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# Polyphenol-enriched extraction from *Thunbergia laurifolia* using natural deep eutectic solvents for enhanced antioxidant and anti-inflammatory activities†

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*Thunbergia laurifolia* Lindl., well known for its detoxifying properties, contains a high concentration of bioactive compounds. The increasing demand for functional foods and age-related therapies emphasizes the importance of enhancing extraction yields using green, sustainable solvents. Natural deep eutectic solvents (NADESs) were employed to enhance extraction yields, and choline chloride-based NADESs and L-proline-based NADESs were compared with traditional 95% ethanol. HPLC analysis revealed that NADES extracts contained significantly higher quantities of polyphenols, approximately three times higher than those detected in the ethanolic extract. The extracts were evaluated for antioxidant and anti-inflammatory activities using DPPH and FRAP assays and NO production in RAW 264.7 macrophage cells. Additionally, the effects of the NADES extracts on inflammatory gene expression were assessed. The results showed that L-proline-based NADES, composed of L-proline and 1,4-butanediol, exhibited enhanced antioxidant activity and effectively inhibited inflammation by reducing the expression of *iNOS*, *COX-2*, *5-LOX*, and *IL-6* genes without causing any adverse effects on RAW 264.7 cells. These findings establish L-proline and 1,4-butanediol as highly effective, universal green solvents for extracting phytochemical compounds with proven safety, making them ideal, practical, ready-to-use candidates.

## 1. Introduction

*Thunbergia laurifolia* Lindl (TL), belonging to the family Acanthaceae, is widely used in Southeast Asian folk medicine owing to its detoxifying properties as an antidote for poisoning caused by toxic drugs, heavy metals, and insecticides. It has anti-mutagenic, anti-oxidant, anti-inflammatory, antipyretic, anti-microbial, hepatoprotective, antinociceptive, antidiabetic, and neuroprotective properties.[1–4] Phenolic acids, flavonoids, iridoid glycosides, carotenoids, and chlorophyll are among the numerous active compounds present in this plant.[5] Among these, phenolic acids and flavonoids are particularly important because they play a critical role in the medicinal properties of this plant.[1] Previous studies have confirmed the presence of detectable levels of rosmarinic acid, caffeic acid, vicenin-2, rutin, and apigenin.[6]

TL is typically consumed as tea and can be used as an ingredient in traditional pills.[7] The traditional method for extracting its phytochemicals involves boiling dried TL leaves in water for 30 minutes to 2 hours, a time-consuming process limited to water-soluble compounds.

The increasing population has fueled increasing interest in nutraceutical functional foods and the enhancement of various health products, emphasizing the extraction of concentrated bioactive compounds to improve their bioavailability and absorption in the body. To maximize productivity, efficient extraction procedures are always being investigated. Traditional solvent-based extraction employs organic and inorganic solvents, including acetone, acetonitrile, and methanol, which are suitable for the extraction of hydrophobic substances. However, traditional solvents have considerable limitations. Many are combustible, volatile, and poisonous, affecting human health and causing environmental concerns. As a result, there has been a growing shift toward greener, sustainable extraction methods that not only improve efficiency but also reduce environmental and health hazards.

Deep eutectic solvents (DESs) are a subclass of ionic liquids. In comparison to conventional ionic liquids, deep eutectic solvents present multiple benefits, such as decreased synthesis costs, diminished toxicity, reduced volatility, and enhanced biodegradability.[8] A DES is composed of two or more solid or liquid components mixed in a specific mole ratio to form a homogeneous solution. This mixture becomes a liquid

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through the interaction forces between a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), involving hydrogen bonding and van der Waals interactions. Therefore, the mixture has a lower freezing point than each component (a depression in the melting point).[9,10] DESs usually comprise quaternary ammonium salts as HBAs and organic compounds containing carboxyl groups as HBDs. The hydrogen bonding between HBA and HBD increases the viscosity of the solvent and gives it a specific polarity, typically between that of ethanol and water. This allows DESs to extract both hydrophilic and hydrophobic compounds simultaneously.[10–12] Natural Deep Eutectic Solvents (NADESs) are a subtype of DESs that serve as green and sustainable alternatives for achieving high extraction yields while maintaining biocompatibility, making them highly suitable for practical applications. These solvents are formed using natural primary metabolites or their derivatives, such as HBDs and HBAs. Additionally, NADESs can act as both solubilizers and stabilizers in pharmaceutical formulations owing to their unique physicochemical properties.[13] A study by Jurić *et al.* (2024) reported that choline chloride-based NADESs significantly enhanced the extractability of rosmarinic acid while increasing the anti-inflammatory potential of the extract.[14]

Beyond their extraction capacities, NADESs improve the pharmacological characteristics of bioactive compounds. Morgana *et al.* (2022) found that an NADES composed of lactic acid, glucose, and water significantly improved the stability of anthocyanins, a highly unstable subclass of flavonoids, in intestinal fluid. This formulation preserved 43% of the compounds, far exceeding the typical retention observed in animal studies (0.26–1.80%) and humans (1.09%).[15] The crude extract of anthocyanin in NADES increased the bioavailability of anthocyanins to 140% compared to the crude extract by the organic solvent in the aqueous solution by the organic solvent (methanol:water:formic acid mixture).[16] A study by Faggian *et al.* (2016) shows the ability of proline-based NADES to increase the solubility of polyphenol rutin,[17] making it more available in oral administration than in water suspension. These findings indicate that NADESs play a vital role in increasing the bioavailability and stability of bioactive compounds throughout an organism's physiological processes.

Macrophage cells, which are innate immune cells, are critical for maintaining tissue homeostasis against infection from extinct stimuli and injury. This cell is the first to respond to lipopolysaccharide (LPS) action by secreting inflammatory mediators, such as reactive oxygen species (ROS), nitric oxide (NO), and pro-inflammatory cytokines, such as interleukin-6 (IL-6), resulting in the development of severe inflammatory and autoimmune disorders. Inflammatory cascade processes can stimulate free radicals, resulting in a series of events that cause oxidative damage to proteins, nucleic acids, and lipids, increasing the risk of cancer and immune-related disorders while compromising cell membrane integrity.[18] Bioactive compounds that strengthen antioxidant and anti-inflammatory defenses are particularly important in this context. Investigating their effects on the inflammatory process in macrophage cells is a key first step in assessing their potential for developing preventive and therapeutic strategies for various disorders.

