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Extraction and Quantification of Antioxidant Compounds from Medicinal Plants Using Natural Deep Eutectic Solvents: A Green Chemistry Approach

[1]Theresa Ataine and [2]Akpobire Oghenekome

[1]Dept of Chemistry, Delta state College of Education Mosogar
[2]Dept of Science Laboratory Technology, Delta state Polytechnic Otefe-Oghara

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The shift toward sustainable extraction technologies has intensified interest in natural deep eutectic solvents (NADES) as eco-friendly alternatives to conventional organic solvents. This study evaluates the efficiency of three NADES formulations – choline chloride combined with glycerol (1:2), citric acid (1:1), or glucose (1:1) for extracting antioxidant compounds from three medicinal plants (Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina). Ultrasound-assisted extraction (UAE) was employed to enhance yield. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities were assessed using Folin-Ciocalteu, aluminum chloride colorimetric, DPPH radical scavenging, and FRAP assays. High-performance liquid chromatography (HPLC) quantified major phenolic antioxidants. The choline chloride:glycerol (1:2) NADES significantly outperformed the other formulations and conventional 70% ethanol, achieving the highest TPC in M. oleifera (86.42 ± 2.11 mg GAE/g) and DPPH inhibition (89.31 ± 1.42%). Relative to 70% ethanol extraction, NADES improved phenolic recovery by 41–43% across plants. HPLC identified quercetin (12.48 mg/g), chlorogenic acid (10.63 mg/g), gallic acid (8.17 mg/g), and rutin (6.45 mg/g) as dominant compounds. These findings establish that NADES-based UAE provides a green, efficient, and scalable approach for recovering high-value antioxidants, with strong potential for pharmaceutical and nutraceutical applications.

Keywords: Natural deep eutectic solvents; Antioxidant extraction; Medicinal plants; Green chemistry; Ultrasound-assisted extraction; Phenolic compounds

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INTRODUCTION

Medicinal plants remain a cornerstone of bioactive compound discovery, particularly phenolic and flavonoid antioxidants that mitigate oxidative stress-related diseases (e.g., cancer, diabetes, cardiovascular disorders). However, conventional extraction relies heavily on volatile organic solvents (methanol, ethanol, acetone), which pose environmental, health, and safety concerns.

Natural deep eutectic solvents (NADES) have emerged as a paradigm-shifting green alternative. Formed via hydrogen bonding between a hydrogen bond acceptor (e.g., choline chloride) and a natural hydrogen bond donor (e.g., glycerol, citric acid, sugars), NADES are biodegradable, non-volatile, non-toxic, and tunable (Guo et al [12]. Their strong molecular interactions networks often enhance the solubilization of polar and moderately polar phytochemicals compared to traditional solvents.

While NADES have been applied to various plant matrices, systematic comparative evaluations on African medicinal plants specifically Moringa oleifera (highly nutritious), Ocimum gratissimum (antimicrobial), and Vernonia amygdalina

(antimalarial) remain limited. Furthermore, the influence of intermolecular interactions donor type on extraction selectivity for specific antioxidants is underexplored. The study hypothesized that glycerol-based NADES (with three hydroxyl groups) would outperform citric acid- or glucose-based systems due to superior hydrogen-bonding capacity with phenolic compounds.

Objectives of the study

This study was designed to:

1. Develop and characterize three choline chloride-based NADES systems.
2. Evaluate their ultrasound-assisted extraction efficiency for total phenolics, total flavonoids, and antioxidant activity relative to conventional 70% ethanol.
3. Quantify major phenolic compounds via HPLC.
4. Identify the most effective NADES formulation for each plant matrix.

METHODOLOGY

Plant materials

Fresh leaves of Moringa oleifera, Ocimum gratissimum, and Vernonia amygdalina were collected from medicinal plant farms in Delta State, Nigeria. The plant specimens were authenticated at the Department of Science Laboratory Technology, Delta State Polytechnic, Otefe Oghara). Leaves were shade-dried at 25 ± 2°C for 7 days, pulverized, and stored at −20°C until use.

