formic acid in water. The solutions were filtered through a 0.22 µm membrane and refrigerated until batch analysis.

Statistical analyses were performed separately for sanguinarine and chelerythrine. Data are expressed as mean ± standard deviation (SD) of three replicate extractions ($n = 3$). The effects of extraction method and temperature on alkaloid recovery were evaluated using two-way analysis of variance (ANOVA), considering extraction method and temperature as fixed factors and including their interaction (extraction method × temperature). When significant differences were detected, multiple comparisons among extraction method–temperature combinations were performed using Tukey's honestly significant difference (HSD) post hoc test. Differences were considered statistically significant at $p < 0.05$. Different letters indicate statistically significant differences among extraction conditions according to Tukey's HSD test (See Table S1).

3.3. Chelerythrine Quantification and Analytical Performance

Chelerythrine was quantified by HPLC-DAD using an external calibration curve prepared from a chelerythrine analytical standard. Eight calibration levels (0.2, 1, 2, 10, 20, 100, 200, and 1000 ppm) were analyzed, with three independent injections performed at each concentration. The calibration curve was constructed by plotting peak area (y) against chelerythrine concentration (x, ppm), and linear regression was performed by the least-squares method.

Linearity was evaluated from the calibration equation and coefficient of determination (R^2). Instrumental repeatability was assessed from the relative standard deviation (RSD) of the peak areas obtained from the three independent injections at each calibration level. The instrumental limits of detection (LOD) and quantification (LOQ) were estimated according to LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ represents the standard deviation of the intercepts obtained from three calibration regressions and S represents the mean slope of the calibration curves.

Chelerythrine concentrations determined in the extracts were subsequently expressed as milligrams of chelerythrine per gram of dry plant material (mg/g DW). Chelerythrine content (mg/g DW) was used as the response variable (D) in the Box–Behnken experimental design. Recovery experiments were not performed in the present study; therefore, the accuracy of the complete analytical procedure was not independently established.

3.4. Statistical Analysis and Box–Behnken Design

A three-factor Box–Behnken design comprising 15 experimental runs, including three replicates at the central point, was used to evaluate the effects of plant material concentration (A, 10–60 mg/mL), extraction temperature (B, 20–40 °C), and DES content in the DES–water mixture (C, 50–80%) on chelerythrine recovery. The response variable was expressed as chelerythrine content per gram of dry plant material (mg/g DW). Values initially obtained as chelerythrine concentrations in the liquid extract (mg/L or ppm) were converted according to Equation (1):

$$D\ (\text{mg/g DW}) = \frac{C_{\text{extract}}\ (\text{mg/L})}{A\ (\text{g/L})} \quad (1)$$

Since a plant material concentration expressed in mg/mL is numerically equivalent to g/L, the conversion was performed by dividing the chelerythrine concentration in ppm by factor A in mg/mL. The experimental data were fitted to a second-order polynomial model:

$$D = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Model adequacy was assessed using analysis of variance, the overall F-test, individual coefficient tests, lack-of-fit, R^2, adjusted R^2, predicted R^2, root mean square error (RMSE), and predicted residual error sum of squares (PRESS). Statistical significance was established at $p < 0.05$. Numerical optimization was restricted to the experimental ranges evaluated for the three factors.

Chelerythrine content was selected as the response variable (D) for the Box–Behnken design and was expressed as milligrams of chelerythrine per gram of dry plant material (mg/g DW). The response therefore represents chelerythrine recovery on a dry-weight basis and does not correspond to the combined concentration of sanguinarine and chelerythrine or to total alkaloid content.

3.5. Acetyl and Butyryl Cholinesterase Inhibition

The inhibitory activity of the alkaloidal extracts against EeAChE and HsBuChE was evaluated using a modified version of Ellman's colorimetric assay. A stock solution of each extract (10,000 ppm in DMSO) was prepared and serially diluted to obtain at least eleven concentrations ranging from 0.001 to 1000 ppm.

Enzymatic assays were conducted using a final enzyme concentration of 0.002 U/mL in 0.1 M phosphate-buffered saline (PBS, pH 8.0) supplemented with 0.1% bovine serum albumin (BSA). In 96 well microplates, 50 µL of extract solution at varying concentrations was mixed with 50 µL of enzyme solution and incubated at 37 °C for 30 min. The reaction was initiated by the addition of 50 µL of substrate acetylthiocholine iodide (ATCI) or butyrylthiocholine iodide (BTCI) to achieve a final concentration of 1 mM, followed by 50 µL of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to a final concentration of 0.5 mM. Absorbance was monitored at 412 nm for 30 min.

