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ORIGINAL RESEARCH PAPER

GLYCEROL-BASED NaDES FOR BIOACTIVE COMPOUND EXTRACTION FROM *PRUNUS SPINOSA* L. FRUIT: FORMULATION, CHARACTERIZATION, AND APPLICATION

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Abstract

The objective of this study was to synthesize and evaluate three innovative Natural Deep Eutectic Solvents (NaDES) for their potential in extracting bioactive compounds from blackthorn (*Prunus spinosa* L.). The NaDES solutions were synthesized by combining glycerol as a hydrogen bond acceptor with fructose to form N1, sucrose to form N2, and tartaric acid to form N3. The characterization involved pH, viscosity, and conductivity measurements, stability evaluations, and Fourier-transform infrared spectroscopy, with extraction efficiency compared against conventional 70% ethanol solvent. Evaluation criteria included total phenolic content, total flavonoid content, and antioxidant activity using the DPPH method. The characterization revealed significant variations in pH, viscosity, conductivity, and chemical structure, directly influenced by the type of hydrogen bond donor employed. Furthermore, TPC was highest in N1, reaching 9.28 mg GAE/g DW, followed by N3, N2, and 70% ethanol. Flavonoid yield peaked in N3 (3.26 mg CE/g DW), followed by N2, N1 and 70% ethanol. The hydroethanolic extract exhibited the highest antioxidant activity at 87.89%. LC-DAD analysis supported these quantification results. These findings underscore the potential of glycerol-based NaDES as a green, non-toxic alternative for extracting bioactive compounds positioning them as promising candidates for future applications in the food, cosmetics, and pharmaceutical industries.

Keywords: Bioactive compounds, Blackthorn, Glycerol, Green Chemistry, NaDES

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Introduction

Prunus spinosa L., commonly known as blackthorn or sloe, contains high levels of beneficial compounds, particularly phenolic compounds like flavonoids, anthocyanins, and phenolic acids, as well as significant levels of vitamin C (Negrean *et al.*, 2023). These bioactive compounds have demonstrated significant antioxidant, anti-inflammatory (Magiera *et al.*, 2022), and antibacterial properties (Pozzo *et al.*, 2020), offering promising potential as natural alternatives to synthetic food additives to enhance shelf life (Lankanayaka *et al.*, 2024). In the cosmetic industry, these compounds have shown efficacy in dermatological care, particularly for reducing skin pigmentation, preventing cutaneous aging, and enhancing skin hydration levels (Michalak *et al.*, 2021). In the light of increasing pressure on the food and cosmetic industry to adopt sustainable and environmentally friendly practices, researchers have turned their attention to alternative green solvents in response to the toxicity of conventional organic solvents such as methanol and acetone. These traditional extraction agents pose significant risks to human health and environmental safety, compelling scientific exploration of more eco-friendly extraction methods (Wang *et al.*, 2024).

Among these innovations, natural deep eutectic solvents (NaDES) have gained significant attention as a promising solution. Typically composed of a hydrogen bond acceptor (HBA), such as glycerol (Hilali *et al.*, 2022), and a hydrogen bond donor (HBD), like carboxylic acids or sugars (Wang *et al.*, 2024), NaDES present two key advantages in extraction processes. First, they significantly enhance the stability of extracts over time by providing superior protection to bioactive compounds. Second, these extracts allow direct utilization without requiring purification or solvent removal steps. This streamlined approach yields a dual benefit: it simplifies the overall process while substantially reducing both economic and energy costs (Koh *et al.*, 2023).

The primary objective of this study is to (i) develop and characterize glycerol-based NaDES using fructose, sucrose, and tartaric acid as hydrogen bond donors, focusing on investigating their physicochemical and structural properties to ensure the extraction process suitability; (ii) apply these NaDES for phenolic compound extraction from *Prunus spinosa* L. fruit, and (iii) to evaluate their extraction efficiency through determination of Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity using DPPH assay to identify the most effective NaDES formulation.

Materials and methods

Raw material

The ripe fruits of *Prunus spinosa* L. were harvested in September in Bejaia (36°29'12.0"N 4°30'37.0" E), Northeastern Algeria. After washing, the fruits were air-dried at room temperature (30±5°C) and relative humidity (45±5%) on perforated sieves until their moisture content was sufficiently reduced. They were then pitted and further dried in a ventilated oven (Memmert UF55, universal oven with forced

air circulation set at 100% fan speed, 2000W, Germany) at 40°C for three days. The resulting dried material was finely ground into a powder using a coffee bean mill (Sonifer SF-3508, 160w, China), sealed in airtight amber containers to protect from moisture and light, and stored in the refrigerator at 4±1°C.

NaDES preparation

Natural Deep Eutectic Solvents (NaDES) were prepared by combining glycerol, selected as the hydrogen bond acceptor (HBA), with tartaric acid, fructose, and sucrose, respectively, which served as hydrogen bond donors (HBD) (Table 1). These components were mixed in a precise molar ratio of 4:1 selected after testing various ratios based on a homogeneous mixture that remained stable after 4 hours of equilibration at room temperature (25±2 °C). The mixtures were then individually stirred using a heating magnetic stirrer plate (CAPPRondo Hotplate Stirrer, CAPP, Denmark) at 600 rpm in a water bath (beaker filled with water) at 80°C until a transparent liquid was formed (Singh *et al.*, 2024). After formation, the NaDES were diluted with 30% (v/v) of ultrapure water obtained from the Milli-Q system supplied by Millipore (Billerica, MA, USA) to reduce their viscosity (Alpat *et al.*, 2023).