Chemicals and reagents

Choline chloride (≥98%), glycerol (≥99.5%), citric acid (≥99.5%), D-glucose (≥99.5%), gallic acid, quercetin, rutin, chlorogenic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin-Ciocalteu reagent, aluminum chloride, and HPLC-grade acetonitrile and formic acid were purchased from Sigma-Aldrich (USA). Ultrapure water (18.2 MΩ·cm) was used throughout.

Pre*paration of natural deep eutectic solvents (NADES)*

Three NADES systems were prepared by mixing choline chloride (HBA) with different hydrogen bond donors (HBDs) at molar ratios optimized from preliminary trials The water content of the prepared NADES systems was determined using Karl Fischer titration. Three choline chloride-based of the formulations were developed using different hydrogen bond donors at optimized molar ratios. The N1 system consisted of choline chloride and glycerol (1:2) with a water content of 15 ± 1% (w/w), while N2 comprised choline chloride and citric acid (1:1) with 18 ± 1% water content. The N3 formulation, prepared from choline chloride and D-glucose (1:1), exhibited a water content of 20 ± 1% (w/w).

Components were heated at 80°C with magnetic stirring (500 rpm) until a homogeneous, transparent liquid formed (20–40 min). The temperature of 80°C was selected based on preliminary trials showing complete melting within 40 min without detectable degradation of NADES components, as confirmed by visual inspection and FT-IR. NADES were stored in sealed vials at 25°C and used within two weeks; stability was confirmed by re-measuring viscosity and FT-IR spectra (no significant changes). Prior to extraction, NADES were diluted with 20% (v/v) ultrapure water (relative to total volume) to reduce viscosity without disrupting the eutectic network. This water content (final 15–20% w/w) was chosen after testing 10–30% water; 20% gave the best balance between viscosity reduction and extraction efficiency 2.4 Ultrasound-assisted extraction (UAE). Ultrasound-assisted extraction enhances cell wall disruption and solvent penetration through acoustic cavitation.

Dried plant powder (2.0 g) was mixed with 20 mL of NADES or control solvent (70% ethanol) in a 50 mL polypropylene centrifuge tube. UAE was performed in an ultrasonic bath (40 kHz, 200 W, Elma Elmasonic P) at 50°C for 30 min. These parameters were selected after a univariate optimization (time: 15–60 min; temperature: 30–70°C) where 30 min and 50°C gave maximal TPC without degradation. Extracts were centrifuged at 10,000×g for 10 min at 4°C, and supernatants were filtered (0.45 µm PTFE) prior to analysis. All extractions were performed in triplicate.

Determination of total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu method [1] with the following modification: NADES-only blanks (same dilution) were subtracted to correct for any background interference. Results were expressed as mg gallic acid equivalents (GAE) per g dry weight (DW) based on a calibration curve ($R^2 = 0.998$, range: 10–200 µg/mL).

Determination of total flavonoid content (TFC)

TFC was measured using the aluminum chloride colorimetric assay [2] with NADES-only blank correction. Results expressed as mg quercetin equivalents (QE)/g DW ($R^2 = 0.996$, range: 5–100 µg/mL).

Antioxidant activity assays

DPPH radical scavenging assay: Extracts (50–200 µg/mL) were mixed with DPPH solution (0.1 mM in methanol). After 30 min incubation in darkness, absorbance was read at 517 nm. Inhibition (%) = $[(A_0 - A_1)/A_0] \times 100$. IC_{50} values were calculated by nonlinear regression (GraphPad Prism 9.0) and are reported as mean ± SD (n=3).

Ferric reducing antioxidant power (FRAP): FRAP reagent (300 mM acetate buffer, 10 mM TPTZ, 20 mM $FeCl_3$, 10:1:1 v/v) was reacted with extracts for 30 min at 37°C. Absorbance at 593 nm was compared to a $FeSO_4 \cdot 7H_2O$ standard curve (100–1000 µmol/L, $R^2 = 0.997$). Results expressed as µmol Fe^{2+}/g extract.