The use of NADES as a solvent for extracting bioactive substances from TL has not been previously documented. Furthermore, there have been few studies on the influence of both the extract and NADES on cells, leaving a considerable gap in understanding their biological impacts. This study focuses on comparing traditional extraction solvents with NADES by comparing chorine chloride-based NADES and L-proline-based NADES to retrieve bioactive compounds from the leaves of TL. Additionally, it determines their bioactivity in anti-inflammatory, cytotoxicity and impact of the composition of NADES on RAW 264.7 cells *via* NO production and inflammatory gene expression (*iNOS*, *5-LOX*, *COX-2*, and *IL-6*). This helps assess the potential of NADES not only as an eco-friendly solvent but also as an effective compound for direct human use, improving stability and enhancing therapeutic effects.

## 2. Materials and methods

### 2.1. Reagents and chemicals

The reference standards utilized in this study were obtained from the following suppliers: vicenin-2 (≥98%), rosmarinic acid (≥95%), caffeic acid (≥95%), and rutin (≥95%) from Chengdu Biopurify Phytochemicals, Ltd (Chengdu, China); apigenin (>95%) from Wako Chemicals (Osaka, Japan); and chlorogenic acid (≥98%) from Chemfaces (Wuhan, China). Acetonitrile (HPLC grade, Far UV, >99.8%) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) supplied the chemicals utilized in the DPPH and FRAP tests. Additional analytical-grade chemicals for the preparation of NADESs were purchased from the suppliers specified as follows: choline chloride from Loba Chemie (Mumbai, MH, India), 1,4-butanediol from Sigma-Aldrich, absolute ethanol and lactic acid from Thermo Fisher Scientific Inc., and L-proline from Himedia (Mumbai, MH, India). RAW 264.7 macrophage cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. NADES preparation

Based on their mentioned extraction capabilities, L-proline and choline chloride-based NADES were chosen as HBAs, combined with two HBDs, namely 1,4-butanediol and lactic acid, with a mole ratio of 1 : 2 and 40% (v/v) water.[19] NADES were prepared by mixing HBA, HBD, and water at the final concentration, followed by stirring in a water bath at 60 °C until a clear solution

Table 1 List of the studied NADESs[a]

| No. | Abbreviation | HBA | HBD |
|---|---|---|---|
| 1 | CB | Choline chloride | 1,4-Butanediol |
| 2 | CL | Choline chloride | Lactic acid |
| 3 | PB | L-Proline | 1,4-Butanediol |
| 4 | PL | L-Proline | Lactic acid |

[a] The mole ratio of HBA to HBD is 1 : 2, with a 40% (v/v) water content.

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was obtained. The particular compositions and abbreviations for HBD and HBA are listed in Table 1.

### 2.3. Physicochemical characterization of the prepared NADESs

The pH of the NADESs was measured using a pH/ORP meter (HI2211, Hanna Instruments). Density was determined gravimetrically by weighing 1 mL of each NADES sample on an electronic analytical balance (AL104, Mettler Toledo Instruments Co., Ltd). Viscosity was measured using a DVNext Rheometer (DVNXLVTJG, AMETEK Brookfield, USA) equipped with an SC4-34 spindle. A 10 mL sample of each NADES was used for viscosity measurement at 30 °C.

### 2.4. Plant material and ultrasound-assisted extraction (UAE)

TL leaves were gathered in the Herbal Garden at the Faculty of Pharmaceutical Sciences, Khon Kaen University (Voucher code NI-PSKKU 157). The leaves were washed and dried at 50 °C until a consistent weight was achieved. The samples were then pulverized by grinding and passed through a 1 mm filter to obtain a fine powder. Each sample was weighed correctly at 50 mg and placed in a microcentrifuge tube. For extraction, 1 mL of the solvents, including 95% ethanol and NADESs, was transferred to tubes containing 50 mg of powder (1 : 2 ratio of solid to liquid). Each tube was then incubated for one hour at 50 °C with vortexing every 20 minutes in an ultrasonic bath (WiseClean WUC-A10H, 40 kHz, 265 W). After that, the sample tubes were centrifuged for 5 min at 4300×*g*; then, the supernatants were collected. The supernatants of the ethanolic extracts were permitted to evaporate at room temperature to produce crude extracts, followed by the addition of 1 mL of absolute ethanol AR to restore the original volume. The experiments were conducted in three replicates.

### 2.5. Chromatographic conditions

The standard compounds (rosmarinic acid, caffeic acid, chlorogenic acid, vicenin-2, rutin and apigenin) were prepared in a previous study.[20] The contents of phenolic acids and flavonoids were determined by HPLC (Agilent 1260 series, USA) using a UV detector at 330 nm and Merck Lichrospher 100 C18 (250 × 4 mm, 5 μm). Gradient elution was conducted at a flow rate of 1 mL $min^{-1}$, with acetonitrile (B) and 0.05% *ortho*-phosphoric acid (A) in water serving as the mobile phase. The initial mobile phase ratio of A : B is 90 : 10, with a gradient of 75 : 25 over 19 minutes. It is then fixed to an isocratic 75 : 25 from 19 to 21 minutes, followed by a gradient to 70 : 30 from 21 to 30 minutes. Finally, the mobile phase is switched to 100% B from 35 to 40 minutes. The phenolic acid and flavonoid contents were calculated using a standard curve and reported in μg g per dry weight (DW).

### 2.6. Antioxidant capacity assay

**2.6 1. Assessment of the free radical scavenging activity by DPPH assay.** The DPPH assay was utilized to determine the free radical scavenging activity of the extracts. Absolute ethanol was used to dilute both the sample and standard components to different concentrations. The NADES were used as the sample control for comparison in each NADES extract sample, with each NADES diluted to match the dilution of its corresponding NADES extract. The procedure followed a previous report,[19] in which 50 μL of the prepared sample and the standard solution were added to a 96-well plate, followed by the addition of 0.5 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution. After the mixture was thoroughly combined, it was incubated in the dark at room temperature for 30 minutes to allow for the reaction to complete. The optical density (OD) was then measured at 517 nm.

The DPPH scavenging percentage was employed to calculate the $IC_{50}$ value, which indicates the sample concentration needed to neutralize 50% of the DPPH radicals, using eqn (1):[19]

$$\text{DPPH radical inhibition (\%)} = [\mathrm{Abs_{DPPH}} - (\mathrm{Abs_s} - \mathrm{Abs_c})/\mathrm{Abs_{DPPH}}] \times 100 \quad (1)$$

where $Abs_{DPPH}$ denotes the absorbance of the DPPH solution in the absence of the sample and $Abs_s$ and $Abs_c$ are the absorbances of the samples with and without the DPPH solution, respectively.