Table 1. The HBA, HBD, Molar Ratios, and Abbreviations of the NaDES Used

Hydrogen bond acceptor (HBA)	Hydrogen bond donor (HBD)	Molar ratio (HBA: HBD)	Abbreviations
Glycerol	Fructose	4:1	N1
Glycerol	Sucrose	4:1	N2
Glycerol	Tartaric acid	4:1	N3

NaDES characterization

Physicochemical analysis

The pH of the NaDES samples was measured at room temperature using a calibrated Edge® pH meter (HI-2002, Hanna Instruments, USA). Viscosity measurements were taken at 25°C using a vibro viscometer SV-10 (sine-wave vibro viscometer, A&D Company, Japan). The conductivity was performed using a WTW inolab® Cond 7110 conductometer (WTW GmbH, Germany) equipped with a TertraCon® 325 immersion electrode with automatic temperature compensation.

NaDES stability

The long-term physical stability of the prepared NaDES (N1, N2, and N3) was evaluated over 90 days. The samples were stored protected from light and at ambient temperature throughout the study. Storage occurred at ambient laboratory temperature (20±5°C) in hermetically sealed containers, eliminating humidity effects. Daily visual monitoring was conducted to detect any signs of precipitation or color change, potential indicators of physical instability.

FT-IR analysis

FTIR spectroscopy analysis was performed using a PerkinElmer Spectrum Two FTIR spectrometer (PerkinElmer Inc., Waltham, MA, USA) for the three prepared

NaDES, as well as their individual components. The spectral range studied was between 4000 and 400 cm^{-1}.

Preparation of extracts

According to the method of Pavlić *et al*. (2022) for each extraction, 1 g of powdered sloe was combined with 20 mL of either NaDES or 70% ethanol solution. The mixture was heated in a water bath (beaker filled with water) at 50°C with continuous stirring at 600 rpm for one hour. Subsequently, the extracts were centrifuged using a Sigma 3-30 KS centrifuge (Sigma, Germany) at 4000 rpm for 15 min, filtered through Whatman No. 1, and stored at 4°C in amber bottles until further use.

Total polyphenol content (TPC)

Total polyphenol content was measured using the Folin-Ciocalteu method described by Ali-Rachedi *et al.* (2018). Each extract (200 µL) was mixed with 1 mL of 10-fold-diluted Folin-Ciocalteu reagent and 800 µL of 7.5% sodium carbonate. After incubating for 30 minutes, absorbance was measured at 765 nm, using a standard curve of gallic acid (0 to 100 µg/mL) for quantification.

Total flavonoid content (TFC)

The flavonoid quantification protocol involved mixing 400 µL of extract, standard, or distilled water (control) with 120 µL of 5% $NaNO_2$ in tubes, following the method by Ali-Rachedi *et al.* (2018). After 5 minutes, 120 µL of 10% aluminium chloride ($AlCl_3$) was added with vigorous agitation. After exactly 6 minutes, 800 µL of 1 M sodium hydroxide (NaOH) was introduced, and the absorbance was immediately measured at 510 nm relative to a control solution. A standard reference curve was generated using catechin solutions with concentrations ranging from 0 to 100 µg/mL.

DPPH Free Radical Scavenging Activity

Antioxidant activity was assessed using the DPPH scavenging method. A mixture of 1450 µL of 0.06 mM DPPH and 50 µL of blackthorn extract was incubated in the dark for 30 minutes (Salem *et al.,* 2022). Absorbance was measured at 515 nm with pure ethanol as a blank. The antioxidant activity was calculated as follows:

$$\text{Antioxidant activity\%} = \frac{(\text{absorbance of negative control} - \text{the absorbance of the assayed extract})}{\text{absorbance of negative control}} \times 100 \quad (1)$$

LC-DAD Analysis

Peaks detection and separation of all extracts (70% ethanol, N1, N2, and N3) were performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD) (Agilent Technologies, Santa Clara, USA) to compare chromatographic profiles and total peaks areas in order to determine the performance of NaDES as efficient extraction solvents in comparison with ethanol. Phenolic compounds were separated on an Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm, Agilent Technologies, USA), using a binary mobile phase system. Mobile phase A consisted of ultrapure water from a Milli-Q system (Millipore, Billerica, MA, USA) containing 0.1% (v/v) formic acid, while mobile phase B comprised acetonitrile with 0.1% (v/v) formic acid. The elution gradient was as follows: 0 min, 3% B; 5 min, 3% B; 20 min, 20% B; 25 min, 25% B; 30 min, 100% B; 35 min, 100% B. The injection volume was set to 40 µL with a flow rate of 0.8 mL/min at room

temperature (23 °C). Absorption spectra were recorded from 240 to 600 nm, and the separation was monitored at 254, 280, 320, 360, and 520 nm.

Statistical analysis

The data for determining bioactive contents are shown as the mean ±SEM from three replicates. Statistical differences among the samples were assessed by the analysis of variance (one-way ANOVA) using JMP (Trial Version 10, SAS, USA) software, followed by Tukey honestly significant difference post hoc test on three replicates. Furthermore, principal component analysis (PCA) was employed to perform a multivariate analysis. This makes it possible to estimate the correlation between the variables under study. This process utilizes a complementary set of scores and loading plots to extract recurring patterns from the data matrix. All computations were carried out with a 95% confidence level, and the significance of the extraction factor was judged by its p-value, which was less than 0.05. The Origin Pro 8.5 software (OriginLab®, Northampton, MA, USA) was used for graphical presentations.