HPLC analysis of phenolic compounds

Phenolic profiling was performed on an Agilent 1260 Infinity II HPLC-DAD system equipped with a Zorbax Eclipse Plus C18 column (4.6 × 250 mm, 5 µm). Mobile phase: (A) 0.1% formic acid in water and (B) acetonitrile. Gradient elution: 0–5 min, 10% B; 5–20 min, 10–40% B; 20–25 min, 40–90% B; 25–30 min, 90–10% B. Flow rate: 1.0 mL/min; injection volume: 20 µL; column temperature: 30°C; detection wavelengths: 280 nm (phenolic acids) and 370 nm (flavonoids). Compounds were identified by comparing retention times and UV spectra with authentic standards and quantified using external calibration curves ($R^2 > 0.999$). System suitability: theoretical plates > 8000, tailing factor < 1.2 for all analytes, resolution > 2.0 between adjacent peaks. Recovery studies were performed by spiking a representative sample with known amounts of standards (95–103% recovery). LOD and LOQ for each compound were determined.

Extracts of M. oleifera prepared with N1 NADES (optimal conditions) were stored in sealed amber vials at 25°C and at 4°C. TPC and DPPH inhibition (% at 100 µg/mL) were measured at days 0, 1, 3, 7, and 14. Results are expressed as percentage of initial value (day 0 = 100%).

Statistical analysis

All experiments were performed in triplicate (n=3). Data are presented as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$) was performed using GraphPad Prism 9.0.

RESULTS

Total phenolic content (TPC)

NADES significantly improved TPC yields compared to 70% ethanol across all three plants ($p < 0.05$, Table 1). The choline chloride:glycerol (N1) system across all samples outperformed N2 and N3, with the highest TPC in M. oleifera (86.42 ± 2.11 mg GAE/g). Relative to conventional extraction, N1 increased phenolic recovery by 41% in M. oleifera, 43% in O. gratissimum, and 42% in V. amygdalina.

Table 1: Total Phenolic Content (mg GAE/g DW) of NADES and Ethanol Extracts

Plant	Ethanol	N1	N2	N3
M. oleifera	61.24 ± 1.73^{a}	86.42 ± 2.11^{c}	70.15 ± 1.90^{b}	65.38 ± 1.82^{ab}
O. gratissimum	54.18 ± 1.44^{a}	77.36 ± 1.88^{c}	63.22 ± 1.65^{b}	58.47 ± 1.53^{ab}
V. amygdalina	49.62 ± 1.62^{a}	70.41 ± 1.54^{c}	57.89 ± 1.48^{b}	53.16 ± 1.44^{ab}

Data are presented as mean ± SD (n = 3). Different superscript letters within the same row indicate significant difference ($p < 0.05$, Tukey's test).