**2.6.2. Determination of antioxidants by ferric reducing antioxidant power assay (FRAP) assay.** The total reducing power of the plant extracts was measured using the FRAP assay, following the method of ref. 21. The FRAP reagent was prepared by combining 100 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) dissolved in 40 mM HCl, and 20 mM $FeCl_3$ in a 10 : 1 : 1 ratio. A standard curve was generated using Trolox concentrations ranging from 3.13 to 100.0 μg $mL^{-1}$. The standards and samples were mixed with the FRAP reagent in a 1 : 9 ratio and incubated for 30 minutes at room temperature. The absorbance was subsequently measured at 595 nm. The standard curve was constructed using Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) at various concentrations to determine the FRAP values, which were presented as Trolox equivalents (TE, μg g per DW).

### 2.7. Evaluation of anti-inflammatory effects through nitric oxide (NO) assay in RAW 264.7 cells

The LPS of Gram-negative bacteria, a widely recognized stimulator of immune cells, was employed as an inflammatory stimulant in accordance with a previous study that tested the anti-inflammatory effects *in vitro* using macrophage cells RAW 264.7 (ref. 22). Dulbecco's Modified Eagle Medium (DMEM), supplemented with penicillin/streptomycin (1% v/v) and fetal bovine serum (FBS) (10% v/v), was used as the culture medium. The cells were cultured under 5% $CO_2$ at 37 °C until they reached a density of $3 \times 10^5$ cells per mL, after which they were used as the inoculum for seeding in 96-well plates. After growing the cells for 24 h, LPS (0.8 μg $mL^{-1}$) and the extracts were co-treated with the cells, compared to the control, and then incubated for an additional 24 h. Subsequently, the media samples were collected and analyzed for NO production using the Griess reagent. The NADES (without extract) were also treated as a sample at the same dilution as the extract to

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evaluate the effect of the NADES. For the ethanolic extracts, the dried crude sample was dissolved in 1% (v/v) DMSO, mixed with DMEM to prepare a stock solution, and then diluted with DMEM to achieve the desired concentration while maintaining the DMSO at 0.1% (v/v). L-NAME (200 μM) was utilized as a positive control. The standard curve for nitric oxide (NO) levels was established by reacting NO standards with the Griess reagent and measuring the absorbance at 562 nm. The NO levels measured in each sample were then converted into percentage inhibition using eqn (2):

$$\%\text{NO inhibition} = [(\text{NO}_{\text{LPS}} - \text{NO}_{\text{Sample}})/(\text{NO}_{\text{LPS}} - \text{NO}_{\text{Control}})] \times 100 \quad (2)$$

### 2.8. Cytotoxicity assessment of NADES and ethanolic extracts from *T. laurifolia* leaf in murine RAW 264.7 macrophages using a cell viability assay

The culture media was discarded after the NO test and replaced with 0.5 mg mL$^{-1}$ of MTT solution, followed by incubation for 2 hours. Subsequently, the media was removed, and isopropanol was used to dissolve the formazan crystals generated by the viable cells. The optical density at 595 nm was measured to calculate cell viability (%), with reference to the value derived from the baseline control (0.1% DMSO).

### 2.9. Real-time PCR for the evaluation of inflammatory gene expression

6-Well plates were employed to scale up the RAW 264.7 cell culture, which was then treated with both LPS and the extract, following the same protocol as the NO assay, with an extract concentration of 250 μg DW per mL. After 24 h of incubation, RNA was purified from the collected cells using TRIzol® LS reagent (Ambion, Life Technologies) following the manufacturer's protocol. The concentration and purity of the extracted RNA were determined using a BioDrop DUO (USA) and then used as a template for cDNA synthesis with a Bio-Rad T100™ Thermal cycler in combination with the ReverTra Ace QPCR RT Master Mix (Toyobo, Japan), following the manufacturer's instructions. Subsequently, cDNA was utilized to analyze the expression levels of inflammatory genes, including inducible nitric oxide synthase (*iNOS*), cyclooxygenase-2 (*COX-2*), 5-lipoxygenase (*5-LOX*), and *IL-6*, by real-time PCR with SYBR Green PCR Master Mix (Bio-Rad) using β-actin as the reference gene. Data analysis was performed using Bio-Rad iQ5 software and expressed as fold changes relative to the control group. Relative gene expression as assessed by the $2^{-\Delta\Delta Ct}$ technique was used to report the expression levels. The primer sequences used in this study can be found in a previous report.[19]

### 2.10. Statistical analysis

The HPLC data, $IC_{50}$ from the DPPH assay, and FRAP values were gathered from three replicates and subjected to analysis through one-way ANOVA, followed by Duncan's multiple range test ($p < 0.05$). The percentage of NO inhibition was determined from six replicates ($n = 6$) and analyzed using one-way ANOVA, followed by Duncan's multiple range test ($p < 0.05$). In the meanwhile, gene expression data were collected from three replicates, and a one-way ANOVA accompanied by Dunnett's test was employed for analysis ($p < 0.001$). Statistical analyses were conducted utilizing SPSS software version 16 (IBM Corp., Chicago, IL, USA).

## 3. Results and discussions

### 3.1. NADES characteristics

All tested NADESs exhibited pH values in the acidic range (<7), as presented in Table 2. Previous studies have suggested that pH plays a critical role in extraction efficiency. Acidic conditions may facilitate the disruption of plant cell walls and enhance the solubilization of biopolymers, thereby improving extraction compared to neutral pH solvents.[23] This may partly explain the superior extraction yields observed with certain NADESs when compared to 95% ethanol, which is typically near neutral in pH. Furthermore, pH influences extraction efficiency and affects the stability of the extracted compounds. As previously reported, acidic environments can promote the protonation of phenolic hydroxyl groups, contributing to structural stability and reducing oxidative degradation. In contrast, alkaline conditions may accelerate the degradation of phenolic compounds through oxidation processes by oxygen.[24]

Interestingly, all tested NADESs containing 40% water exhibited relatively low viscosities, approaching that of water (approximately 0.8 cP at 30 °C).[25] Viscosity is a critical factor influencing extraction efficiency, as there appears to be an optimal viscosity range that maximizes yield. Variations in the optimal condition, whether above or below, may lead to reduced extraction efficiency. As viscosity significantly influences mass transfer, solute solubility, dispersion, and system stability, a reduction in viscosity can enhance diffusion and facilitate more effective solute–solvent interactions.[26] However, NADESs with higher viscosity may enhance extraction owing to their strong solvation power and increased chemical affinity for the target compounds. However, their high viscosity can limit mass transfer and hinder simple diffusion. In our study, the use of UAE at a controlled temperature of 50 °C helps mitigate this issue by reducing viscosity during the process, thereby allowing the high solvating capacity to coexist with enhanced mass transfer. Moreover, the water content in NADES significantly influences viscosity and, consequently, affects extraction efficiency. An appropriate water concentration can enhance

Table 2 Physicochemical characteristics of the NADESs[a]

| NADESs | pH | Color | Density (g mL$^{-1}$) | Viscosity (cP) |
|---|---|---|---|---|
| CB | 4.25 | Colorless | 1.054 ± 0.007 | 7.910 ± 0.014 |
| CL | 1.50 | Colorless | 1.115 ± 0.007 | 7.040 ± 0.069 |
| PB | 5.70 | Colorless | 1.092 ± 0.003 | 15.560 ± 1.021 |
| PL | 2.85 | Colorless | 1.183 ± 0.004 | 14.280 ± 0.170 |

[a] The mole ratio of HBA to HBD is 1 : 2, with 40% (v/v) water content. Viscosity values were measured at 30 °C.