Results and discussion

Physicochemical characterization of NaDES

pH

The pH of NaDES plays a crucial role in their selection for bioactive compound extraction, as it helps prevent undesirable reactions within the solution (Koh *et al.*, 2023). In this context, the three prepared NaDES were analysed using a pH meter, with measurements performed in triplicate, as shown in Table 2. The pH values were determined to be 3.45, 3.08, and 0.87 for N1, N2, and N3, respectively, indicating pronounced acidity. These findings align with previous research by Alfaleh and Sindi (2024) and Popovic *et al.* (2022), confirming that NaDES containing organic acids as hydrogen bond donors exhibit significantly higher acidity compared to sugar-based systems. The pronounced acidic nature of these NaDES bestows several notable advantages. Their environmental compatibility positions them as benign extraction media (Hayyan *et al.*, 2012), minimizing potential ecological impacts. Moreover, their inherent antimicrobial properties render them effective preservation environments, expanding their potential application (Rachmaniah *et al.*, 2020). The acidic characteristics of these NaDES additionally suggest their potential suitability for the extraction of anthocyanins from botanical matrices (Foroutani *et al.*, 2023), where lower pH enables remarkable anthocyanin stability during extraction and minimizes color degradation (Ettoumi and Fang, 2024).

Dynamic Viscosity

The dynamic viscosity of NaDES represents one of the most significant challenges to their widespread industrial application (Ayres *et al.*, 2023)**.** High viscosity adversely affects mass transfer, dissolution kinetics, and extraction processes while increasing pumping costs (Koh *et al.*, 2023)**.** Therefore, measuring the viscosity of the prepared NaDES was crucial to assess their practical viability. As shown in Table 1, the measured viscosity values for N1, N2, and N3 were 128, 156.33, and 89.5

mPa·s, respectively. These results are significantly lower compared to those reported by Dai *et al.* (2013), Gómez *et al.* (2019), and Pan *et al.* (2021) for choline chloride-based NaDES incorporating fructose, sucrose, and tartaric acid, which exhibited viscosities of 2504, 712.83, and 430.3 mPa·s, respectively. Our findings demonstrate that glycerol-based NaDES exhibit substantially lower viscosities. Furthermore, in this study, we incorporated 30% water to modulate NaDES viscosity, as previous research has demonstrated that water weakens the hydrogen bonding between NaDES components, thereby reducing viscosity (Koh *et al.*, 2023).

Conductivity

The high conductivity of NaDES has been demonstrated to enhance polyphenol extraction efficiency by promoting molecular mobility and facilitating targeted compound interactions. The conductivity of three NaDES was measured and found to be 7.23, 5.6, and 324.33 $\mu S \cdot cm^{-1}$ for N1, N2, and N3, respectively. The NaDES based on glycerol and tartaric acid showed the highest conductivity value compared to other NaDES, which confirms its suitability for extraction. Moreover, a notable observation emerged regarding the conductivity values appearing inversely proportional to viscosity values. In other words, NaDES with high viscosity exhibit low conductivity, a finding that corroborates previous reports by Craveiro *et al*. (2016).

Table 2. Physicochemical Properties of the Prepared NaDES

System	Abbreviation	Visual appearance	pH	Viscosity (mPa·s)	Conductivity $\mu S \cdot cm^{-1}$	Stability (days)
Glycerol-Fructose	N1	Transparent viscous liquid	3.45±0.03	128±0.7	7.23±0.3	> 90
Glycerol-Sucrose	N2	Transparent viscous liquid	3.08±0.03	156.33±0.9	5.6±0.2	> 90
Glycerol-Tartaric acid	N3	Transparent viscous liquid	0.87±0.04	89.5±0.2	324.33 ± 1.1	> 90

Stability

The three prepared solvents exhibited remarkable stability after 90 days of storage at room temperature (20±5°C). No changes in color or signs of browning were observed, and they remained colorless and translucent, with no evidence of turbidity or precipitation. These findings indicate that the solvents retained their chemical integrity and showed no signs of degradation over the storage period. This stability is a critical attribute for their effective application in extraction processes.

FTIR

The three synthesized NaDES, along with pure hydrogen bond donors and acceptors, were examined using FTIR. It was employed to confirm the formation of hydrogen bonds through intermolecular interaction. The FTIR spectrum of N1, alongside the spectrum of its individual components, is presented in Figure 1A. In the glycerol

spectrum, a broad band was observed in the high wavenumber region (3000-3730cm^{-1}), attributed to hydroxyl groups (-OH) stretching vibrations. Additionally, minor peaks at 2940 and 2880 cm^{-1} correspond to (C-H) stretching vibrations, indicating the aliphatic nature of glycerol (Mendelovici *et al.*, 2000).