To minimize potential interference arising from the intrinsic color, turbidity, or non-enzymatic absorbance of the extract, a sample blank without enzyme was included at each concentration tested. The absorbance of each blank was subtracted from that of the corresponding enzymatic reaction before calculating enzyme inhibition. A 0.2% DMSO solution in PBS was used as the negative control, while berberine and physostigmine served

as positive controls. Enzymatic activity was expressed as a percentage relative to the activity measured for negative control. Half-maximal inhibitory concentrations (IC_{50}) were estimated by nonlinear regression of the concentration–response curves (log[inhibitor] versus response) using GraphPad Prism 8. Results were obtained from three independent experiments, each conducted with at least three technical replicates.

3.6. Aβ$_{1–42}$ Peptide Aggregation Inhibition Assay

The inhibition of $A\beta_{1–42}$ peptide aggregation was evaluated using the fluorescent dye thioflavin T (ThT), which binds preferentially to amyloid fibrils. Fluorescence measurements were performed using a Varioskan Lux (Thermo Scientific, Waltham, MA, USA) microplate reader at excitation and emission wavelengths of 440 and 484 nm, respectively. Assays were conducted in black 96-well plates to minimize optical interference and protect photosensitive compounds.

A stock solution of *Bocconia frutescens* root extract, obtained using the DES system showing the best extraction performance, was prepared at 500 µg/mL in methanol, from which 12 serial dilutions were prepared. Each assay well contained 25 µL of sample, 25 µL of ThT solution, and 50 µL of $A\beta_{1–42}$ peptide solution. Tannic acid was used as a positive control under the same experimental conditions, whereas methanol and PBS (10 mM phosphate buffer, pH 8) were included as negative controls.

For each extract concentration, a corresponding blank was prepared containing the same amount of extract and ThT, while the 50 µL of $A\beta_{1–42}$ peptide solution was replaced with an equal volume of PBS. Thus, the extract blanks contained extract, ThT, and PBS but no $A\beta_{1–42}$ peptide, while maintaining the same final assay volume and ThT concentration as the corresponding test samples. The fluorescence signal obtained from each extract blank was subtracted from that of the corresponding $A\beta_{1–42}$-containing sample before calculating the percentage of aggregation inhibition, thereby correcting for extract-associated background fluorescence under the assay conditions.

Fluorescence intensity was recorded every 4 min over a period of 2–4 h. Each concentration was evaluated in triplicate, and the background-corrected fluorescence values were used to calculate the percentage of $A\beta_{1–42}$ aggregation inhibition and the half-maximal inhibitory concentration (IC_{50}).

4. Conclusions

This study demonstrates that deep eutectic solvents constitute an efficient and sustainable alternative to conventional extraction methods for the recovery of neuroactive alkaloids from *Bocconia frutescens*. Among the solvent systems evaluated, the citric acid–glucose (1:1) DES showed superior extraction performance, enabling the selective recovery of the benzophenanthridine alkaloids sanguinarine and chelerythrine. The optimization of extraction conditions through a Box–Behnken experimental design revealed that the plant material-to-solvent ratio, extraction temperature, and DES concentration significantly influenced alkaloid recovery, leading to a four-fold increase in extraction yield under optimized conditions.

The alkaloid-rich extract obtained using the optimized DES-based extraction methodology established through the Box–Behnken design exhibited remarkable multitarget potential neuroprotective activity, including potent inhibition of acetylcholinesterase and butyrylcholinesterase, as well as strong anti-amyloidogenic activity against $A\beta_{1–42}$ peptide aggregation. These biological effects are particularly relevant in the context of Alzheimer's disease, where the simultaneous modulation of multiple pathological pathways is increasingly recognized as a promising therapeutic strategy.

Beyond its biological relevance, the green analytical chemistry metrics GAPI and AGREE demonstrate that the obtention of extracts from *B. frutescens* through the approach

developed herein aligns with the principles of green analytical chemistry by reducing the sample size, the number of steps involved in the sample preparation and the reliance on non-renewable volatile organic solvents, while improving the safety of operators, the extraction efficiency and selectivity. Overall, the results position *B. frutescens* as a valuable source of multifunctional potential neuroprotective alkaloids and highlight DES-assisted extraction as a promising platform for the sustainable production of phytopharmaceutical ingredients with potential applications in neurodegenerative disease management. Future studies should focus on the isolation of the active constituents, characterization of synergistic interactions, and validation of the observed potential neuroprotective effects in cellular and in vivo models.

The optimized DES system enabled the recovery of an alkaloid-rich extract displaying cholinesterase-inhibitory and anti-Aβ aggregation activities. However, the extent to which these effects are attributable to the concentrations of sanguinarine and chelerythrine, differences in the overall phytochemical composition, or interactions among extract constituents remains to be established through concentration-normalized comparative assays.