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extraction yield, as demonstrated in a previous study,[19] which reported that NADESs with lower water content led to decreased total flavonoid extraction. To enhance extraction yield, a water content of 40% (v/v) was selected based on previous reports identifying it as optimal.[19] The extraction capacities of the different NADESs showed a similar trend in yield, which may be attributed to their comparable viscosity ranges.

### 3.2. HPLC profiling of extracts of *T. laurifolia* leaf obtained using different solvents

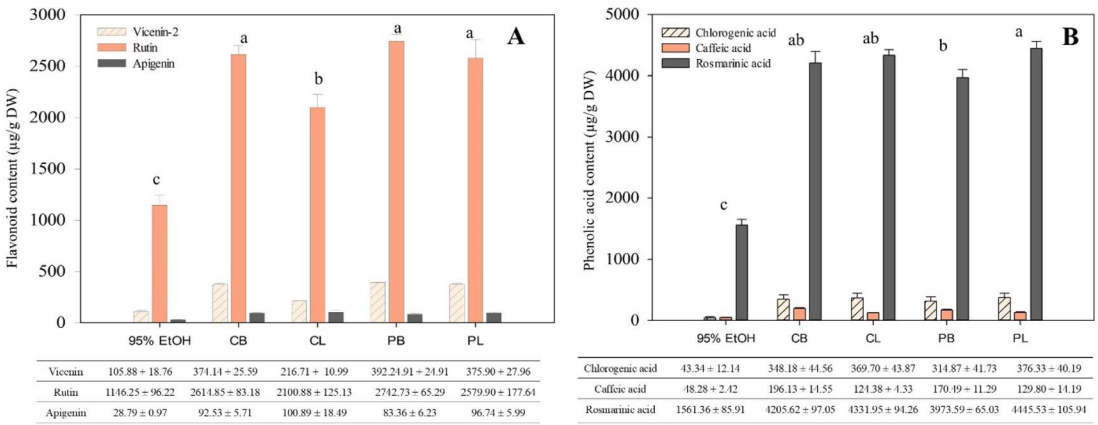
Four NADES formulations were selected to evaluate their efficacy in extracting bioactive compounds from TL leaves compared to the conventional solvent (95% ethanol). HPLC analysis (Fig. 1 and S1†) demonstrated that the NADES extracts contained significantly higher total phenolic and flavonoid contents compared to the ethanolic extract. Notably, all extracts consistently contained rutin and rosmarinic acid as the predominant bioactive compounds. This finding aligns with a previous report by Pattananandecha *et al.* (2021), which identified rosmarinic acid as a major component in TL leaf extracts.[27] Under this condition, NADES can improve the yield of total phenolic content, resulting in rutin and rosmarinic acid levels that are 2.4 times and 2.9 times higher, respectively, than those obtained with 95% ethanol.

### 3.3. Antioxidant capacity assay using DPPH and FRAP

Since there were no significant differences in the total flavonoid and phenolic contents among the NADES extracts, antioxidant activity was assessed further. The FRAP (total reduction capacity) and DPPH (radical scavenging activity) tests were used to evaluate the antioxidant effectiveness of the extracts obtained with various solvents. The results, presented in Fig. 2, indicate a strong correlation between the DPPH and FRAP assay outcomes. All NADES extracts exhibited lower $IC_{50}$ values for antioxidant activity compared to the ethanolic extract, aligning with the Trolox Equivalent Antioxidant Capacity (TEAC) measured by the FRAP assay. PB demonstrated the best antioxidant properties, showing a significantly lower $IC_{50}$ in the DPPH assay and a comparable (non-significant) TE value to extracts obtained with other NADES in the FRAP assay. This was supported by HPLC analysis of bioactive compounds, which revealed that the PB extract had a higher total flavonoid content, with greater amounts of rutin and vicenin-2 compared to the other extracts. Several studies have reported the antioxidant properties and ROS-reducing effects of rutin[28,29] and vicenin-2.[30,31] Vicenin-2 has been shown to prevent oxidative stress, ROS generation, and apoptosis induced by UVB in human dermal fibroblasts.[32] Meanwhile, rutin has demonstrated the ability to reduce oxidative stress, as evidenced by the DPPH assay, contributing to its potential anticancer effects.[33] This was supported by the findings of Lumsangkul *et al.* (2024), indicating that TL extract may alleviate aflatoxicosis induced by aflatoxin B1, a toxin known to cause oxidative stress, leading to growth impairment and liver toxicity in ducks.[34]

### 3.4. Evaluation of the anti-inflammatory properties of the extracts in RAW 264.7 murine macrophages *via* nitric oxide (NO) inhibition assay

With regard to biological activity, the extract obtained from PB showed significantly stronger NO inhibition compared to the positive control (200 μM L-NAME) and other extracts, as shown in Fig. 3. This impact was attributed to its antioxidant activity, with the PB-extracted compound containing the highest amounts of flavones, particularly rutin and vicenin-2. When combined with other phenolic acids, especially rosmarinic acid, which constitutes the largest proportion of the extract, these compounds have been reported to present various biological activities, including antioxidant, anti-inflammatory, anticarcinogenic, anti-aging, and angiogenic effects.[27] Oral administration of rosmarinic acid markedly attenuated carbon tetrachloride-induced elevations in NO, superoxide dismutase (SOD) activity, oxidative DNA damage, and pro-inflammatory cytokine levels in mice.[35] Injection of LPS was used to observe the modulation of proinflammatory cytokines by rosmarinic




| | 95% EtOH | CB | CL | PB | PL |
|---|---|---|---|---|---|
| Vicenin | 105.88 ± 18.76 | 374.14 ± 25.59 | 216.71 ± 10.99 | 392.24.91 ± 24.91 | 375.90 ± 27.96 |
| Rutin | 1146.25 ± 96.22 | 2614.85 ± 83.18 | 2100.88 ± 125.13 | 2742.73 ± 65.29 | 2579.90 ± 177.64 |
| Apigenin | 28.79 ± 0.97 | 92.53 ± 5.71 | 100.89 ± 18.49 | 83.36 ± 6.23 | 96.74 ± 5.99 |

| | 95% EtOH | CB | CL | PB | PL |
|---|---|---|---|---|---|
| Chlorogenic acid | 43.34 ± 12.14 | 348.18 ± 44.56 | 369.70 ± 43.87 | 314.87 ± 41.73 | 376.33 ± 40.19 |
| Caffeic acid | 48.28 ± 2.42 | 196.13 ± 14.55 | 124.38 ± 4.33 | 170.49 ± 11.29 | 129.80 ± 14.19 |
| Rosmarinic acid | 1561.36 ± 85.91 | 4205.62 ± 97.05 | 4331.95 ± 94.26 | 3973.59 ± 65.03 | 4445.53 ± 105.94 |