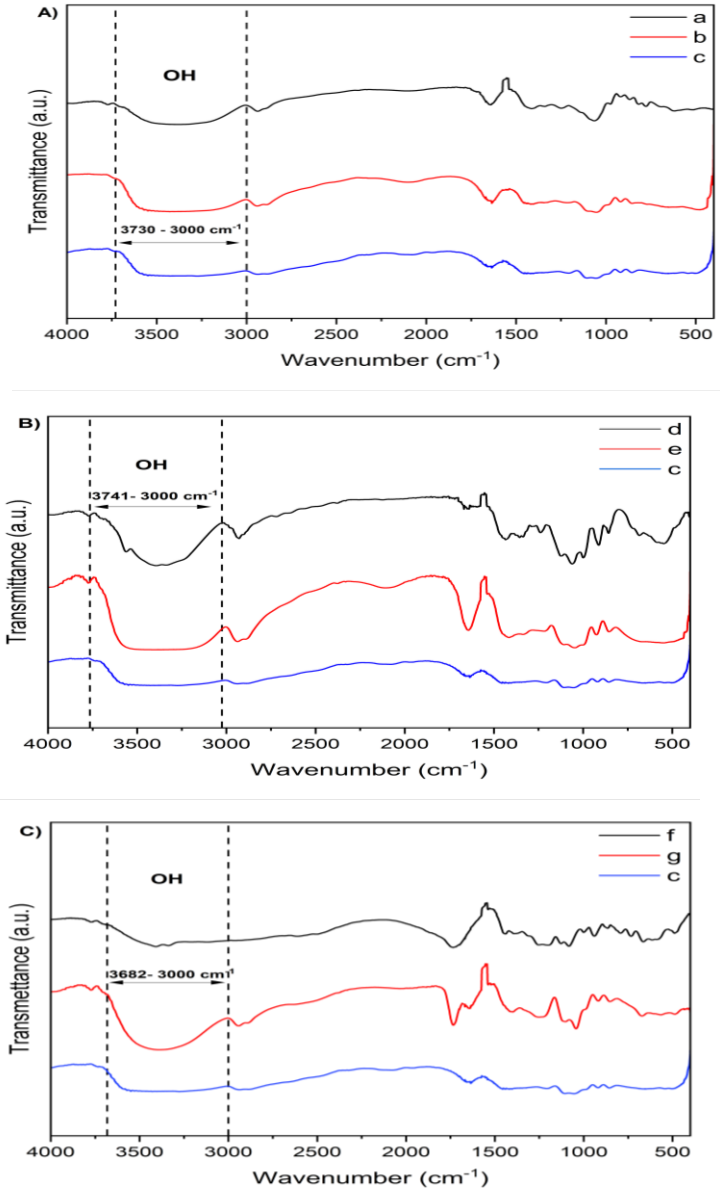

Figure 1. FTIR spectra for A) Fructose (a), glycerol (c), and glycerol-fructose (b) System. B) Sucrose (d), glycerol (c), and glycerol-sucrose (e) System. C) Tartaric acid (f), glycerol (c), and glycerol-tartaric acid (g) System.

However, for the fructose spectrum, broad band extends across the 3000-3730 cm^{-1} region, corresponding to (O-H) stretching vibrations characteristic of the abundant hydroxyl groups in the molecule. Weak peaks located between 2850 and 3000 cm^{-1} indicate (C-H) stretching vibration typical of carbohydrate structures, while the medium peak at 1064 cm^{-1} is attributed to (C-O) stretching vibrations (Wang *et al.*, 2010).

The comparative analysis between the glycerol-fructose mixture and the separated spectra of pure glycerol and fructose reveals notable differences. The characteristic hydroxyl band exhibits a bathochromic shift towards lower wavenumbers and increased intensity compared to individual component spectra. This observation strongly suggests hydrogen bond formation (Caprin *et al.*, 2024) between glycerol and fructose molecules within the NaDES mixture.

Figure 1B presents the FTIR spectral analysis of the glycerol-sucrose mixture (N2) alongside its individual component spectra. For the sucrose spectrum, the spectral region between 3741 and 3000 cm^{-1} represents a distinctive and broad intense band, characterized by a slightly pointed peak associated with (O-H) stretching vibrations (Magazù *et al.*, 2018)**.** This spectral feature directly corresponds to the eight hydroxyl functional groups present in the sucrose molecule, highlighting its rich hydrogen-bonding capabilities. At 2928 cm^{-1}, a distinct weak peak represents C-H stretching vibrations, indicating the presence of methyl and methylene groups within the molecular structure. The medium peak at 1050 cm^{-1} corresponds to C-O stretching vibrations, definitively identifying the glycosidic bonds inherent in the sucrose molecule (Patra *et al.*, 2010; J. Wang *et al.*, 2010).

Regarding the glycerol-sucrose NaDES (N2), the spectral region between 3741 and 3000 cm^{-1}, corresponding to (O-H) bond vibrations, exhibits a distinctive profile compared to its individual components. The most notable changes include a shift of peak positions toward lower wavenumber and a significant band broadening (Ryeon Ryu *et al.*, 2011), accompanied by the loss of the fine structure originally visible in the sucrose spectrum. These spectral modifications strongly support the formation of the NaDES system. Furthermore, in the 1200-900 cm^{-1} region, associated with (C-C) and (C-O) vibrations, a marked decrease in peak intensities is observed. This reduction can be attributed to the new intermolecular interactions established within the system, providing additional evidence for the successful formation of the NaDES complex.

The vibrational mode characteristics of N3 (glycerol-tartaric acid system) and its individual components are presented in Figure 1 C.

For tartaric acid, the characteristic hydroxyl (-OH) stretching vibrations were observed between 3682 and 3000 cm^{-1}. A prominent peak at 1732 cm^{-1} indicates the presence of carbonyl (C=O) moieties. Additionally, the weak peaks observed between 1335 and 1500 cm^{-1} are attributed to the symmetric and asymmetric stretching vibrations of the carboxylate (-COO) group.

The NaDES system (N3) spectrum exhibits significant modifications compared to its pure components. Notable spectral changes include the disappearance of the fine

structure characteristic of tartaric acid and a pronounced broadening of the OH stretching band. The latter demonstrates increased intensity in the 3682-3000 cm^{-1} region, accompanied by a slight bathochromic shift. These spectral modifications collectively indicate the establishment of stronger hydrogen bonding interactions within the system.

Moreover, glycerol, when mixed with fructose, sucrose, and tartaric acid, forms polar solutions N1, N2, and N3 due to their hydroxyl groups capable of forming hydrogen bonds. This characteristic makes these NaDES capable of dissolving various polar substances.

