

Article

Smart-NADES Licorice Extract as a Pharmacologically Active Phytocomplex: From Antioxidant Synergism to Anti-Inflammatory Hydrogel Efficacy

Veronika A. Shikova ¹, Olga N. Pozharitskaya ², Elena V. Flisyuk ¹, Dmitry Yu. Ivkin ³ and Alexander N. Shikov ^{1,*}

¹ Department of Pharmaceutical Formulations, St. Petersburg State Chemical Pharmaceutical University, 14a Prof. Popov Str., 197022 Saint-Petersburg, Russia; veronika.shikova@spcpcu.ru (V.A.S.); elena.flisyuk@pharminnotech.com (E.V.F.)

² Murmansk Marine Biological Institute of the Russian Academy of Sciences (MMBI RAS), 17 Vladimirskaia Str., 183038 Murmansk, Russia; olgapozhar@mail.ru

³ Center of Experimental Pharmacology, St. Petersburg State Chemical Pharmaceutical University, 14 Prof. Popov Str., 197022 Saint-Petersburg, Russia; dmitry.ivkin@pharminnotech.com

* Correspondence: alexs79@mail.ru

Abstract

Background/Objectives: The pharmacological management of inflammation remains a clinical challenge, driving the demand for advanced topical formulations. This study utilizes a “smart-NADES” (natural deep eutectic solvent) paradigm to develop an innovative anti-inflammatory topical system. The aim was to evaluate the integrated antioxidant potential, alongside the in vitro and in vivo anti-inflammatory activities, of a novel smart-NADES licorice root extract and its hydrogel formulation. **Methods:** A NADES system composed of *D*-sorbitol and *L*-lactic acid (3:1) was employed for licorice root extraction. The antioxidant capacity was assessed using three independent assays and integrated via the Relative Antioxidant Capacity Index (RACI), while phytochemical interactions were quantified using the Chou–Talalai Combination Index (CI). In vitro anti-inflammatory activity was evaluated via protein stabilization capacity, and in vivo efficacy was validated using a formalin-induced paw edema model in mice. **Results:** The NADES extract contained glycyrrhizic acid levels of 6.3 ± 0.3 mg/g and showed strong antioxidant synergism in the DPPH assay ($CI = 0.49 \pm 0.07$). The extract exhibited potent total antioxidant capacity ($IC_{50} = 11.9 \pm 0.6$ μ g/mL) with a superior RACI score (1.23). In vitro protein stabilization ($IC_{50} = 63 \pm 5$ μ g/mL) was comparable to diclofenac sodium. The 5% hydrogel numerically surpassed the commercial 2% diclofenac Emulgel (67.5% vs. 32.9% inhibition, respectively) at 24 h, although the direct pairwise comparison did not reach statistical significance ($p = 0.186$). **Conclusions:** The developed smart-NADES licorice hydrogel represents an effective, green formulation with pronounced topical anti-inflammatory properties, establishing a robust pharmacological rationale for advanced topical drug delivery.

Keywords: *Glycyrrhiza glabra*; glycyrrhizic acid; natural deep eutectic solvents; Chou–Talalai method; Relative Antioxidant Capacity Index (RACI); topical drug delivery; green extraction

Academic Editor: Fabrizio Schifano

Received: 5 July 2026

Revised: 28 July 2026

Accepted: 29 July 2026

Published: 30 July 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

The pharmacological management of inflammation remains a clinical challenge, primarily due to the well-documented adverse effects associated with use of conventional non-steroidal anti-inflammatory drugs (NSAIDs), such as gastrointestinal ulceration, cardiovascular risks, and nephrotoxicity [1]. This limitation has intensified the search for safer, multi-targeted therapeutic alternatives derived from natural sources. *Glycyrrhiza glabra* L. roots (licorice) are a validated anti-inflammatory agent, whose efficacy is attributed to a complex phytochemical matrix, predominantly comprising triterpene saponins (such as glycyrrhizic acid, GA), and phenolic compounds (including flavonoids) [2,3]. Consequently, designing new natural formulations for topical application remains a focused area of modern pharmaceutical research [4,5].

Recently, natural deep eutectic solvents (NADESs) have emerged as a class of sustainable solvents derived from renewable, biocompatible natural metabolites such as sugars, polyols, and organic acids [6–8]. NADESs offer distinct pharmaceutical advantages, including superior extraction efficiency for biomolecules, high biodegradability, and inherent dermatological safety [8]. Within this field, the innovative “smart-NADES” concept has rapidly gained traction [9]. In a smart-NADES platform, the deep eutectic mixture serves a dual technological role: it functions as an efficient extraction solvent during raw material processing and transitions directly into a functional excipient or active vehicle in the final dosage form [9]. This paradigm effectively eliminates the necessity for conventional downstream purification, toxic solvent evaporation, and complex reconstitution steps, thereby ensuring streamlined manufacturing and lower manufacturing energy consumption.

From a pharmacological standpoint, the anti-inflammatory activity of licorice is considered to be closely linked with its potent antioxidant capacity [10]. Peripheral inflammation is aggravated by excessive local generation of reactive oxygen species (ROS) at the site of tissue injury, which contributes to oxidative damage [3]. Given this chemical diversity, a comprehensive assessment of its antioxidant potential cannot be adequately achieved using a single chemical assay. Due to the distinct reaction kinetics and mechanisms of action of different antioxidant compounds, a multi-mechanistic approach is required to accurately characterize the integrated redox capacity of the extract. Therefore, it is rational to combine complementary assays—such as DPPH (hydrogen atom transfer), FRAP (single-electron transfer), and phosphomolybdenum total antioxidant capacity (TAC)—providing a holistic evaluation of the antioxidant pharmacology of the licorice extract [11,12].

The pharmacological efficacy of the licorice phytocomplex is not merely the sum of its individual components. The co-occurrence of amphiphilic saponins and hydrophobic flavonoids enables potent synergistic interactions. Thus, quantifying these interactions via the Chou–Talalay Combination Index (CI) is pharmacologically critical, as it defines the extract as “a functionally optimized multi-target synergistic network”, where the combined effect ($CI < 1$) significantly exceeds the theoretical efficacy of its isolated constituents [13].

Validating a novel topical formulation requires a stepwise approach, beginning with translation from in vitro screening to robust in vivo models that replicate the complex nature of clinical inflammation. In the present study, the egg albumin thermal denaturation assay was employed as an initial in vitro screening tool to assess the protein-stabilizing capacity of the extract, which serves as a valuable indicator of potential anti-inflammatory activity [14]. To test this hypothesis within a physiologically complex environment, the formalin-induced paw edema test was selected for in vivo evaluation. This model is particularly informative as it distinctly separates the early neurogenic phase (0–1 h) from the late tissue-mediated phase (up to 24 h) characterized by sustained leukocyte

infiltration and prostaglandin synthesis [15], allowing for the assessment of whether the redox-active phytocomplex translates into sustained macro-inhibition of acute exudative inflammation.

Despite these advantages, preclinical evaluation of smart-NADES configurations in semi-solid topical dosage forms remains limited, and traditional organic extracts continue to dominate dermatological drug research [16]. Based on our previous extensive multi-system screenings, the specific *D*-sorbitol–lactic acid matrix utilized in this work was pre-selected due to its optimized phytochemical recovery and established dermal safety profile. Given the limitations of classical synthetic NSAIDs and the lack of pharmacological evaluation of smart-NADES systems, this study aims to: (i) characterize the integrated antioxidant pharmacology and calculate synergism of NADES licorice extract using DPPH, FRAP, and TAC assays, alongside the Chou–Talalay method; (ii) assess its in vitro protein-stabilizing capacity; and (iii) validate the functional anti-inflammatory efficacy of the formulated NADES hydrogels using the formalin-induced paw edema model, establishing a clear pharmacological and technological rationale for this green, multi-target unified system.