Fig. 1 .Quantitative profiling of flavonoids and phenolic acids extraction yields (mg g $DW^{-1}$) across various solvent types, showing (A) flavonoid content and (B) phenolic content. Identical letters (a–c) suggest no significant difference, whereas distinct letters signify significant differences among treatments ($p < 0.05$, $n = 3$) as determined by Duncan's test. The NADES formulas are as follows: CB: choline chloride–1,4-butanediol, CL: choline chloride–lactic acid, PB: L-proline–1,4-butanediol, PL: L-proline–lactic acid.

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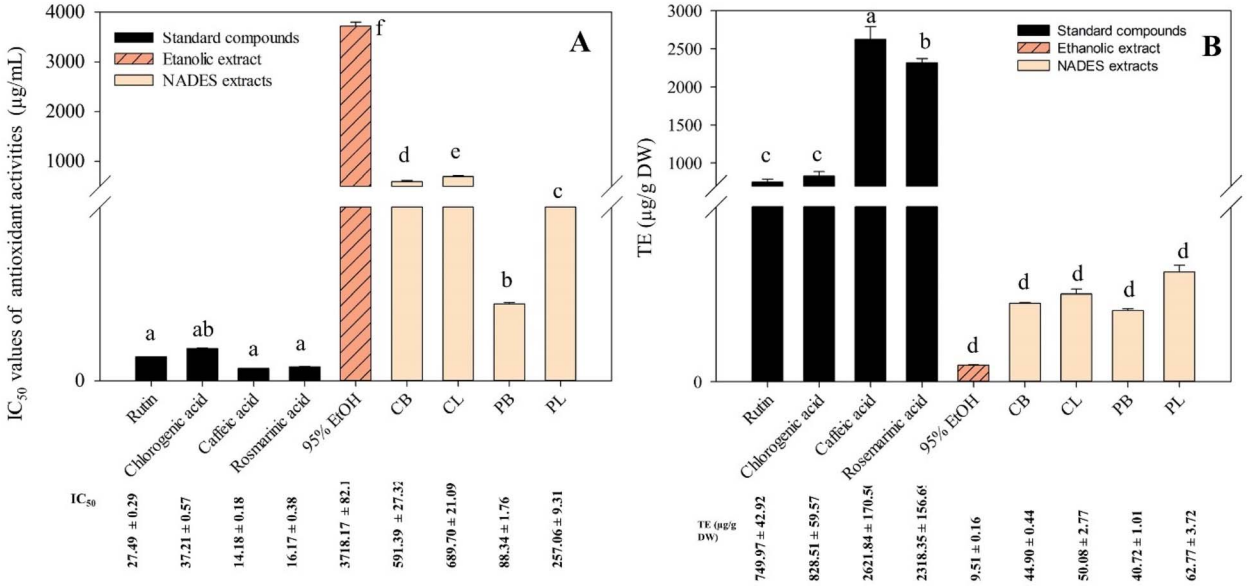


Fig. 2 Antioxidant activity assessed using DPPH ($IC_{50}$ values) (A) and FRAP (TE μg g per DW) (B), compared across different extraction solvents. Identical letters (a–f) suggest no significant difference, whereas distinct letters signify significant differences among treatments ($p < 0.05$, $n = 3$) as determined using Duncan's test. The NADES formulas are as follows: CB: choline chloride–1,4-butanediol, CL: choline chloride–lactic acid, PB: L-proline–1,4-butanediol, PL: L-proline–lactic acid.

acid. It was found that LPS-induced neuroinflammation in the adult zebrafish model could be prevented by rosmarinic acid, which reduced the levels of tumor necrosis factor-α (TNF-α) and interleukin-1 beta (IL-1β).[36] Rutin is a major component of the extract and has been widely reported for its diverse bioactivities, including anti-inflammatory, antitumor, antiallergic, antiviral, anticarcinogenic, and antidiabetic effects, as well as its potential to alleviate Alzheimer's disease.[29,37] Rutin exhibits potent anti-inflammatory and antioxidant properties that play a crucial role in combating organ inflammation induced by toxins and chemical exposure.[38] An *in vivo* study in rats[39] demonstrated that co-treatment with rutin successfully mitigated Cu-induced brain damage by reducing inflammation and oxidative stress. Similarly, Bitencourt *et al.* (2024) reported that an aqueous extract containing rutin and chlorogenic acid exhibited strong anti-inflammatory effects, alleviating edema, inflammation, and myonecrosis caused by *Bothrops jararaca* snake venom.[40] However, our study suggests that extracts rich in diverse bioactive compounds may exert enhanced anti-inflammatory effects through synergistic interactions.

The cell viability assay demonstrated that the extracts derived from ethanol and NADES had no negative effect on the

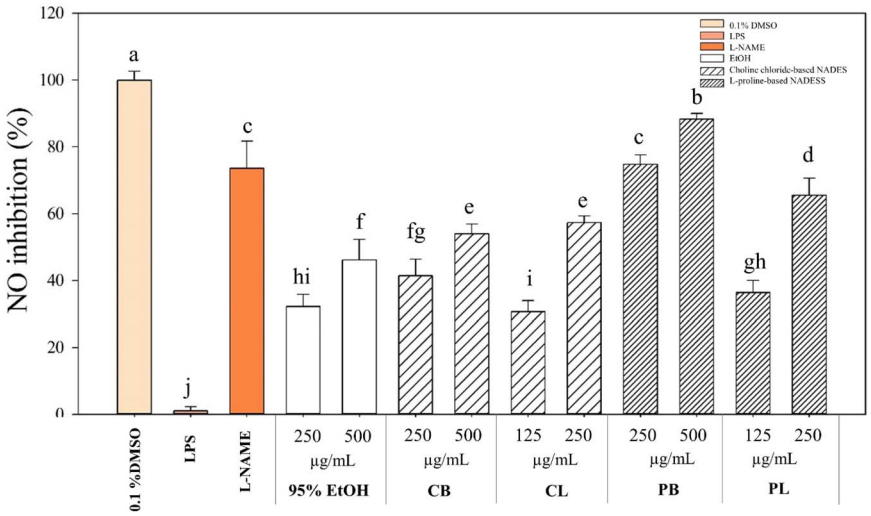


Fig. 3 Nitric oxide inhibition (%) by TL leaf extracts from different extraction solvents. According to Duncan's test, distinct letters indicate significant differences between treatments ($p < 0.05$, $n = 6$), whereas identical letters (a–j) indicate no significant difference, where different letters denote significant differences between treatments . The standard deviation (SD) is shown by the error bars. LPS: 0.8 μg $mL^{-1}$, L-NAME: 200 μM.