Quantification of TPC

According to Mane *et al.* (2015) results, the solution of 70 % aqueous ethanol, was evaluated as a reference extraction solvent. This last was used in our study in comparison with N1, N2, and N3 NaDES, for TPC from *Prunus spinosa* L. fruit. The one-way ANOVA analysis of the obtained results discloses a correlation coefficient (R^2) of 0.99 for TPC (Table 3).

Table 3. One-way ANOVA analysis results

TPC			**TFC**			**DPPH FRSA**		
R^2	Mean	Prob.>F	R^2	Mean	Prob.>F	R^2	Mean	Prob.>F
0.99	8.12	<.0001*	0.95	2.71	<.0001*	0.81	82.80	0.0026*

Since the extraction of polyphenols is affected by several factors, such as the degree of conjugation and the existence of numerous hydroxyl groups, it is vital to test and adjust the ideal solvent before the main procedure. For this purpose, the NaDES N1, N2, and N3 were prepared using the same molar ratios of glycerol combined with fructose, sucrose, and tartaric acid, respectively. The TPC values obtained with the studied NaDES are shown in Figure 2. The highest rate was obtained using N1, followed by N3, N2, and 70% ethanol solution, with the values of 9.28, 8.76, 8.33, and 6.08 mg GAE/g DW, respectively. The reproducibility of the obtained TPC rates follows a regression coefficient of 0.99. Therefore, the reference solvent had the lowest extraction capacity of the TPC. It is important to mention that physical and chemical characteristics of NaDES, such as hydrogen bonding, polarity, and viscosity, correlate with their capacity to extract bioactive compounds from the natural product matrix. More specifically, the polarity and viscosity of NaDES significantly influence their effectiveness in extracting secondary metabolites. In addition, although the literature showed that there is no solvent that is generally accepted as the best for extracting polyphenols, it is usually accepted that solvents with a higher polarity frequently extract polyphenols the best because of the high solubility of polyphenols in these solvents. This suggests that the selected NaDES provide high solubility of *Prunus spinosa* L. fruit polyphenols. Furthermore, solvation power is determined by solvent polarity, which depends on the

composition of the individual constituents of NaDES and is believed to be related to the molecular structure of the hydrogen bond donor (HBD). It is increased by increasing intermolecular interactions.

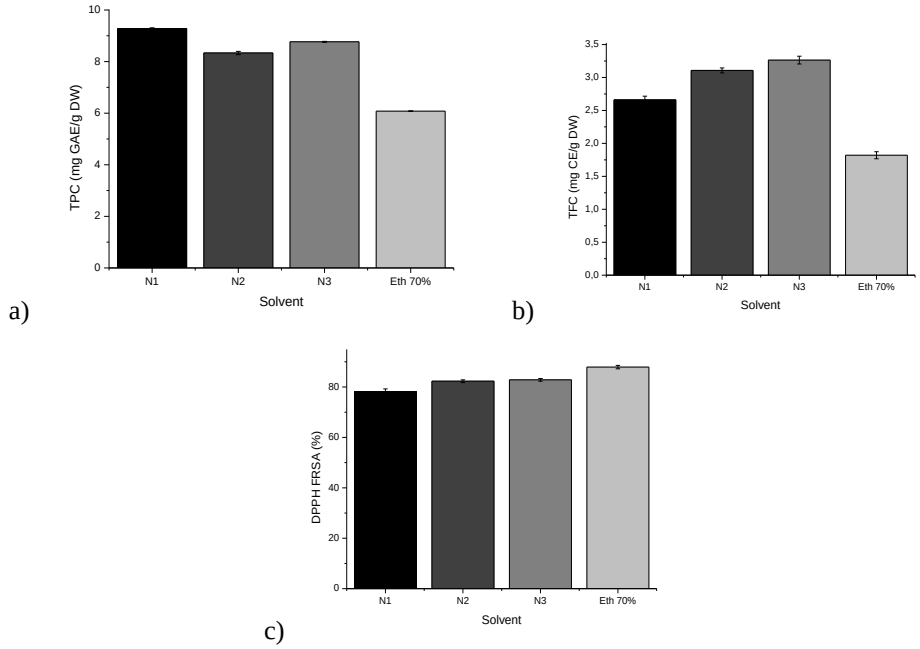

Figure 2. TPC a), TFC b), and DPPH antioxidant activity c) evaluation of *Prunus spinosa* L. fruit extract using different solvents

In NaDES preparation, the combination of intramolecular hydrogen bonds and intermolecular interactions resulted in hydrogen bonding. Glycerol acts as a hydrogen bond acceptor (HBA). However, fructose, sucrose, and tartaric acid act as hydrogen bond donors (HBD). The ability of N1, N2, and N3 NaDES solvents to extract bioactive compounds is due to the similarity of polarity between the available polyphenols and the solvents used. Furthermore, as a sugar alcohol, glycerol has lower polarity if compared to organic acid-based NaDES, amino acid-based NaDES, and sugar-based NaDES, which have higher solvent viscosity and polarity. Thus, NaDES have the benefit of being customizable; meaning that by altering their constituents, their selectivity for a natural active ingredient can be altered. For this purpose, glycerol was combined with fructose, sucrose, and tartaric acid.