2. Materials and Methods

2.1. Materials and Reagents

The roots of *Glycyrrhiza glabra* L. were provided by Krasnogorskleksredstva JSC (Krasnogorsk, Russia). *D*-sorbitol (>98.0%) and *L*-lactic acid (88.0–92.0%, with the remaining 8.0–12.0% consisting of purified water) were obtained from Panreac Química SLU (Barcelona, Spain). Formalin, potassium ferricyanide and potassium sorbate were purchased from Vekton JSC (St. Petersburg, Russia), ammonium molybdate from Reakhim JSC (Moscow, Russia), 0.9% sodium chloride solution from Grotex LLC (St. Petersburg, Russia), Carbopol® 940 from Noveon (Lubrizol Advanced Materials, Cleveland, OH, USA), and triethanolamine from Sigma-Aldrich (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. NADES Preparation and Extraction of Plant Material

The NADES was prepared using the heating method [17], as previously described. *D*-Sorbitol and *L*-lactic acid were mixed at a molar ratio of 3:1 (*D*-sorbitol/*L*-lactic acid) and heated at 70 °C for 60 min under continuous agitation at 400 rpm. Subsequently, 30% water (*v/v*) was added to the mixture until a clear homogeneous liquid was formed. The addition of 30% *v/v* water was used to optimize mass transfer, and previous physical-chemical evaluations of similar lactic acid-based systems confirmed that core supramolecular structures and hydrogen-bonding networks are successfully preserved within these hydration parameters [18]. For the extraction process, 1 g of crushed licorice roots was mixed with 25 g of the prepared NADES. The mixture was stirred at 400 rpm for 30 min at 40 °C, and the raw liquid extract was separated from the plant residue by centrifugation at 3000 rpm for 15 min at room temperature (22 ± 2 °C). To ensure consistency in subsequent preclinical testing and to exclude inter-batch variability, a single batch of extract was prepared under previously selected conditions [7] and its safety profile was consistent with previous data [8].

2.3. Characterization of NADES Extract and Quantitative Analysis of Glycyrrhizic Acid

The NADES extract was analyzed by Liquid Chromatography–Mass Spectrometry (LC-MS/MS) and metabolites profile was described in our previous publication [8].

The content of glycyrrhizic acid (GA) in the NADES extract was determined spectrophotometrically, following a modified procedure in accordance with the State Pharmacology

poeia of the Russian Federation, 14th Edition (FS.2.5.0040.15 “Glycyrrhizae radices”), modified by V.A. Kurkin et al. [19]. Briefly, approximately 2.0 g of the extract was refluxed with 3% trichloroacetic acid in acetone. This specific acid–acetone treatment and subsequent precipitation with ammonia allows for the selective isolation of GA as its monoammonium salt, thereby eliminating potential spectral interferences from co-extracted UV-absorbing polyphenolic matrix components. After filtration and precipitation with ammonia, the resulting GA ammonium salt was dissolved in purified water and subjected to serial dilutions. The absorbance was measured at 260 nm using a Shimadzu UV-1240 spectrophotometer (Kyoto, Japan) using purified water as a blank. The GA content (C_{GA} , %) was calculated using the following equation:

$$C_{GA} = \frac{A \cdot M \cdot V_{\text{total}} \cdot F \cdot 100}{m \cdot \epsilon \cdot 1000} \cdot 1.0257 \quad (1)$$

where A is the absorbance of the test solution; M is the molecular weight of glycyrrhizic acid (822.9 g/mol); V_{total} is the total volume of the initial solution (mL); F is the dilution factor; m is the sample mass (g); ϵ is the molar extinction coefficient of GA ammonium salt (11,000 L·mol^{−1}·cm^{−1}) at 260 nm; and 1.0257 is the coefficient for converting the molecular mass of monoammonium salt of glycyrrhizic acid to free acid.

2.4. Determination of Total Flavonoid Content

The total flavonoid content (TFC) in the NADES licorice extract was determined using the aluminum chloride colorimetric assay [20]. Briefly, a 1.0 mL aliquot of the extract was mixed with 5 mL of a 10% aluminum chloride (AlCl₃) solution in ethanol and one drop of glacial acetic acid. The mixture was adjusted to a final volume of 25 mL with ethanol in a volumetric flask. A blank solution was prepared similarly, replacing the AlCl₃ solution with an equal volume of ethanol. Following 30 min of incubation at room temperature, the absorbance was measured at $\lambda = 410$ nm using a Shimadzu UV-1240 spectrophotometer (Kyoto, Japan). The TFC was calculated based on a five-point calibration curve of standard rutin ($R^2 \geq 0.99$). The results are expressed as milligrams of rutin equivalents per gram of NADES extract weight (mg RE/g). All measurements were performed in triplicate.

2.5. DPPH Radical-Scavenging Activity

The radical-scavenging activity (RSA) of the liquid NADES licorice extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, as described by Gupta et al. [12] with modifications. A 0.1 mM working solution of DPPH was prepared in methanol. Briefly, 1.0 mL of the liquid extract at various concentrations was thoroughly mixed with 3.0 mL of the DPPH solution. The reaction mixture was shaken vigorously and incubated in the dark at room temperature for 30 min. The absorbance was then measured at $\lambda = 517$ nm with a Shimadzu UV-1240 spectrophotometer (Kyoto, Japan), using methanol as the blank. The antioxidant potency was expressed as the IC₅₀ value, defined as the extract concentration required to inhibit 50% of the initial DPPH radicals. To ensure standardized global comparisons, the results were also converted into Ascorbic Acid (AA) Equivalent Antioxidant Capacity (AEAC) per 100 g of NADES extract using the following equation [14]:

$$\text{AEAC (mg AA/100 g)} = \frac{\text{IC}_{50}(\text{AA})}{\text{IC}_{50}(\text{sample})} \times 10^5 \quad (2)$$

where IC₅₀ (AA) is the IC₅₀ of the AA standard, and IC₅₀ (sample) is the IC₅₀ of the NADES licorice extract.

2.6. Assessment of Synergistic Antioxidant Interactions

To determine the Combination Index (CI) using the Chou–Talalay method [13], the dose–response curves were generated by testing a serial dilution matrix of the whole NADES licorice extract. The individual concentrations of GA and flavonoids at each point were calculated based on their spectrophotometrically quantified levels in the stock extract and the respective dilution factors, ensuring identical testing conditions. The CI was calculated using the following equation:

$$CI = \frac{C_{GA}}{IC_{50(GA)}} + \frac{C_{flav}}{IC_{50(flav)}} \quad (3)$$

where C_{GA} and C_{flav} are the molar concentrations (μM) of glycyrrhizic acid and flavonoids, respectively, present in the NADES extract at its experimentally determined IC_{50} level. The terms $IC_{50(GA)}$ and $IC_{50(flav)}$ are the individual IC_{50} values of the reference standards (ammonium glycyrrhizinate and rutin, respectively), which were measured concurrently within the same experimental batch under identical operational conditions using the blank NADES matrix as a diluent to ensure absolute matrix matching.

All calculations, including the conversion of mass concentrations ($\mu\text{g/mL}$) to molar concentrations (μM) using the respective molecular weights ($M_{GA} = 822.9 \text{ g/mol}$ and $M_{rutin} = 610.52 \text{ g/mol}$), were performed using Microsoft Excel 365 (Microsoft Corp., Redmond, WA, USA) without external statistical add-ins. The nature of the interaction was interpreted as follows: $CI < 1$ indicates synergism, $CI = 1$ represents additivity, and $CI > 1$ signifies antagonism. To visually confirm these phytochemical interactions, algebraic isobolograms were constructed by plotting the concentrations of the individual components required to achieve a 50% effect against their combination.

2.7. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing capacity of the liquid NADES licorice extract was evaluated based on the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of potassium ferricyanide [12]. Briefly, 1.0 mL of the NADES extract at various concentrations was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of a 1% (w/v) potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] solution. The mixture was incubated in a water bath at 50°C for 20 min. To terminate the reaction, 1.5 mL of a 10% (w/v) trichloroacetic acid solution was added, followed by incubation at room temperature for 10 min. Subsequently, a 2.5 mL aliquot from the upper layer was collected and mixed with 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) ferric chloride (FeCl_3) solution. After a 10 min incubation period, the absorbance was measured at $\lambda = 700 \text{ nm}$ using a Shimadzu UV-1240 spectrophotometer (Kyoto, Japan) using the same phosphate buffer as a blank. Ascorbic acid (0.98–1000 $\mu\text{g/mL}$) and ammonium glycyrrhizinate (1.9–2000 $\mu\text{g/mL}$) served as reference standards. To maintain methodological consistency across all antioxidant evaluations, these reference compounds were analyzed concurrently with the extract samples within the same experimental batch. The antioxidant potency was expressed as the IC_{50} value, defined here as the sample concentration required to reach an optical density of 0.5 [21]. All assays were performed in triplicate.