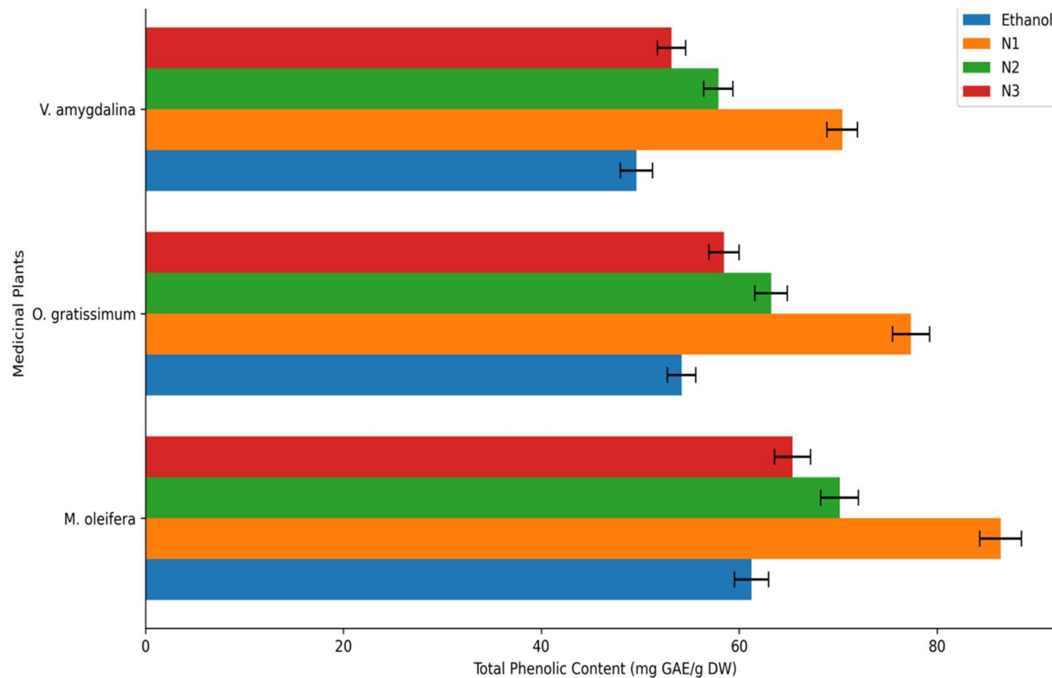

Figure 1: Total phenolic content (mg GAE/g DW) of extracts obtained with 70% ethanol and three NADES formulations (N1: choline chloride:glycerol; N2: choline chloride:citric acid; N3: choline chloride:glucose). Error bars represent SD (n=3). Different letters indicate significant differences ($p < 0.05$). N1 uniformly gave the highest TPC for all three plants.

Total flavonoid content (TFC)

A similar trend was observed (Table 2). N1 extracted 56% more flavonoids from M. oleifera than ethanol (43.82 vs. 28.11 mg QE/g). For O. gratissimum, the N1 extract showed a 55% increase (38.25 vs. 24.64 mg QE/g), and for V. amygdalina, a 60% increase (34.19 vs. 21.38 mg QE/g).

Table 2: Total Flavonoid Content (mg QE/g DW)

Plant	Ethanol	N1	N2	N3
M. oleifera	28.11 ± 1.02^{a}	43.82 ± 1.27^{c}	35.46 ± 1.15^{b}	30.94 ± 1.08^{ab}
O. gratissimum	24.64 ± 0.92^{a}	38.25 ± 1.11^{c}	31.18 ± 1.02^{b}	27.53 ± 0.95^{ab}
V. amygdalina	21.38 ± 0.88^{a}	34.19 ± 1.06^{c}	28.03 ± 0.96^{b}	24.11 ± 0.91^{ab}