According to Peng *et al.* (2015), the viscosity of the prepared NaDES increases with the amount of glycerine used. Additionally, the properties of NaDES can be adjusted by adding water. For this reason, 30% of water has been incorporated in the process of preparation of NaDES to modulate the viscosity. Moreover, adding water may also increase the polarity of the solvent. However, it has been found that breaking the hydrogen bond between HBD and HBA and reducing the interactions between the NaDES and the target molecules can result from adding too much water. Thus,

the extraction yields can be greatly increased by using a solvent with appropriate viscosity and polarity. This was the case for N1, N3, and N2, which exhibited high polyphenol extraction rates. This performance is attributed to the high viscosity and polarity of their respective components: fructose, tartaric acid, and sucrose.

Compared to other organic solvents, such as methanol, NaDES is less harmful to humans and more environmentally friendly. It is a perfect solvent for extracting a wide range of compounds with varied polarity since it is easy to adjust its extraction capacity by adding water (Le *et al.*, 2018)**.** Despite this, the extraction rate of polyphenols from *Prunus spinosa* L. fruit using a 70% aqueous ethanol solution was lower than NaDES solvents (Figure 2.a.). This demonstrates the superior performance of NaDES in the extraction of polyphenols from *Prunus spinosa* L. fruit compared with chemical solvents. The obtained data are clearly consistent with values reported in the literature (Giamperi *et al.*, 2009).

Quantification of TFC

Regarding flavonoid extraction using the studied NaDES and the 70 % aqueous ethanol solution, the one-way ANOVA analysis of the obtained results discloses a correlation coefficient (R^2) of 0.95 for TFC (Table 3).

Figure 2.b. represents the Extraction yield of flavonoids from blackthorn using the different studied solvents. A high extraction yield of 3.26 mg CE/g DW was obtained using N3. A very similar value was recorded with N2, reaching 3.1 mg CE/g DW. This was followed by the yield obtained with N1, which amounted to 2.66 mg CE/g DW. In addition, the least efficient solvent was 70% aqueous ethanol with an extraction rate of 1.82 mg CE/g DW.

In the case of flavonoid extraction using the selected NaDES, the hydroxyl and carbonyl groups contained in the sugar-based NaDES initiated the hydrogen bonding interactions with the flavonoids contained in blackthorn fruit, which have moderate-to-high polarities. The findings of the current study support the general preference for sugars, organic acids, and polyalcohols as building blocks for NaDES synthesis in flavonoid extraction from plant matrices (Wei *et al.*, 2015; Koutsoukos *et al.*, 2019).

Spectroscopic analysis confirmed the presence of hydrogen bonding interactions within the NaDES attributed to the abundance of hydroxyl groups (–OH) in their components, including water. It is therefore highly likely that, during the extraction process, these hydroxyl groups interacted with those of the flavonoids in blackthorn fruit, forming strong hydrogen bonds. However, due to the higher viscosity of NaDES compared to conventional solvents such as ethanol, mass transfer during extraction may be somewhat hindered (Che Zain *et al.*, 2021)**.**

Antioxidant activity

The DPPH antioxidant evaluation of *Prunus spinosa* L. fruit extract using different NaDES solvents and the 70% ethanol solution is illustrated in Figure 2.c. The best performance was achieved by the 70% aqueous ethanol solution with a rate of 87.89%. The NaDES solutions had close rates to those obtained with the 70% ethanol solution but also close to each other.

The levels obtained were approximately 82.83%, 82.28%, and 78.18% for N3, N2, and N1, respectively. It is well known that in order to create a stable end product that does not start or spread more lipid oxidation, antioxidants donate hydrogen from hydroxyl groups to stop the oxidation of free radical chains (Sherwin, 1978). In this study, all extracts demonstrated significant inhibitory activity against the DPPH radical. The best performance was obtained with the 70% ethanol solution with an inhibition rate of 87.89%. The obtained data show that 70% aqueous ethanol extract, N3, N2, and N1 extracts demonstrated a high level of free radical scavenging activity. The activities obtained for these solvents were not significantly different (P>0.05). The findings showed that the examined extracts have the highest free radical scavenging activity and are primary antioxidants and free radical inhibitors that react with free radicals (Akowuah *et al.*, 2005)**.**

In studying the antioxidant activity of the extracts, polarity may play a role in the inhibition of free radicals, but there are other factors to consider. The solubility of antioxidant compounds in ethanol may be higher, even if its polarity is lower, due to its ability to penetrate cellular structures and dissolve a wide range of polar and non-polar compounds. It is also possible that ethanol interacts differently with plant matrices, thereby promoting the extraction of certain specific antioxidants that are highly active. In other words, polarity is just one factor among many, including the chemical structure of the antioxidants and the nature of the plant matrix (Boeing *et al.*, 2014)**.**

PCA analysis

In order to evaluate the relationship between the content of TPC, TFC and AA of the extracts obtained using the different NaDES and the reference solvent expressed by the different tests carried out, PCA analysis was performed. The multidimensional structure of the data was reduced by PCA and produced a two-dimensional map that explained the variation that was seen *(*Figure 3). The two-dimensional map was illustrated as loading (b) and scores (a) plots.

According to the loadings plot (Figure 3a), the first principal component accounted for 80.4% of the 98.4% cumulative variance percentages that were found, while the second principal component accounted for 18% of the variance. Moreover, TPC and TFC were both located on the positive axis of the first component (PC1), so it can be inferred from the PCA that they are both positively correlated. However, the antioxidant activity determined by DPPH was positioned on the negative axis, and right alongside the TFC. This indicates an inverse correlation between TFC and the antioxidant activity. At the same time, there is no correlation between TPC and antioxidant activity. These findings do not match those obtained by Marčetić *et al.* (2022)**,** who have found strong positive correlations between antioxidant activity and the total phenolic content. Additionally, they did not observe any significant correlations between antioxidant activity and TFC, using a 50% ethanol solution. Moreover, Giamperi *et al.* (2009) have also found a strong correlation between antioxidant capacity and total phenols in *Prunus spinosa* L. fruit juice.