2.8. Total Antioxidant Capacity Assay

The total antioxidant capacity (TAC) of the liquid NADES licorice extract was evaluated using the phosphomolybdenum method [22]. The assay is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compounds present in the extract and the subsequent formation of a green phosphate/ Mo(V) complex at an acidic pH. A 100 μL aliquot of the sample (prepared via serial two-fold dilutions of the NADES extract) was combined with 3.0 mL of the specific reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and

4 mM ammonium molybdate). Ascorbic acid (200–1000 µg/mL) was used as the reference standard, evaluated concurrently with the extract solutions.

The reaction tubes were tightly capped and incubated in a water bath at 95 °C for 90 min. After cooling to room temperature, the absorbance was measured at $\lambda = 695$ nm using a Shimadzu UV-1240 spectrophotometer (Kyoto, Japan) using reagent solution without extract as a blank. The relative reducing effect (%) was calculated using the following equation [23]:

$$\text{Relative reducing power (\%)} = \frac{A_{\text{sample}} - A_{\text{min}}}{A_{\text{max}} - A_{\text{min}}} \times 100 \quad (4)$$

where A_{sample} is the absorbance of the test sample, and A_{min} and A_{max} are the minimum and maximum absorbance values observed in the assay, respectively. The IC_{50} value, representing the concentration (in µg/mL) required to achieve 50% of the maximal reducing capacity, was determined graphically from the dose–response curves.

2.9. Relative Antioxidant Capacity Index (RACI) Calculation

To integrate the results obtained from the different antioxidant assays (DPPH, FRAP, and TAC) and provide a comprehensive ranking of the antioxidant potential, the Relative Antioxidant Capacity Index (RACI) was calculated. The RACI is a multidisciplinary statistical index derived from the standard score (Z-score) according to the following equation [24]:

$$Z = \frac{x - \mu}{\sigma} \quad (5)$$

where x represents the raw data (expressed as $1/IC_{50}$ to ensure that higher values correspond to higher antioxidant activity); μ is the mean of the $1/IC_{50}$ values across all methods; and σ is the standard deviation of the mean. The RACI for each sample was determined by averaging the Z-scores obtained from the three independent antioxidant assays. This dimensionless index allows for a robust comparison of antioxidant capacity across different chemical systems and reaction mechanisms.

2.10. Evaluation of Protein Stabilization Capacity via Inhibition of Albumin Denaturation

The anti-inflammatory potential of the NADES licorice extract was evaluated using the protein denaturation inhibition assay, as described by [25] with slight modifications. This method assesses the ability of the extract to stabilize proteins against thermal stress. The reaction mixture (5.0 mL total volume) consisted of 0.2 mL of fresh egg albumin, 2.8 mL of isotonic saline solution, and 2.0 mL of the NADES extract at various concentrations. A control solution was prepared using 4.8 mL of 0.9% NaCl and 0.2 mL of egg albumin. The mixtures were incubated at 37 ± 2 °C for 15 min and then heated at 70 ± 2 °C for 10 min to induce denaturation. After cooling to room temperature, the turbidity was measured spectrophotometrically at $\lambda = 660$ nm using 0.9% NaCl as a blank. The percentage of protein denaturation inhibition was calculated as follows:

$$\text{Protein denaturation inhibition (\%)} = 100 \times \left(1 - \frac{A_t}{A_c}\right) \quad (6)$$

where A_t is the absorbance of the test sample, and A_c is the absorbance of the control. The IC_{50} value—representing the concentration (expressed in µg/mL)—required to inhibit 50% of albumin denaturation, was determined from the dose–response curves. Diclofenac sodium (Hemofarm LLC, Obninsk, Russia) was used as a reference standard for comparison.

2.11. Preparation of Smart-NADES Licorice Gels

To evaluate the therapeutic potential of the extract in a topical dosage form, three gel formulations were developed containing 2%, 5%, and 10% (*w/w*) of the smart-NADES licorice root extract. Carbopol® 940 was used as the primary gelling agent. Briefly, the polymer was dispersed in purified water and allowed to hydrate for 2 h at room temperature. Subsequently, the smart-NADES licorice extract was incorporated into the hydrated polymer matrix under continuous stirring at 300 rpm. To achieve optimal viscosity and rheological properties suitable for skin application, the pH of the system was adjusted to 5.5–6.0 using triethanolamine, and monitored using a digital pH meter AMT28F (Amtast USA Inc., Lakeland, FL, USA) at 25 ± 1 °C. Potassium sorbate (0.1%, *w/w*) was added as a pharmaceutical preservative.

To assess the preliminary pharmaceutical suitability for *in vivo* trials, the sedimentation and aggregative stability of the resulting formulations were thoroughly evaluated after 3 months of storage under refrigerated conditions (4–6 °C). This quality screening included visual monitoring and centrifugal testing (3000 rpm for 30 min). The resulting gels were translucent and homogeneous, and exhibited no signs of phase separation, syneresis, or discoloration under the evaluated storage parameters prior to the *in vivo* experiments.

2.12. *In Vivo* Anti-Inflammatory Activity: Formalin-Induced Paw Edema

2.12.1. Animals and Ethical Statements

Female outbred mice (24–30 g, 5–6 weeks old, total $n = 25$) were obtained from the Rappolovo Laboratory Animal Nursery (Leningrad Region, Russia). Females were selected to minimize baseline behavioral variability and ensure a stable, homogeneous inflammatory outcome during primary screening, aligned with protocols reporting strong reproducible peripheral responses in this specific cohort [26]. Furthermore, utilizing female mice was vital to eliminate critical baseline confounding variables; unlike males, group-housed females do not exhibit high territorial aggressiveness, dominance fighting, and mutual biting inside cages, which cause minor mechanical micro-trauma, scratches, and secondary non-specific localized skin inflammation that act as housing-induced stressors and compromise experimental reproducibility. Rigorous meta-analyses have also established that female rodents do not display greater overall data variance than males across physiological timelines [27,28]. The animals were acclimatized for 10 days under controlled environmental conditions (12 h light/dark cycle, 22 ± 2 °C, $60 \pm 10\%$ humidity) with *ad libitum* access to a standard laboratory diet (Volosovo, Russia) and purified water. All procedures were performed in strict accordance with the European Union legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU) [29] and the Russian Manual for Laboratory Animals [30]. The group size ($n = 5$) was chosen as the mandatory absolute minimum required to achieve acceptable statistical power while strictly complying with the 3Rs principles (Reduction, Refinement, Replacement) aimed at minimizing animal use in exploratory pharmacological screens. The experimental protocol was approved by the Bioethical Commission of Saint Petersburg State Chemical and Pharmaceutical University (Protocol No. Mice-01-Inflam/01, dated 1 April 2024).

2.12.2. Experimental Design and Edema Induction

Mice were randomly assigned to five groups ($n = 5$ each):

- Untreated Control: received subplantar formalin injection without subsequent therapy;

- Three Experimental groups: treated with the developed smart-NADES licorice root extract gels at concentrations of 2%, 5%, or 10% (*w/w*);
- Reference Group: treated with a commercial 2% diclofenac gel (Voltaren Emulgel, GlaxoSmithKline Consumer Healthcare, Brentford, UK).

Acute peripheral inflammation was induced by a single subplantar injection of a 2% formalin solution (20 µL) into the right hind paw. Immediately following the induction, 0.050 ± 0.005 g of the respective gel formulations was applied topically to the affected plantar surface. Paw thickness (mm) was measured using a digital caliper (resolution: 0.01 mm, Guanglu, Guilin, China) prior to induction (baseline) and at 1, 2, and 24 h post-injection. The 24 h endpoint was selected to evaluate the late, tissue-mediated phase of the formalin-induced inflammatory response [31]. To minimize inter-observer variability, all serial measurements were performed by the same operator in a blinded manner. The primary endpoint evaluated was mechanical paw thickness changes; detailed tissue histology and cytokine expression parameters were omitted in this preliminary screening matrix and are planned for subsequent validation phases.