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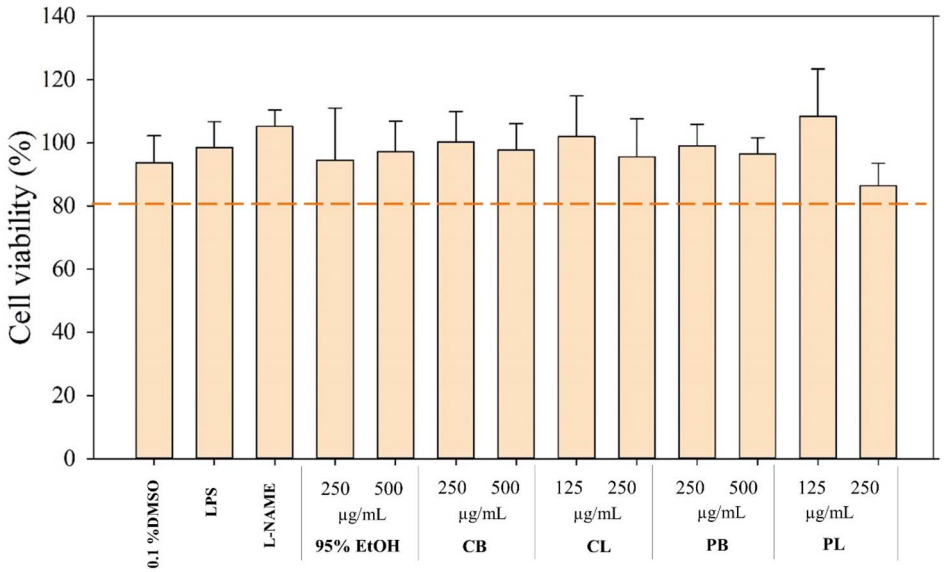


Fig. 4 Cell viability in LPS-stimulated RAW macrophage cells 24 h after treatment with TL leaf extracts using different solvents.

cells at low concentrations, as shown in Fig. 4. This was supported by a previous report by Senghoi *et al.* (2024), who found that increasing the concentration of aqueous extract affects cell viability.[1] However, for the NADES formulation that contains lactic acid, including PL and CL, the cytotoxicity on the cell occurs at a lower concentration than the other solvents. This may be caused by lactic acid having an effect on the pH of the extract and subsequently affecting the pH of the media, which can affect cell events. It contains more bioactive compounds owing to its extractability.

### 3.5. Analysis of inflammatory gene expression in NO-stimulated RAW 264.7 murine macrophages subjected to treatment with extracts from selected NADES and ethanol

Considering the observed reduction in NO, it is essential to investigate the effects of the ethanolic extract and selected NADESs on the expression of inflammatory genes to further clarify their potential anti-inflammatory properties. The results show that the expression of *iNOS* was directly related to the NO level, as shown in Fig. 5A. The PB extract significantly reduced the expression of the *iNOS* gene more than the ethanolic and PL

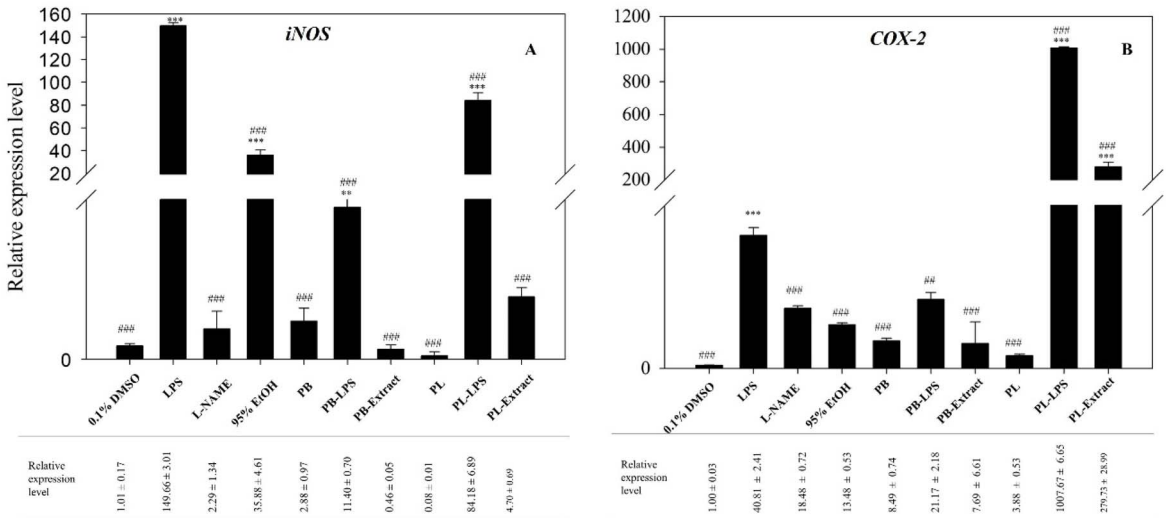


Fig. 5 RNA expression levels of *iNOS* (A) and *COX-2* (B) in RAW 264.7 cells treated with LPS, extracts, and L-NAME (200 μM) from different solvents, including ethanol, PB, and PL, are shown as relative fold changes normalized to β-actin in the control group (0.1% DMSO). Extracts were applied at a concentration of 250 μg DW per mL, with each NADES diluted to the same extent. The data are presented as mean ± SD. One-way ANOVA followed by Dunnett's post hoc test was used to assess statistical significance. The significance thresholds were defined as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the untreated control (0.1% DMSO); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with LPS-treated cells.