This study demonstrated that DES-assisted extraction is an effective strategy for recovering the benzophenanthridine alkaloids sanguinarine and chelerythrine from *Bocconia frutescens*. The resulting alkaloid-rich extract exhibited marked in vitro inhibitory activity against AChE and BChE and produced a concentration-dependent reduction in $A\beta_{1–42}$-associated ThT fluorescence. However, because potential interference with the ThT assay cannot be completely excluded, complementary fluorescence-independent methods are required to confirm direct inhibition of amyloid aggregation.

The observed biochemical activities may reflect both the physicochemical selectivity of the DES extraction system and the greater quantity of alkaloids recovered. Nevertheless, these findings do not demonstrate cellular or in vivo neuroprotection, pharmacological efficacy, safety, bioavailability, blood–brain barrier permeability, or therapeutic effectiveness against Alzheimer's disease. Further studies involving isolated constituents, concentration-normalized comparisons, cell-based assays, toxicity and pharmacokinetic evaluations, and appropriate in vivo models are required to establish the biological and pharmacological relevance of the extract. Thus, the present results provide a foundation for further investigation of *B. frutescens* alkaloids but should not be interpreted as evidence supporting their use in the prevention, management, or treatment of Alzheimer's disease.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules31224102/s1, The Supplementary Materials contain tables with replicate data from the different experiments and the corresponding statistical analyses, analytical validation parameters for chelerythrine quantification, and the results of the direct bioautography assay assessing cholinesterase inhibition by *Bocconia frutescens* extracts.

Author Contributions: V.N.: Investigation, data curation. J.M.: Investigation, data curation. C.O.-P. Investigation, conceptualization, methodology, review and editing, funding acquisition, project administration M.Á.-M.: Investigation, conceptualization, methodology, formal analysis, funding acquisition, writing—original draft preparation, review and editing, project administration. All authors have read and agreed to the published version of the manuscript.

Funding: We gratefully acknowledge the financial support from the Ministerio de Ciencia, Tecnología e in-novación-MINCIENCIAS, Instituto Colombiano de Crédito Educativo y Estudios Técnicos en el Exterior-ICETEX and Universidad Nacional de Colombia (grant number RC 2022-745).

Institutional Review Board Statement: Ministerio de Ambiente y Desarrollo Sostenible by Permission No. 121 of 22 January 2016 (modification Otrosí No. 21) and permission request to access genetic resources and their derivative products No. RGE 46 (Article 6, Law 1955 2019).

Informed Consent Statement: Not applicable. This study did not involve human participants.

Data Availability Statement: The data presented in this study are available within the article. Additional data supporting the findings of this study are available from the corresponding authors upon reasonable request.

Acknowledgments: The authors thank MINCIENCIAS–MEN–ICETEX for financial support and the Ministerio de Medio Ambiente for the Framework Agreement for Access to Genetic Resources and Their Derivatives No. 121 of 2016, Amendment No. 21. During the preparation of this manuscript, the authors used ChatGPT for the purpose of managing bibliographic references. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

Aβ 1–42	Amyloid beta 1–42
AChE	Acetyl Cholinesterase
AD	Alzheimer disease
AGREE	Analytical greenness
ANOVA	Analysis of variance
ATCI	Acetylthiocholine iodide
B. frutescens	*Bocconia frutescens*
BBB	Blood–brain barrier
BBD	Box–Behnken Design
BChE	Butyryl Cholinesterase
BSA	Bovine Serum Albumin
BTCI	Butyrylthiocholine iodide
ChCl	Choline chloride
DESs	Deep eutectic solvents
DMSO	Dimethyl sulfoxide
DTNB	5,5-Dithiobis-(2-nitrobenzoic acid)
DW	Dry weight
EeAChE	*Electric eel* acetylcholinesterase
ESI	Electrospray ionization
EtOH	Ethanol
GAC	Green analytical chemistry
GAPI	Green analytical procedure index
HSBuChE	Horse serum butyrylcholinesterase
IC_{50}	50% inhibitory concentration
MAO-A/B	Monoamine oxidase A/B
min	Minutes
mAU	Milli absorbance unit
m/z	Mass-to-charge ratio
nm	Nanometers
PBS	Phosphate-buffered saline
ppm	Parts per million
ROS	Reactive oxygen species
RSM	Response Surface Methodology
SPME	Solid-phase microextraction
Th-T	Thioflavin T
TLC	Thin-layer chromatography
UHPLC-DAD	Ultra-high-performance liquid chromatography–diode array detector
UHPLC-ESI-HRMS	Ultra-high-performance liquid chromatography–electrospray ionization–high-resolution mass spectrometry
UV–Vis	Ultraviolet–visible spectroscopy

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