2.12.3. Data Analysis

The anti-inflammatory efficacy of the formulations was quantified by calculating [32] the percentage of inflammation inhibition according to the following equation:

$$\text{Inhibition (\%)} = \left(1 - \frac{S_{t,\text{drug}} - S_{0,\text{drug}}}{S_{t,\text{control}} - S_{0,\text{control}}} \right) \times 100\% \quad (7)$$

where S_t and S_0 represent paw thickness at time t and baseline, respectively.

Additionally, the Paw Edema Index (I_{PE}) was calculated as [15]:

$$I_{PE} = \frac{S_{t,\text{drug}} - S_{t,\text{control}}}{S_{t,\text{drug}}} \quad (8)$$

2.13. Statistical Analysis

All spectrophotometric and turbidimetric measurements were performed in triplicate ($n = 3$). Statistical significance was determined using one-way ANOVA and two-way repeated-measures analysis of variance (RM-ANOVA) to evaluate longitudinal group-by-time interactions, followed by Tukey's post hoc test for multiple comparisons using Jamovi v. 2.7.24 (The Jamovi Project, Sydney, Australia). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Quantitative Analysis of Glycyrrhizic Acid in the NADES Extract

Spectrophotometric analysis revealed that the GA content in the NADES extract was 6.3 ± 0.3 mg/g. When calculated on a dry raw material weight basis, the total GA yield reached 116.5 ± 3.1 mg/g, ensuring compliance with standardized reporting units and eliminating percentage-based variance.

3.2. Total Flavonoid Content in NADES Extract

The TFC expressed as rutin equivalent in the NADES licorice extract was determined to be 11.0 ± 0.3 mg RE/g.

3.3. Radical-Scavenging Activity (DPPH Assay)

The evaluation of the anti-radical activity (ARA) of the liquid NADES licorice extract demonstrated distinct, dose-dependent inhibition of DPPH radicals (Figure 1). The maximum radical-scavenging capacity of the extract reached approximately 90%.

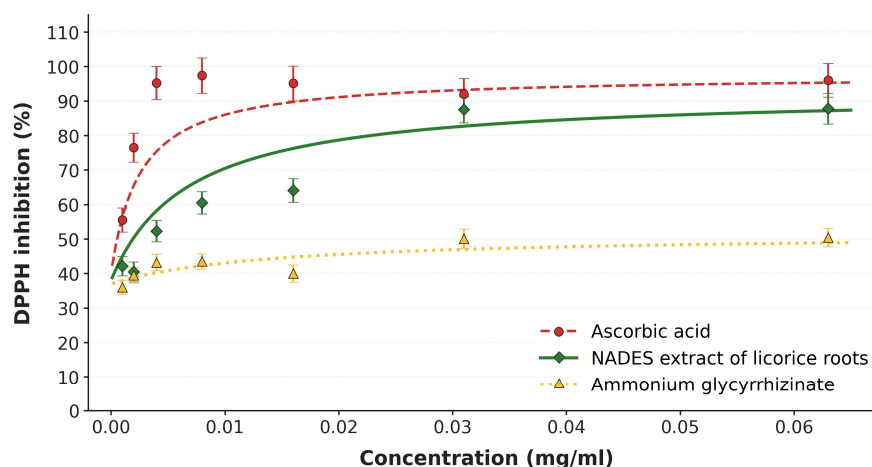


Figure 1. DPPH radical-scavenging activity of the NADES extract of licorice roots compared to the reference compounds.

The calculated IC_{50} values and AEAC scores are summarized in Table 1. Notably, the NADES extract exhibited an IC_{50} of $12.6 \pm 0.4 \mu\text{g/mL}$ when expressed as GA equivalent, and $21.8 \pm 0.7 \mu\text{g/mL}$ as rutin equivalent. The antioxidant potency of the NADES extract in solution was numerically higher than that of pure ammonium glycyrrhizinate ($IC_{50} = 178.0 \pm 8.1 \mu\text{g/mL}$; $p < 0.05$).

Table 1. Antioxidant parameters of NADES licorice extract and reference compounds ($M \pm SD$).

Sample	$IC_{50}^a, \mu\text{g/mL}$	AEAC (mg AA/g)
NADES licorice extract (as GA equivalent) ^b	12.6 ± 0.4	174.6
NADES licorice extract (as rutin equivalent) ^c	21.8 ± 0.7	100.9
Ascorbic acid	2.2 ± 0.1	—
Ammonium glycyrrhizinate	178.0 ± 8.1	12.4
Quercetin ^d	26.0 ± 3.2	84.6

^a IC_{50} values represent the concentration of the whole NADES licorice extract required to achieve 50% antioxidant inhibition, normalized based on its specific internal chemical components to allow direct matrix and synergy modeling. ^b Calculated based on the absolute mass concentration of GA present in the serial dilutions of the stock extract (stock content: $6.3 \pm 0.3 \text{ mg/g}$). ^c Calculated based on the absolute mass concentration of total flavonoids, expressed as rutin equivalents, present in the serial dilutions of the stock extract (stock content: $11.0 \pm 0.3 \text{ mg RE/g}$). ^d Data obtained from previous literature benchmarks [14] for historical baseline comparison.

3.4. Evaluation of Synergistic Phytochemical Interactions

To calculate the ratio of GA and TFC as rutin (R) equivalents, both quantitative determination results were converted to percentages (%): GA (0.63%), TFC (1.10%). The complex licorice metabolome, characterized by the co-occurrence of triterpene saponins and flavonoids, requires a robust mathematical approach to evaluate its combined therapeutic potential. To quantify these interactions, the Chou–Talalai median-effect principle was applied, providing a standardized Combination Index (CI) to differentiate among synergism ($CI < 1$), additivity ($CI = 1$), and antagonism ($CI > 1$) [13]. The synergistic interaction was explicitly confirmed via isobolographic analysis (Figure 2). The experimental data point representing the 50% inhibitory concentration (IC_{50}) of the NADES extract is located numerically below the theoretical line of additivity, demonstrating a potent synergistic effect with a calculated CI value of 0.49 ± 0.07 . This finding indicates that the actual anti-

radical potency of the combined phytochemicals within the NADES matrix is approximately 2.04 times higher than the predicted additive sum of its individual components.

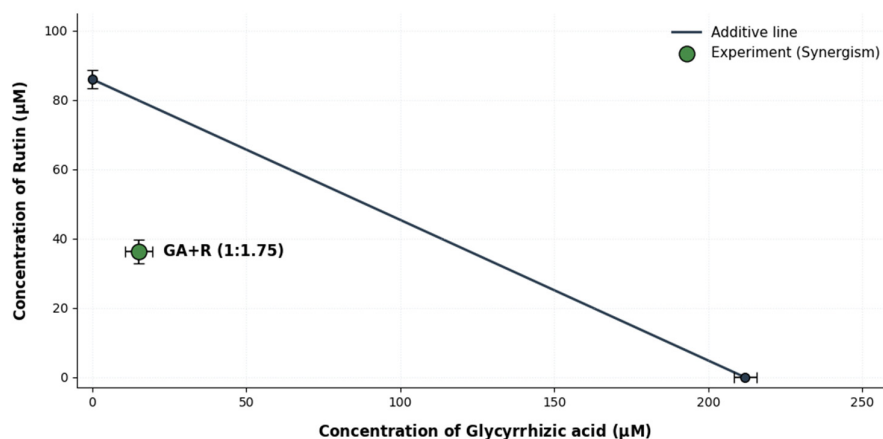


Figure 2. Isobologram analysis of the interaction between GA and total flavonoids (expressed as rutin equivalents, RE) in the DPPH radical-scavenging assay. The fixed ratio of GA to flavonoids in the extract was 1:1.75, established based on the quantitative content of GA (0.63%) and TFC (1.10%) as R equivalents. The diagonal line represents the theoretical line of additivity ($CI = 1$). The experimental data point (filled circle) corresponding to the IC_{50} of the smart-NADES licorice root extract is positioned numerically below the additivity line, confirming a strong synergistic interaction ($CI = 0.49 \pm 0.07$). Concentrations are plotted in molar units (μM) to ensure a direct molecular potency comparison.

3.5. Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was applied to evaluate the electron-donating capacity of the liquid NADES extract. This mechanism involves the reduction of the Fe^{3+} /ferricyanide complex to the ferrous (Fe^{2+}) form, resulting in the formation of a characteristic Prussian blue complex [33].