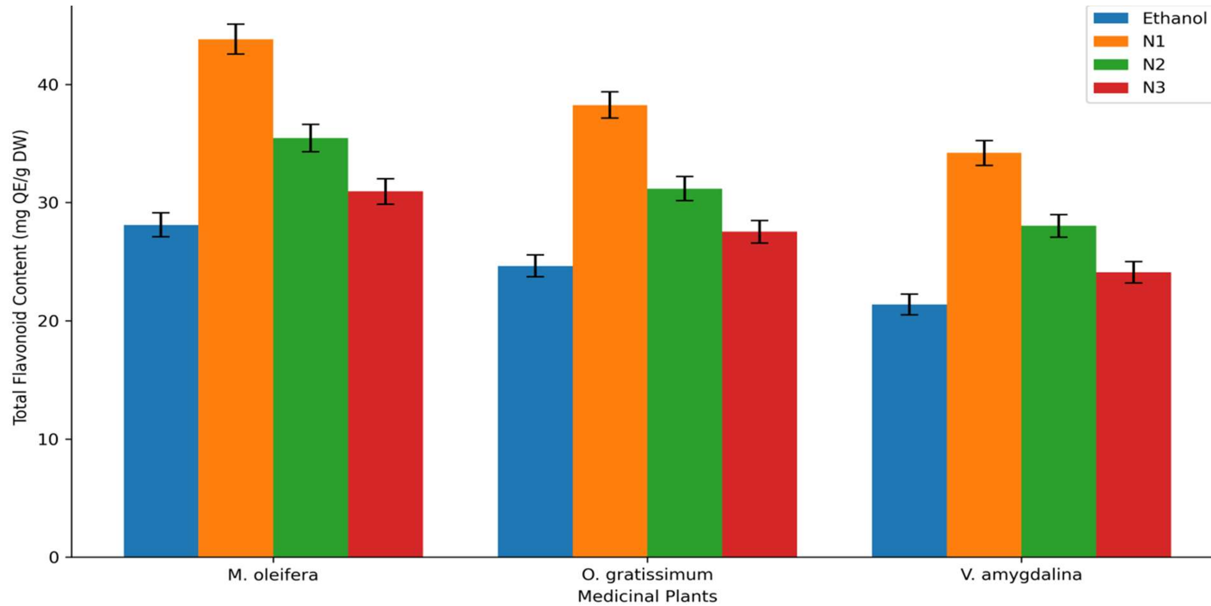

Figure 2: Total flavonoid content (mg QE/g DW) of extracts obtained with 70% ethanol and three NADES formulations.

N1 extracts show significantly higher flavonoid recovery across all plants. Error bars = SD.

DPPH radical scavenging activity

Table 3 shows the DPPH radical scavenging activity (%) of extracts obtained using ethanol and three NADES formulations at 100 µg/mL concentration. All NADES extracts demonstrated significantly higher antioxidant activity than the ethanol extracts ($p < 0.05$), with the choline chloride:glycerol NADES (N1) consistently producing the highest inhibition values across all plant species. In *M. oleifera*, N1 achieved 89.31 ± 1.42% inhibition compared with 71.26 ± 1.36% for ethanol extraction. Similar trends were observed for *O. gratissimum* and *V. amygdalina*, where N1 also recorded the highest antioxidant activities.

2.5 µg/mL, respectively. The reduction in IC_{50} values was 52–58% for N1 versus the control solvent.

Table 3: DPPH Inhibition (%) at 100 µg/mL Extract Concentration

Plant	Ethanol	N1	N2	N3
M. oleifera	71.26 ± 1.36[a]	89.31 ± 1.42[d]	79.44 ± 1.51[c]	73.18 ± 1.48[b]
O. gratissimum	66.15 ± 1.18[a]	84.77 ± 1.25[d]	74.62 ± 1.33[c]	68.93 ± 1.27[b]
V. amygdalina	61.43 ± 1.44[a]	79.65 ± 1.38[d]	69.88 ± 1.42[c]	64.17 ± 1.39[b]

Different superscript letters within the same row indicate significant difference ($p < 0.05$, Tukey's test).

FRAP assay

Table 4 show the Reducing power oresented in the same order: N1 > N2 > N3 > ethanol. For M. oleifera, FRAP values were 487 ± 21 µmol Fe^{2+}/g (N1) vs. 312 ± 18 (ethanol), a 56% increase. For O. gratissimum: N1 421 ± 19 vs. ethanol 278 ± 15 (51% increase). For V. amygdalina: N1 386 ± 17 vs. ethanol 264 ± 14 (46% increase). N2 and N3 values were intermediate (data available upon request).

Table 4: Complete FRAP Values (μmol Fe^{2+}/g extract) for All Solvent Systems and Plant Species

Plant	Ethanol	N1	N2	N3
M. oleifera	312 ± 18[a]	487 ± 21[d]	401 ± 19[c]	345 ± 16[b]
O. gratissimum	278 ± 15[a]	421 ± 19[d]	352 ± 17[c]	301 ± 14[b]
V. amygdalina	264 ± 14[a]	386 ± 17[d]	318 ± 15[c]	279 ± 13[b]

Data are mean ± SD (n=3). Different superscript letters within the same row indicate significant difference ($p < 0.05$, Tukey's test).