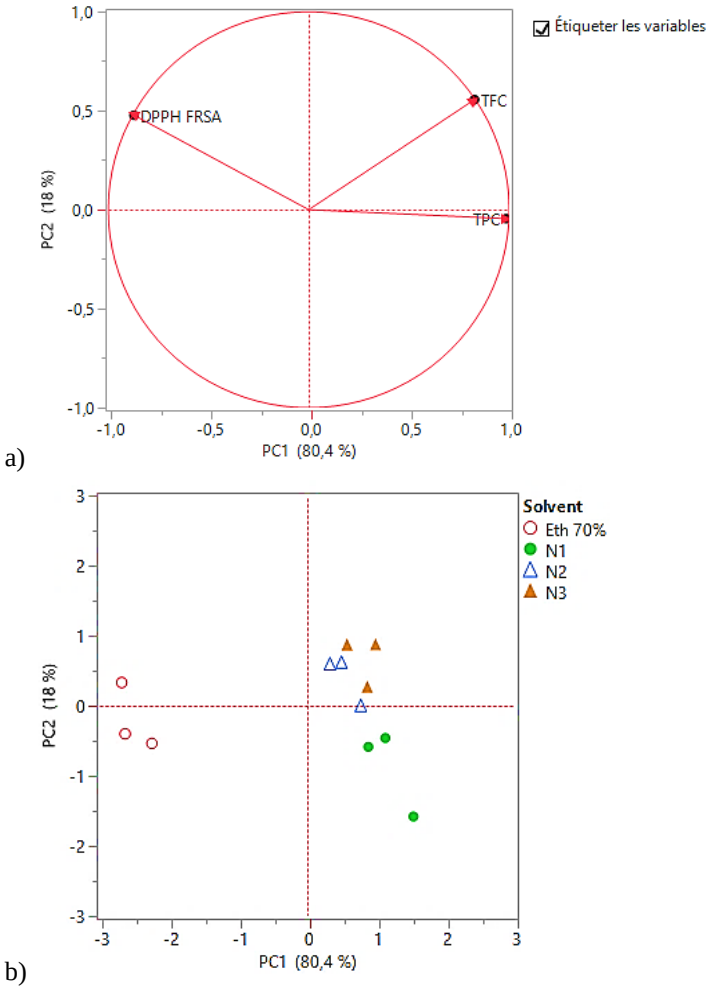

Figure 3. Analysis (PCA) results: scores (a) and loading (b) plots

On the other hand, according to the scores plot *(*Figure 3 (b)), the studied variables cluster close to each other, especially those concerning TPC and TFC values. However, the studied variables for the 70% aqueous ethanol solution appear positioned on the same side of the DPPH antiradical activity parameter and on the opposite side of the NaDES variables.

Additionally, according to the score plot (b), the studied NaDES were positioned in the positive quadrant alongside TFC and TPC. This indicates a strong correlation between the studied NaDES and both flavonoids and polyphenols extraction from *Prunus spinosa* L. fruit. For more accuracy, it is clear from the score plot (b) that N3 and N2 have a strong correlation with TFC and therefore have a greater impact on the extraction of flavonoids. However, N1 was strictly correlated with TPC, indicating its performance in extracting polyphenols.

LC-DAD Chromatographic Profiles

LC-DAD analysis at multiple wavelengths showed that different solvents not only extract different compounds but also had different extraction power for the extracted compounds. The five wavelengths used for monetarizing the separation of the compounds present in the extracts (254, 280, 320, 360, and 520 nm) were selected for the detection of aromatic compounds, phenolic acids, hydroxycinnamic acids, flavonoids, and anthocyanins, respectively (Ignat *et al.*, 2011; Pallauf *et al*., 2008). The obtained chromatographic profiles (Figure 4) visually confirm these extraction differences. Statistical analysis using Tukey's test reveals distinct homogeneous groups according to wavelengths. Ethanol 70% demonstrated the weakest extraction performance, yielding only 230 peaks with a total chromatographic area of 8626.27 mAU. This poor efficiency was further validated statistically (group d: 78 peaks at 254 nm) (Table 4), suggesting its limited capacity for phenolic compound extraction.

Table 4. Number of detected peaks in ethanol 70% and NaDES extracts by LC-DAD at different wavelengths

Wavelength (nm)	**N1**	**N2**	**N3**	**Eth 70%**
254	90 ± 1 ^{a}	85 ± 1 ^{b}	74 ± 1 ^{c}	78 ± 0 ^{d}
280	85 ± 0 ^{a}	79 ± 1 ^{c}	81 ± 1 ^{b}	61 ± 0 ^{d}
320	57 ± 1 ^{a}	51± 0 ^{b}	51 ± 1 ^{b}	45 ± 1 ^{c}
360	34 ± 1 ^{a}	32 ± 0 ^{b}	32 ± 1 ^{b}	34 ± 1^{a}
520	18 ± 0 ^{a}	11 ± 0 ^{d}	17 ± 0 ^{b}	12 ± 0 ^{c}
Total peaks	284	258	255	230

NaDES N1 performed best for total compound extraction, producing 284 peaks (23.5% more than ethanol) with a total area of 10593.5 mAU (Figure 4B). It ranked in the top statistical group (group a: 90 peaks at 254 nm), explaining its high total phenolic content. The peaks were evenly distributed across the chromatogram, showing that N1 extracts many different families of phenolic compounds.