The calculated IC_{50} values for the NADES extract and the respective reference standards are summarized in Table 2. Notably, when expressed as GA equivalents, the liquid NADES extract ($IC_{50} = 470 \pm 16 \mu g/mL$) demonstrated an 11.4-fold higher reducing power compared to pure ammonium glycyrrhizinate ($IC_{50} = 5363 \pm 141 \mu g/mL$; $p < 0.05$).

Table 2. Ferric reducing antioxidant power (IC_{50}) of NADES licorice extract and reference standards ($M \pm SD$).

Sample	IC_{50} , $\mu g/mL$
NADES licorice extract (as GA equivalent)	470 ± 16
NADES licorice extract (as rutin equivalent)	832 ± 28
Ascorbic acid	45 ± 2
Ammonium glycyrrhizinate	5363 ± 141

In contrast to the DPPH assay, the isobolographic analysis for the FRAP test revealed a different interaction pattern. The calculated Combination Index ($CI = 9.54 \pm 0.33$) indicates a distinct antagonism between GA and flavonoids within this specific single-electron transfer (SET) model (Figure 3).

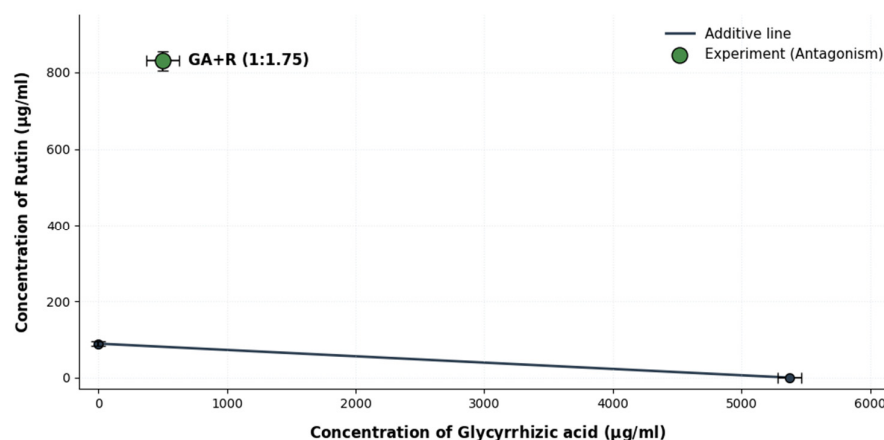


Figure 3. Isobologram analysis of the interaction between glycyrrhizic acid (GA) and total flavonoids (as rutin equivalent) in the FRAP assay. The fixed ratio of GA to flavonoids in the extract was 1:1.75, established based on the quantitative content of GA (0.63%) and TFC (1.10%) as R equivalents. The diagonal line represents the theoretical line of additivity ($CI = 1$). The experimental data point for the liquid NADES extract is located numerically above the additivity line, indicating a marked antagonistic interaction ($CI = 9.54 \pm 0.33$) under the specific single-electron transfer conditions of the ferric reducing assay. Concentrations are plotted in molar units (μM) to ensure a direct molecular potency comparison.

3.6. Total Antioxidant Capacity (TAC)

The TAC was evaluated using the phosphomolybdenum assay, which measures the combined reducing power of both lipophilic and hydrophilic antioxidants. The process involves the reduction of $Mo(VI)$ to $Mo(V)$ by the bioactive compounds present in the sample and the subsequent formation of a characteristic green phosphate/ $Mo(V)$ complex under acidic conditions.

The NADES licorice extract demonstrated a high antioxidant activity, with an IC_{50} of $11.9 \pm 0.6 \mu g/mL$ (Figure 4). This low IC_{50} threshold reflects an intensive reducing capacity within the strongly acidic parameters of the assay system, comparing favorably with the reference standard, ascorbic acid ($IC_{50} = 71.8 \pm 3.4 \mu g/mL$; $p < 0.05$).

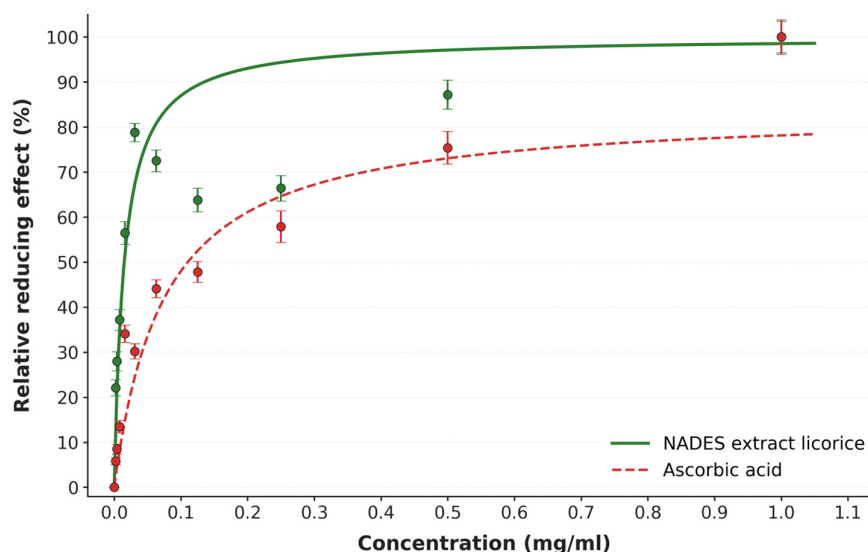


Figure 4. Dose–response curves of the relative reducing effect in the TAC assay for NADES licorice extract and ascorbic acid. The relative reducing power (%) is plotted against the logarithm of sample

concentration ($\mu\text{g/mL}$). The data points represent the mean values of three independent experimental replicates ($M \pm \text{SD}$).

3.7. Integrated Assessment via Relative Antioxidant Capacity Index (RACI)

To provide an objective comparison of the antioxidant potency across different chemical environments and reaction mechanisms, the results from the DPPH, FRAP, and TAC assays were integrated into a single dimensionless index, the RACI. This statistical approach eliminates discrepancies arising from the different units, endpoints, and scales of measurement by converting the raw $1/\text{IC}_{50}$ data into standardized Z-scores (Table 3).

Table 3. Standardized Z-scores and mean RACIs for the integrated assessment of antioxidant activity.

Sample	Z _{DPPH}	Z _{FRAP}	Z _{TAC}	RACI (Mean Z)
NADES licorice extract (as GA equivalent)	−0.11	−0.01	3.82	1.23
NADES licorice extract (as rutin equivalent)	−0.11	−0.01	2.29	0.72
Ascorbic acid	1.91	0.17	−4.43	−0.78
Ammonium glycyrrhizinate	−0.15	−0.01	−2.36	−0.84
Rutin	−0.17	−0.01	−2.82	−1.00

All antioxidant activities exhibited a clear concentration-dependent correlation, where increasing concentrations of the NADES extract consistently led to a proportional rise in antioxidant efficacy.

3.8. In Vitro Anti-Inflammatory Activity

Protein denaturation is a well-established hallmark of inflammatory processes, leading to the loss of biological function and triggering immune responses in skin tissues [24]. In the present study, the anti-inflammatory activity was evaluated through the inhibition of egg albumin thermal coagulation.

As shown in Figure 5, the NADES licorice extract demonstrated a notable, concentration-dependent inhibitory effect against protein denaturation, achieving a maximum stabilization capacity of approximately 87%.