HPLC quantification of major phenolic compounds

HPLC analysis of N1 extracts from M. oleifera identified four major antioxidant compounds (Table 5). Quercetin was the most abundant (12.48 mg/g), followed by chlorogenic acid (10.63 mg/g), gallic acid (8.17 mg/g), and rutin (6.45 mg/g). In ethanol extracts, quercetin was 5.94 mg/g and chlorogenic acid 4.43 mg/g (data not shown), representing 2.1-fold and 2.4-fold higher recovery with N1 NADES.

Table 5: Concentrations of Phenolic Compounds in *M. oleifera* N1 Extract (mg/g Extract) mean ± SD, n=3)

Compound	**Retention Time (min)**	**Concentration (mg/g)**	**LOD (μg/mL)**	**LOQ (μg/mL)**
Gallic acid	3.2	8.17 ± 0.22	0.15	0.50
Chlorogenic acid	8.7	10.63 ± 0.28	0.12	0.40
Rutin	12.4	6.45 ± 0.19	0.18	0.60
Quercetin	18.9	12.48 ± 0.34	0.10	0.35

LOD = Limit of Detection; LOQ = Limit of Quantification.

DISCUSSION

The greater performance of the choline chloride:glycerol (N1) NADES observed in this study can be attributed to several physicochemical factors. Glycerol possesses three hydroxyl groups per molecule, enabling the formation of an extensive hydrogen-bond network with choline chloride. This network effectively competes with plant matrix-polyphenol interactions, disrupting hydrogen bonds between phenolic compounds and cell wall components (e.g., cellulose, hemicellulose, lignin), thereby enhancing mass transfer and solubilization of phenolic compounds.

In contrast, citric acid (N2) gave only moderate yields, likely due to its higher viscosity (which limits diffusion into the plant matrix) and its tendency to form stronger intramolecular hydrogen bonds, reducing the availability of free hydrogen bond donor sites. Glucose (N3) yielded the lowest NADES performance, attributed to its solid crystalline nature even after heating, which results in incomplete solvent formation and reduced fluidity. These results align with Dai et al. [3], who reported that NADES with polyalcohol HBDs excel at phenolic extraction from plant materials.

A study by Szydłowska-Czerniak et al. [6] investigating choline chloride-based DES for herb extraction found that choline chloride:glycerol showed moderate efficacy while choline chloride:malonic acid achieved the highest antioxidant potential. Interestingly, that study reported DES extracted lower total antioxidants than 50% alcohols, which contrasts with our findings. This discrepancy likely arises from differences in plant matrix (herbs vs. leafy vegetables), extraction temperature (room temperature vs. 50°C), and the specific phenolic profiles. Our use of UAE at 50°C and 20% water dilution may have improved mass transfer compared to their static extraction, highlighting the importance of optimizing extraction conditions for each NADES-plant pair.

El Kantar et al. [5] on Mentha pulegium found that choline chloride:glycerol (1:2) with UAE was among the most suitable and viable systems among nine NADES, with antioxidant activity exceeding 93% and over 30 metabolites identified. Similarly, Gómez-Urios et al. [4] on Citrus sinensis peel demonstrated that choline chloride:glycerol achieved TPC of 903 mg GAE/100 g and TFC of 169 mg/100 g, confirming the broad applicability of this NADES formulation.