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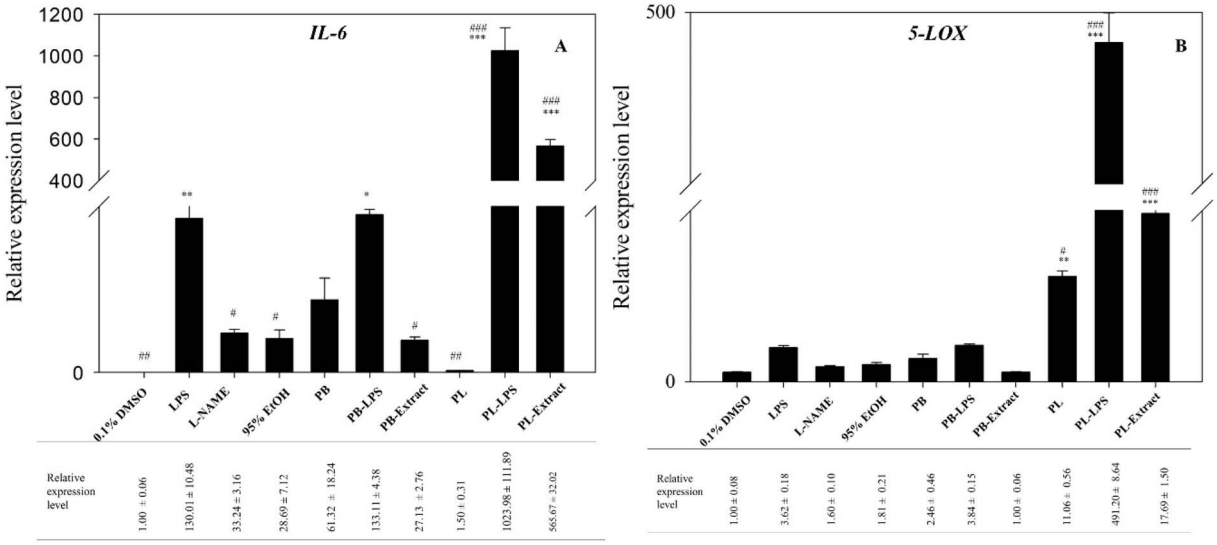


Fig. 6 RNA expression levels of *IL-6* (A) and *5-LOX* (B) in RAW 264.7 cells treated with LPS, L-NAME (200 μM), and extracts from different solvents, including ethanol, PB, and PL, are shown as relative fold changes normalized to β-actin in the control group (0.1% DMSO). Extracts were applied at a concentration of 250 μg DW per mL, with each NADES diluted to the same extent. The data are presented as mean ± SD. One-way ANOVA followed by Dunnett's post hoc test was used to assess statistical significance. The significance thresholds were defined as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the untreated control (0.1% DMSO); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with LPS-treated cells.

extracts. Surprisingly, the expression of the *iNOS* gene in the LPS-NADES system was lower than in the traditional 0.1% DMSO system. This indicates that only NADES (PB and PL) can help reduce the expression of the *iNOS* gene. However, when focusing on the expression of *COX-2*, *IL-6*, and *5-LOX*, as shown in Fig. 5B and 6, the PL-LPS and PL extracts significantly increased the expression of *COX-2* compared to the other treatments.

This likely suggests that the combination of PL and LPS may enhance certain factors influencing gene expression, as noted in a previous study, which reported that lactic acid combined with LPS can activate cellular stress pathways and alter membrane permeability.[19] Additionally, NADES (PL) alone appears to elevate *5-LOX* expression likely owing to the lactic acid concentration contributing to the acidity of the medium. An excess of $H^+$ ions from acidic NADES may adsorb onto the cell membrane surface, altering lipid conformation, phase transition, and membrane structure.[13] These changes can ultimately influence cellular processes by membrane-associated proteins, such as cell signaling, enzyme localization, and membrane permeability, thereby affecting gene expression.

Interestingly, the PB extract remains effective in reducing the expression of all target genes likely owing to its high total phenolic content. However, in the LPS-PB group, NADES alone (without the extract) also reduced the expression levels of most of the studied genes, except for *IL-6*, when compared to the LPS-treated control (0.1% DMSO). This may be because NADES contains L-proline, an amino acid that plays a role in protein structuring, synthesis, and metabolism. Furthermore, it plays a role in wound healing, antioxidative reaction, and immunological response.[41] There are many reports about the effectiveness of proline on anti-inflammatory activity. A report from previous research found that L-proline-based-cyclic dipeptides from *Pseudomonas* sp. (ABS-36) were able to inhibit pro-inflammatory cytokines.[42] The co-administration of proline and LPS can mitigate the effects of LPS by reducing inflammation and oxidative stress and altering energy metrics in the cerebral cortex and cerebellum of rat brain.[43]

However, a different effect is observed in PL owing to the presence of lactic acid, which has more deleterious effects on cells than other components. This effect is not solely attributed to the acidity resulting from the pH of NADES but also to the significant influence of lactic acid on the inflammatory response of macrophage cells, as mentioned in a previous study.[44] Lactic acid plays both anti-inflammatory and pro-inflammatory roles through the lactic acid signaling pathway, which directly influences macrophage activity and phenotypic shifts, making it a key regulator in inflammation-related disorders. Its effects on the inflammatory process are strongly concentration-dependent, exhibiting both positive and negative influences. Lactic acid can alleviate inflammation in low quantities; however, accumulation at tumor sites or at high concentrations has been found to increase tumor angiogenesis and promote tumor progression.

The high phenolic content of TL leaf extracts supports their efficacy in inhibiting the expression of inflammatory genes. Our findings are consistent with a previous report, demonstrating that TL leaf extract exhibits protective effects against beta-amyloid (Aβ)-induced neurotoxicity in SH-SY5Y cells by lowering oxidative stress, caspase-3/7 activation, and LDH release.[45] Rosmarinic acid, a major component of the extract, has been widely reported as a potent anti-inflammatory agent. Oral administration of rosmarinic acid to mice with $CCl_4$-induced liver tissue injury reduced many inflammatory-related genes,

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notably hepatic ROS, NO, tumor necrosis factor-α (TNF-α), and IL-6. Furthermore, rosmarinic acid increased the levels of important antioxidants, such as hepatic glutathione (GSH), SOD, and catalase (CAT), as well as the protein expressions of oxidative stress defense enzymes. Meanwhile, *in vitro* studies suggest that rosmarinic acid pretreatment significantly inhibits *IL-6, iNOS*, and *COX-2* expression, as evidenced by immunohistochemistry staining of CCl4-treated hepatocytes.[35] Vicenin-2 exhibits anti-inflammatory effects by suppressing COX-2, IL-6, and TNF-α phosphorylation in *Helicobacter pylori*-infected gastric epithelial cells. It also enhances antioxidant proteins, such as nuclear factor erythroid 2-related factor 2 (Nrf2) and tensin homolog (PTEN), contributing to its protective properties.[46] Additionally, Hu *et al.* (2025) reported that pre-treating human dermal fibroblasts (HDF-1 cells) with vicenin-2 before UVB irradiation protects the cells from UVB damage. This treatment prevents the expression of matrix metalloproteinases, which play a role in inflammatory responses, while acting as potent protectors against UVB-induced oxidative stress and photoaging signaling.[47] Rutin has also been reported to modulate key inflammatory genes, including *p38-MAPK, COX-2, iNOS, NF-κB,* and *IL-6*, while effectively reducing ROS-induced inflammation and oxidative stress in rats.[29] Meanwhile, apigenin (Salehi *et al.*, 2019), caffeic acid (Zielińska *et al.*, 2021), and chlorogenic acid (Hwang *et al.*, 2014) have been reported to exhibit anti-inflammatory effects by downregulating the expression of *TNF-α, IL-6, iNOS*, and *COX-2*.[48–50]