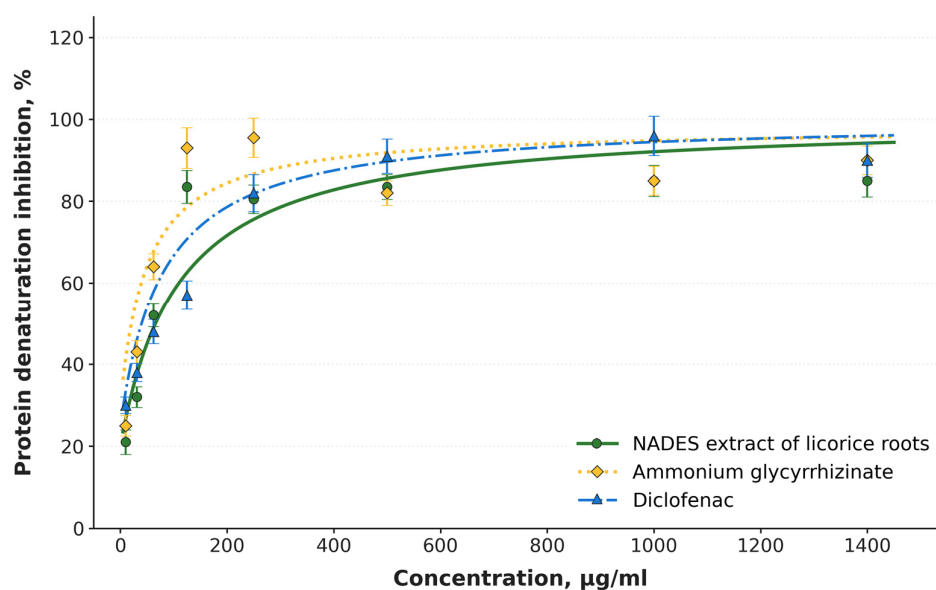


Figure 5. Dynamics of thermal denaturation inhibition of egg albumin by NADES extract of licorice roots (expressed as glycyrrhizic acid), ammonium glycyrrhizinate, and diclofenac.

The statistical analysis of the calculated IC_{50} values presented in Figure 6 reveals that the inhibitory activity of the NADES extract ($63 \pm 5 \mu\text{g/mL}$, expressed as GA equivalent) was statistically equivalent to that of the reference NSAID, diclofenac sodium ($70 \pm 4 \mu\text{g/mL}$; $p > 0.05$). Although pure ammonium glycyrrhizinate exhibited the highest individual potency ($48 \pm 3 \mu\text{g/mL}$; $p < 0.05$), the multicomponent NADES extract provides a superior biocompatible and non-toxic pharmaceutical matrix for subsequent topical applications.

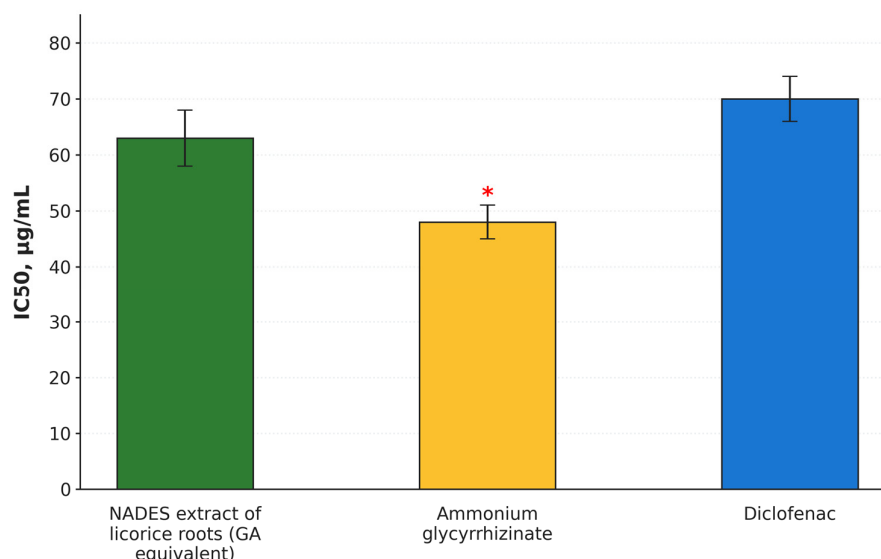


Figure 6. Calculated IC_{50} values of the NADES extract of licorice roots (expressed as GA equivalent) compared with the reference compounds, ammonium glycyrrhizinate and diclofenac sodium. Data are presented as mean $\pm$ standard deviation ($M \pm SD$, $n = 3$). (*) indicates statistically significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post hoc multiple comparisons test.

3.9. In Vivo Anti-Inflammatory Activity

To validate the therapeutic potential of the “smart-NADES” concept, three Carbopol-based topical gel formulations containing 2%, 5%, and 10% (w/w) of the NADES licorice root extract were evaluated. These formulations integrated the liquid smart-NADES extract as a functional Active Pharmaceutical Ingredient (API). The utilization of the “smart-NADES” approach enabled a highly simplified manufacturing protocol: the unified extractant–solvent system served directly as the vehicle within the Carbopol® 940 polymer matrix for the final dosage form, completely eliminating the need for intermediate energy-intensive purification and solvent evaporation stages.

The in vivo anti-inflammatory efficacy was evaluated using the classic formalin-induced paw edema model, which characteristically replicates a biphasic inflammatory response. The early phase (0–1 h) is primarily driven by neurogenic pathways, whereas the late phase (up to 24 h) involves tissue-mediated processes known from the literature characterized by the continuous release of pro-inflammatory cytokines (such as $\text{TNF-}\alpha$ and IL-6) and prostaglandins [11]. The percentage of paw edema inhibition across the experimental groups is summarized in Table 4.

Table 4. Inhibition (%) of formalin-induced paw edema in mice ($M \pm SD$, $n = 5$).

Experimental Groups	1 h	2 h	24 h
Gel with 2% NADES licorice extract	32.9 ± 7.5	16.2 ± 6.5	10.1 ± 5.7

Gel with 5% NADES licorice extract	45.9 ± 4.3	27.4 ± 5.7	67.5 ± 3.5
Gel with 10% NADES licorice extract	44.2 ± 8.1	17.3 ± 5.5	25.0 ± 5.4
Voltaren Emulgel (2% diclophenac)	53.9 ± 6.3	25.2 ± 4.2	32.9 ± 5.3

Throughout the entire observational period, no adverse reactions or local intolerance to the developed gels was observed. There was a complete absence of visible skin irritation, erythema, or edema on the healthy areas of the skin, which preliminarily confirms the excellent dermatological safety profile of both the raw smart-NADES extract and the final formulated hydrogels.

The anti-inflammatory dynamics were systematically assessed using the Paw Edema Index (I_{PE}) as the definitive dependent variable. Statistical analysis via one-way ANOVA followed by Tukey's post hoc multiple comparisons test revealed distinct, concentration-dependent therapeutic windows for the studied formulations.

At 1 h post-induction (early neurogenic phase), significant intergroup differences were detected ($F = 2.77$; $p = 0.041$). While the commercial reference drug Voltaren Emulgel demonstrated a statistically significant edema reduction relative to the untreated control group ($p = 0.040$), the 5% NADES gel achieved a therapeutic effect highly comparable to the reference standard ($p = 0.999$) and a strong trend toward reduction relative to the control group ($p = 0.086$). At the 2 h mark, corresponding to the peak of acute peripheral edema development, the intergroup differences were not statistically significant ($F = 2.34$; $p = 0.073$), indicating a uniform intensity of the peak acute inflammatory response across all treated cohorts (Figure 7a).

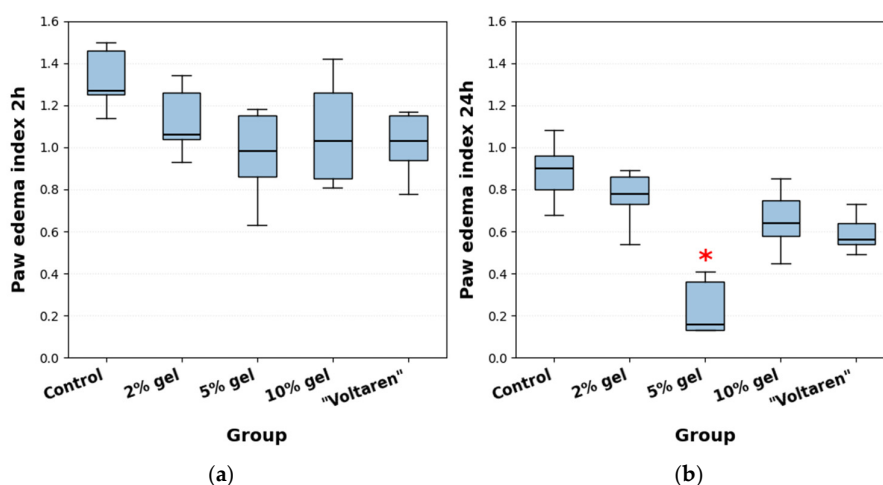


Figure 7. Paw edema index (I_{PE}) in mice at (a) 2 h and (b) 24 h post-formalin induction. Data are presented as $M \pm SD$ ($n = 5$). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Columns marked with different letters/symbols represent significant differences ($p < 0.05$). (*)—statistically significant differences relative to the control group.