NaDES N3 showed a different pattern. Although it produced fewer peaks (255), it achieved the highest total chromatographic area (20484.8 mAU), which is 2.37 times higher than ethanol. This high area comes from very intense peaks, especially between 25-30 minutes at 254 nm.

NaDES N2 presented 258 total peaks with a total chromatographic area of 9199.63 mAU (Figure 4 D). At 254 nm, N2 exhibited 85 peaks (group b), while at 280 nm it showed 79 peaks (group c). The chromatographic profile displayed well-defined peaks with lower intensity compared to N1 and N2.

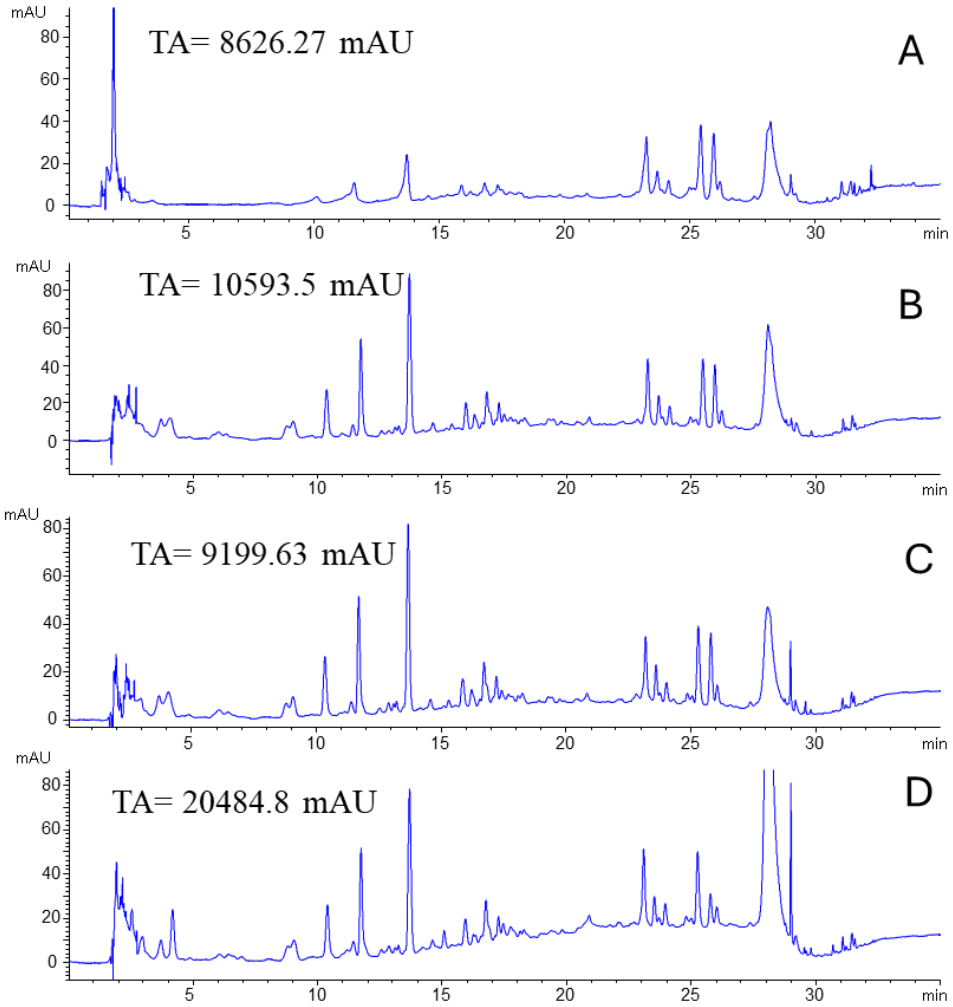

Figure 4. LC-DAD chromatograms of ethanol 70% (A) and NaDES extracts N1 (B), N2 (C), N3 (D) with corresponding total areas (TA) at 254 nm

The chromatographic analysis provides complementary insights to the quantification results. For total phenolics, the quantification hierarchy N1 > N3 > N2 > Ethanol aligns with the ability of N1 ability to extract the highest diversity of phenolic compounds (284 peaks), indicating broad-spectrum extraction efficiency. For Total flavonoids, the quantification order N3 > N2 > N1 > Ethanol reflects the selective extraction approach of N3, where fewer but more concentrated compounds contribute significantly to the total flavonoid content.

This shows that NaDES work differently: N1 extracts many diverse compounds while N3 extracts specific compounds very efficiently showing a higher selectivity for these compounds. The chromatographic data suggest that NaDES represent an effective and environmentally friendly alternative to conventional solvents.

Conclusions

This study demonstrates the promising potential of glycerol-based Natural Deep Eutectic Solvents (NaDES) as a non-toxic and environmentally friendly approach for extracting bioactive compounds from blackthorn fruit. The results revealed exceptional long-term stability, with reduced viscosity, suggesting promising industrial-scale applicability, while structural characterization via Fourier Transform Infrared (FTIR) spectroscopy confirmed the formation of hydrogen bonds through molecular interactions.

The glycerol-fructose combination emerged as the most efficient system for polyphenol extraction, whereas the glycerol-tartaric acid mixture exhibited superior performance in flavonoid extraction. These findings demonstrate the innovative dimensions of our deep eutectic solvents as viable alternatives in food, pharmaceutical, and cosmetic applications. Future research could explore the capacity of the prepared NaDES for polyphenol extraction using other alternative methodologies, such as ultrasound and microwave-assisted techniques and could investigate the potential of the glycerol-tartaric acid solvent for anthocyanin extraction.

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