A recent study by Vongthip *et al.* (2025) highlighted the efficacy of TL leaf extract in protecting HT-22 cells from glutamate-induced oxidative stress and mitophagy-mediated cell death. Pre-treatment with TL leaf extract reduced intracellular ROS and significantly enhanced the expression of key antioxidant enzymes, including SOD, CAT, and glutathione peroxidase. Additionally, TL leaf extract inhibited the activation of autophagic proteins and increased the expression of the mitochondrial protein TOM20, suggesting preserved mitochondrial integrity and function.[51] Meanwhile, a report by Senghoi *et al.* (2024) found that TL leaf extract prepared with 50% ethanol effectively inhibits the release of the pro-inflammatory mediator NO and protects against LPS-induced apoptosis in RAW 264.7 macrophage cells.[1]

When focusing on the improvement of bioavailability, NADES can contribute to increasing the extracted products and enhancing their solubility. Moreover, it can prolong shelf life and even reduce the impact of the digestive process on degradation.[16,52] These factors help enhance pharmacological properties. As reported by Wang *et al.* (2022), poor bioavailability of rutin was observed in aqueous extracts, which consequently limited membrane permeability.[29] Therefore, using NADES as both an extractor and carrier can enhance pharmacological properties. The *in vivo* evaluation of a hydrogel containing rutin demonstrated superior therapeutic efficacy compared to free rutin in a colitis mouse model. It effectively suppresses the overexpression of inflammatory cytokines, such as TNF-α and IL-6.[29] The hydrogel's viscous properties are expected to prolong drug retention at inflammatory sites, a feature closely linked to the unique properties of NADES, further supporting its potential as a versatile delivery system for improved therapeutic outcomes.

As highlighted by da Silva *et al.* (2020), NADES can efficiently deliver biocompatible blueberry extracts that reduce protein oxidation and NO overproduction in ethanol-treated rats, offering gastroprotective effects. Pretreatment with NADES-based blueberry extract significantly protected against gastric lesions caused by ethanol, demonstrating the potential of NADES as a solvent that eliminates the need for solvent removal.[53] Thus, PB-NADES could offer enhanced performance, enabling direct application. Further research is crucial to optimize the dosage for animal models or *in vivo* assays, paving the way for more effective and direct therapeutic use.

NADESs can be utilized mainly as green extraction solvents to increase the yield of bioactive substances. Following extraction, both the NADES and the target molecules can be retrieved *via* various approaches. This approach aligns with the principles of green chemistry by promoting sustainability. The reusability of NADESs enhances extraction efficiency while simultaneously encouraging cost-effectiveness and environmental sustainability. Purification of the extracted compounds and recovery of NADES can be achieved using various methods, including liquid–liquid extraction (LLE), solid-phase extraction (SPE) using macroporous resins, adsorption with sorbent materials, and the use of antisolvents, such as water, nanofiltration, electrodialysis, or a combination of these techniques.[54,55] Khan *et al.* (2025) demonstrated that NADES composed of betaine and acetic acid in a 1 : 4 mole ratio with 25% water, used to extract flavonoids from *Fagonia cretica* leaves, could be recycled by passing the spent NADES through porous activated carbon. This adsorption step recovered the flavonoids and allowed for the recovery of 89.78% of the NADES; moreover, the recycled NADES retained 92% of its original extraction efficiency after six cycles.[56] Four macroporous resins were tested for their ability to recover flavonoids from *Acanthopanax senticosus* extracts produced with a 1 : 1 mole NADES of glycerol–levulinic acid. AB-8 resin was the most successful at adsorption and desorption, with a total flavonoid recovery rate of 71.56 ± 0.256%. Furthermore, the recovered NADES can be efficiently reused for two extraction cycles without a substantial loss of efficacy.[57] Similarly, a study on glycyrrhizic acid employed a choline chloride–lactic acid NADES for the extraction from *Glycyrrhiza glabra* utilizing macroporous DIAION™ SP700 resin, which demonstrated effective adsorption and desorption capacities, achieving over 90% NADES recovery across two resin cycles.[58] A combined method of bipolar membrane electrodialysis (BME), followed by ultrafiltration, was employed to recover both NADES and flavonoids from *Dendrobium officinale* using choline chloride and lactic acid as the solvent system. This approach resulted in a purified flavonoid glycoside content that was approximately 59 times higher while enabling the recovery of the original NADES composition.[55] For scalability assessment, a pilot-scale study demonstrated that a 1,4-butanediol/ChCl-based NADES could be effectively recovered using AB-8 macroporous resin, achieving a recovery yield of 72.36% after extracting flavonoids from sea buckthorn leaves.[59] These results highlight the potential

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applicability of NADESs in industrial-scale extraction processes. Nevertheless, high viscosity remains a significant limitation during the scale-up. Notably, the low viscosity of the NADESs employed in this study may help overcome this limitation.

## 4. Conclusions

Choline chloride-based and proline-based NADES significantly enhance the extraction yield of polyphenols from TL leaves when compared to conventional solvents (95% ethanol) using UAE under the same conditions. Notably, proline-based NADES demonstrated superior efficacy in antioxidant activity and in inhibiting NO production at equivalent concentrations. Interestingly, PB-NADES exhibited a beneficial effect on inflammatory gene expression, even when applied alone. In contrast, PL showed a negative effect on cell viability at high concentrations and induced overexpression of the inflammatory genes when co-incubated with LPS. All our findings suggest that the PB formulation is the most effective green extraction solvent for enhancing bioactive compounds, particularly phenolic and flavonoid compounds, in plant materials. The investigation of gene expression and cell studies revealed that the extract containing PB was potent in inhibiting the inflammatory process while exhibiting no adverse effects on RAW 264.7 cells. This establishes PB as a highly effective, universal green solvent for extracting phytochemical compounds with proven safety, making it an ideal candidate for practical, ready-to-use applications.

## Data availability

All the data underlying the results are available as part of the article, and no additional source data were required.

## Author contributions

Chonticha Srimawong: writing – original draft, investigation, data curation, and formal analysis. Padiphat Torkaew: investigation, data curation, and formal analysis. Waraporn Putalun: review & editing, conceptualization, supervision, and visualization.

## Conflicts of interest

There are no conflicts to declare.

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