At the 24 h endpoint (late tissue-mediated phase), highly significant intergroup differences emerged ($F = 6.03$; $p < 0.001$). The 5% NADES gel proved to be statistically superior to both the untreated control ($p = 0.002$) and the 2% gel formulation ($p = 0.002$). Notably, its long-term sustained anti-inflammatory performance at this terminal stage numerically surpassed that of the commercial reference, Voltaren Emulgel (Figure 7b), although this direct pairwise comparison did not reach statistical significance ($p = 0.186$) due to the biological variability of the outbred cohort.

4. Discussion

4.1. Extraction Efficiency of the Smart-NADES System

The “smart-NADES” concept describes a dual-function eutectic system that operates as an extraction solvent during raw material processing and transitions directly into a functional active vehicle in the final formulation [9]. Our results provide experimental evidence supporting this green chemistry paradigm. The NADES matrix composed of *D*-sorbitol and *L*-lactic acid (3:1 molar ratio) achieved a GA yield of 116.5 ± 3.1 mg/g and a TFC of 11.0 ± 0.26 mg RE/g (expressed as rutin equivalents). This indicates efficient, simultaneous co-extraction of both polar triterpene saponins and less polar phenolic compounds from the roots of *G. glabra* [7].

The total GA yield obtained via the sorbitol–lactic acid system matches or exceeds several traditional industrial hydroethanolic extraction protocols, where standard 30:70 ethanol/water mixtures typically yield lower baselines of GA from licorice roots under similar kinetic conditions [34]. While highly concentrated industrial extractives can reach raw GA levels of up to 155.0 mg/g, these conventional manufacturing loops rely heavily on harsh organic solvents, hazardous alkalization stages, and energy-intensive concentration steps [35–37]. In contrast, the NADES approach achieved competitive GA extraction values without the need for volatile organic media.

With respect to the hydration parameters, the incorporation of 30% *v/v* water was employed to improve mass transfer during extraction. Although excessive dilution may impair eutectic interactions, prior physicochemical studies of lactic acid-based NADESs have shown that the key supramolecular architecture, hydrogen-bonding network, and functional stability remain effectively intact within these defined hydration limits [18].

Regarding the extraction of the polyphenolic fraction, our smart-NADES system yielded a notable TFC of 11.0 ± 0.26 mg RE/g within a completely “ready-to-use”, non-toxic NADES vehicle, which is highly significant [38]. This structure entirely eliminates the necessity for subsequent downstream solvent removal, vacuum drying, and chemical reconstitution steps in active base matrices. Because parallel extractions with conventional hydroalcoholic or glycerolic controls were not performed under identical parameters in this exploratory trial, definitive claims regarding baseline “green superiority” cannot be made. However, the data confirm that the matrix provides a practical and stable phytochemical profile necessary for subsequent incorporation into topical hydrogels.

4.2. Antioxidant Activity and Synergistic Mechanisms

The liquid NADES licorice extract exhibited high antioxidant activity across multiple independent chemical assays, with the integrated RACI analysis confirming its effective overall performance (RACI = 1.23) compared to the tested reference standards, including ascorbic acid (RACI = −0.78) (Table 3) [39]. The extract demonstrated potent DPPH radical-scavenging activity ($IC_{50} = 12.6 \pm 0.4$ µg/mL), which is highly efficient compared to previously reported conventional aqueous and ethanolic licorice root extracts that typically possess IC_{50} values in a much higher range of 1420–1630 µg/mL [12]. Notably, the multi-component NADES extract demonstrated a strong radical-scavenging capacity within the range of quercetin ($IC_{50} = 26.0 \pm 3.2$ µg/mL), despite the fact that pure individual ammonium glycyrrhizinate showed much weaker radical-scavenging capacity ($IC_{50} = 178.0 \pm 8.1$ µg/mL) [14]. The pronounced antioxidant capacity of licorice is primarily attributed to its diverse flavonoid constituents, which were previously identified via HPLC-MS/MS screening of this specific NADES matrix [8]. However, the finding that the total NADES extract substantially outperforms pure GA-based activity strongly implies a collective contribution among the phytocomplex components within the eutectic system [33].

The Chou–Talalai Combination Index analysis ($CI = 0.49 \pm 0.07$) provided definitive quantitative evidence of strong synergism between GA and TFC as R equivalent in the DPPH radical-scavenging model [13]. Isobolographic analysis further confirmed that the actual anti-radical potency of the complex within the extract is approximately 2.04 times higher than the predicted additive sum of its individual components. This low CI value strongly suggests that the specific green solvent medium (*D*-sorbitol/*L*-lactic acid, 3:1) establishes an optimal supramolecular environment for the co-action of saponins and flavonoids.

We hypothesize that this marked synergism is primarily driven by the advanced co-solubilization environment established within the “smart-NADES” matrix. The dense hydrogen-bonded supramolecular network effectively prevents the self-aggregation of hydrophobic flavonoids [40]. Structural features and amphiphilic properties of flavonoids like rutin, specifically in polyol-based NADES, facilitate co-solubilization and prevent premature precipitation [41]. These systems enable efficient green recovery of compounds from plant materials [42] and allow for the direct incorporation of crude extracts into cosmetic formulations, reducing the need for energy-intensive purification [43]. The synergistic profile provides a compelling pharmacological rationale for utilizing the total, non-fractionated NADES extract rather than isolated compound sub-fractions, as the unified system clearly outperforms the sum of its independent parts in neutralizing oxidative stress.

It must be noted, however, that while the integrated RACI provides a standardized holistic ranking, its mathematical averaging via Z-scores tends to neutralize individual directional extremes. Specifically, the pronounced antagonistic behavior observed in the FRAP assay ($CI = 9.54$) resulted in a near-zero Z-score (-0.01), mathematically masking a biologically relevant negative interaction. This complete inversion from strong synergism (in the DPPH assay) to antagonism (in the FRAP assay) is directly attributable to the specific chemical environment required by the SET model. Flavonoids are well-documented to form highly stable coordination complexes with transition metal ions, including iron [44]. Under the distinctly acidic conditions of the FRAP assay (pH 3.6), this immediate chelation likely sequesters the active ortho-hydroxyl groups of the flavonoids, thereby minimizing their electronic availability for the reduction of the Fe^{3+} -ferricyanide complex [33]. Furthermore, certain flavonoids can catalyze the auto-oxidation of Fe^{2+} ions under specific conditions, effectively establishing a competitive reaction pathway that interferes with the core reduction signaling [45]. Importantly, while mathematical antagonism was observed in the isolated FRAP model, it does not diminish the practical therapeutic potential of the developed extract. As highlighted in recent critical analyses of antioxidant methodologies [46–48], multi-assay combinations feature distinct, non-interchangeable chemical environments operating via different hydrogen atom transfer (HAT) and single-electron transfer (SET) mechanisms. Therefore, global indexes like the RACI should be interpreted with caution alongside individual assay kinetics, as mechanism-specific interactions (such as acid-induced iron chelation in the FRAP model) can be underrepresented in the integrated score.

Despite this mechanism-specific competitive interaction, the total NADES extract remains more effective than pure GA alone, confirming that the “smart-NADES” architecture successfully preserves the baseline biological activity of licorice saponins compared to conventional, less stable formulations.

4.3. Total Antioxidant Capacity and Comparative Efficacy

The TAC assay is a powerful tool for evaluating the total reducing potential of complex plant extracts in an intensely acidic environment. In this test, the liquid NADES extract achieved an IC_{50} of $11.9 \pm 0.6 \mu\text{g/mL}$, which reflects a high density of active reducing

agents. The calculated AEAC of 174.6 mg AA/g is consistent with the high extraction efficiency demonstrated by eutectic media for polyphenolic complexes. This value compares favorably with organic solvent-based extracts previously reported in the literature. Notably, conventional hexane and dichloromethane extracts of licorice roots have been reported to show TAC values of 74.9 and 159.2 mg AA/g, respectively. Our data indicate that the bio-based sorbitol–lactic acid system achieves competitive reducing values, rivaling the extraction capacity of chlorinated industrial solvents like dichloromethane without the associated environmental and toxicological drawbacks [49].