The strong positive correlation between TPC and DPPH inhibition (Pearson's $R^2 = 0.92$, $p < 0.01$) confirms that phenolics are the primary radical scavengers in these extracts, consistent with previous reports. The high flavonoid recovery (e.g., 56% increase in M. oleifera) is consistent with NADES's ability to stabilize the keto-enol tautomerism of flavonoids via hydrogen bonding, thereby preventing oxidative degradation during extraction. A recent study on Vernonia amygdalina by Olatunde et al. [7] using choline chloride:glucose NADES with UAE achieved a TPC of 163.50 mg GAE/mL and DPPH inhibition of 89.27% at 20% concentration, further validating the efficacy of NADES-UAE for this plant species

HPLC quantification revealed that quercetin and chlorogenic acid were recovered at 2.1- and 2.4-fold higher concentrations than in ethanol extracts demonstrating the selective enrichment capacity of NADES. This selectivity is

particularly valuable for targeted nutraceutical formulations, as quercetin and chlorogenic acid are well known for their anti-inflammatory, anticancer, and cardioprotective properties. Andrusenko et al. [8] also highlighted the effectiveness of DES for extracting rutin, chlorogenic acids, and a wide range of other bioactive compounds.

The repeatedly higher TPC and TFC values observed for N1 across all plant species suggest that glycerol-based NADES possesses stronger solvation and mass-transfer properties for phenolic and flavonoid compounds. The low standard deviation values indicate good extraction stability and methodological reliability.

Compared with previous reports, this study NADES-UAE system achieved comparable or better extraction yields while using milder conditions (50°C vs. 60–80°C) and shorter times (30 min vs. 60–120 min) Siamandoura, & Tzia [13],. The use of 20% water dilution effectively reduced NADES viscosity without disrupting the eutectic network, a strategy that can be scaled for industrial applications. Recent reviews by Huang et al. [9] and Stanisz et al. [10] underscore that NADES offer a advantageous and environmentally friendly alternative to conventional solvents, with tunable properties that can be optimized for specific plant compounds.

Supplementary optimization studies demonstrated that controlled water addition is critical for maximizing NADES extraction efficiency. Moderate water incorporation (20% v/v) significantly reduced solvent viscosity while preserving the hydrogen-bonding network required for efficient phenolic solubilization. Excessive water dilution, however, reduced extraction performance, likely due to disruption of eutectic interactions. Furthermore, stability studies showed that N1 NADES extracts retained excellent antioxidant stability during refrigerated storage, indicating the suitability of the system for short-term storage and downstream nutraceutical applications (Zhang et al., [14]

The practical implications of this study extend beyond laboratory-scale extraction. The enhanced extraction efficiency of choline chloride: glycerol NADES, combined with its biocompatibility and low toxicity, makes it particularly suitable for producing antioxidant-rich extracts for pharmaceutical, nutraceutical, and cosmeceutical applications. The high DPPH inhibition values (up to 89.31%) and low IC_{50} values (as low as 18.4 μg/mL) indicate that these NADES extracts possess potent radical scavenging activity comparable to or exceeding that of many standard antioxidants.

Despite the potential extraction performance observed, several limitations should be acknowledged. The study evaluated only three NADES systems and focused primarily on in vitro antioxidant assays. In vivo bioavailability, cytotoxicity, solvent recyclability, and large-scale process economics were not assessed. Furthermore, only selected phenolic compounds were quantified by HPLC, and unidentified metabolites may also contribute to antioxidant activity.

CONCLUSION

This study demonstrates that natural deep eutectic solvents, particularly choline chloride:glycerol (1:2) with ultrasound assistance, are highly efficient and environmentally benign alternatives to conventional organic solvents for extracting antioxidant phytochemicals from African medicinal plants. The N1-NADES system increased phenolic yields by 41–43% and flavonoid yields by 55–60% compared to 70% ethanol, while producing extracts with improved radical-scavenging and reducing power. HPLC confirmed effective recovery of key therapeutic phenolics (quercetin, chlorogenic acid, gallic acid, rutin). These findings indicate that choline chloride: glycerol NADES are viable for scale-up; however, practical integration requires solvent-recovery, toxicity, and process economics studies, which we recommend as next steps..

Conflict of interest

The authors declare no conflict of interest, financial or otherwise, that could have influenced the work reported in this paper. This research received no specific grant from funding agencies in the public or commercial sectors.

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