The pronounced reducing capacity in the TAC assay likely stems from the enhanced thermodynamic stability and concurrent dissolution of both hydrophilic saponins and hydrophobic flavonoids within the eutectic matrix. In a deep eutectic system, target biomolecules are integrated into a dense network of hydrogen bonds established between the hydrogen bond donor (lactic acid) and acceptor (sorbitol). This specific microenvironment has been shown in similar plant matrices to minimize the oxidative degradation of labile phenolic structures during thermal processing, preserving their baseline reducing potential [45]. The integrated RACI assessment (Table 3) confirmed that the NADES licorice extract (expressed as GA equivalent) achieved a highly positive overall score (RACI = 1.23). When expressed as rutin equivalents, the extract maintained a positive activity score (RACI = 0.72), largely driven by its performance in the TAC assay. This phenomenon aligns with previous reports demonstrating that the electronic transfer and reduction kinetics of specific flavonoids are highly accelerated within strongly acidic sulfuric acid media, such as the TAC reagent environment [45,50].

In contrast, while ascorbic acid excelled in the DPPH radical-scavenging assay ($Z_{\text{DPPH}} = 1.91$), its performance in the TAC assay led to a lower overall integral index (RACI = -0.78). This discrepancy highlights the inherent limitations of relying on a single chemical assay to evaluate multi-component antioxidant capacity and reinforces the scientific importance of utilizing a multi-methodological approach, as emphasized by several authors in recent phytopharmacological screenings [39,49]. Pure individual standards of flavonoids and ammonium glycyrrhizinate consistently displayed negative Z-scores across the evaluated assays, indicating that their isolated activities fall below the system's baseline average. The lowest integral index was observed for pure ammonium glycyrrhizinate (RACI = -0.84), correlating with its weak independent reducing capacity in metal-based single-electron transfer assays, which has been previously documented in the literature [50].

Ultimately, these statistical findings suggest that the sorbitol–lactic acid phytocomplex acts as a unified antioxidant system. The collective presence of bioactives provides a broader spectrum of redox protection than isolated single-component standards, supporting the therapeutic strategy of using whole eutectic extracts rather than fractionated isolates in topical applications [45,50].

4.4. *In Vitro* Anti-Inflammatory Activity and Potential Mechanisms

The inhibition of protein denaturation serves as a well-established surrogate marker for evaluating anti-inflammatory pathways, as thermal stabilization helps minimize the loss of biological function and subsequent inflammatory responses in damaged skin tissues [24,25]. In the present study, the NADES licorice extract demonstrated a concentration-dependent inhibitory effect against protein denaturation, achieving a maximum stabilization capacity of 87%. This performance is consistent with the 81% inhibition previously reported for conventional hydroalcoholic licorice root extracts within the identical egg albumin denaturation model [24], thereby confirming that the eutectic NADES composition successfully preserves the intrinsic *in vitro* anti-inflammatory potential of the plant matrix.

Comparative statistical analysis revealed that the protein-stabilizing efficacy of the NADES extract ($IC_{50} = 63 \pm 5 \mu\text{g/mL}$) was comparable to that of the reference NSAID, diclofenac sodium ($IC_{50} = 70 \pm 4 \mu\text{g/mL}$; $p > 0.05$), under the specific tested parameters. Although pure individual ammonium glycyrrhizinate expectedly exhibited the lowest absolute IC_{50} value ($48 \pm 3 \mu\text{g/mL}$), the multicomponent NADES extract offers a sustainable, biocompatible, and non-toxic pharmaceutical matrix for topical dermal delivery. Data available in the recent literature regarding the anti-inflammatory activity of pure glycerolic extracts of licorice roots demonstrate IC_{50} values in the range of 30–50 $\mu\text{L/mL}$ [51]. Direct comparative evaluation is limited because liquid extracts are measured in volumetric units ($\mu\text{L/mL}$), whereas our results are expressed as dry mass concentrations ($\mu\text{g/mL}$). However, our results correlate well with these benchmarks, effectively validating the extraction capability of the sorbitol–lactic acid system. Crucially, the blank NADES solvent matrix exhibited no biologically relevant stabilization activity ($IC_{50} \approx 28,000 \mu\text{g/mL}$), definitively ensuring that the observed anti-inflammatory effects are derived solely from the co-extracted phytochemical complex.

The *in vitro* anti-inflammatory activity of the NADES extract ($IC_{50} = 63 \pm 5 \mu\text{g/mL}$) was found to be tightly cross-correlated with its potent radical-scavenging ability (DPPH, $IC_{50} = 12.6 \pm 0.4 \mu\text{g/mL}$) and its total antioxidant capacity (TAC, $IC_{50} = 11.9 \pm 0.6 \mu\text{g/mL}$). Based on these overlapping kinetic indices, it can be hypothesized that the total antioxidant capacity exerts a significant influence on the overall anti-inflammatory potential of the phytocomplex. This correlation suggests that the underlying mechanisms of inflammation suppression by this smart-NADES extract are governed in part by direct free-radical neutralization [51]. The results of this analysis confirm that the sorbitol–lactic acid matrix maintains the biological integrity of the constituents, making it a promising candidate for incorporation into topical hydrogels.

4.5. *In Vivo* Efficacy and Prolonged Topical Anti-Inflammatory Action

The formalin-induced paw edema model characteristically replicates a complex, biphasic inflammatory cascade: the early phase (0–1 h) is primarily driven by neurogenic pathways and the immediate release of histaminergic mediators, whereas the late phase (up to 24 h) involves tissue-mediated processes governed by the continuous release of pro-inflammatory cytokines and prostaglandins [15,31]. Our *in vivo* evaluation revealed a favorable therapeutic profile for the developed NADES-based topical hydrogels.

During the early neurogenic phase of acute inflammation (1 h), the commercial reference drug Voltaren Emulgel exhibited the highest absolute inhibition level (53.9%), which is consistent with the rapid, well-documented inhibitory effect of classical NSAIDs on early-stage arachidonic acid cascade mediators. In contrast, the phytocomplex components within the NADES matrix likely require a longer latency period to develop their full therapeutic potential. Nevertheless, the hydrogel formulated with 5% NADES licorice extract demonstrated a comparable protective effect at this early stage ($p = 0.999$ vs. Voltaren Emulgel), showing a trend toward acute edema mitigation relative to the untreated control group.

An interesting dynamic emerged at the terminal 24 h endpoint, which directly corresponds to the late tissue-mediated phase of the formalin-induced inflammatory response. At this stage, the hydrogel containing 5% NADES extract exhibited a time-dependent increase in therapeutic efficacy, achieving 67.5% inhibition of peripheral edema. This terminal result numerically exceeded the performance of the commercial reference, Voltaren Emulgel (32.9%). Two-way repeated-measures ANOVA confirmed a highly significant “Group $\times$ Time” interaction ($p < 0.01$), confirming that the smart-NADES extract provides a sustained therapeutic effect over time.

At the 2 h mark, which corresponds to the physiological peak of acute edema development, the intergroup differences were not statistically significant ($F = 2.34$; $p = 0.073$), indicating a uniform intensity of peak acute inflammation across all treated cohorts. However, at the 24 h endpoint, highly significant global intergroup differences emerged ($F = 6.03$; $p < 0.001$). The 5% NADES gel proved to be statistically superior to both the untreated control group ($p = 0.002$) and the lower-dose 2% gel formulation ($p = 0.002$), although the direct pairwise comparison did not reach statistical significance ($p = 0.186$). Notably, the direct post hoc pairwise comparison between the 5% NADES gel and Voltaren Emulgel fell within a strong statistical trend but did not reach significance ($p = 0.186$) due to the inherent biological variance in outbred animal models and the minimal group size ($n = 5$) required by modern 3Rs reduction ethics. The overall longitudinal performance highlights the promising drug-release kinetics of the eutectic system.

The formulated hydrogels exhibited high macro-structural homogeneity and physical integrity. The stable polymer architecture of neutralized Carbopol® 940 effectively retained the liquid NADES extract within its continuous matrix, preventing active component precipitation and ensuring appropriate technological properties for topical application.

The recent literature describes various applications of soft topical dosage forms incorporating conventional licorice root extracts (such as methanolic or aqueous preparations), which have demonstrated baseline anti-inflammatory activity in both in vivo and ex vivo models. For instance, Semenescu et al. recently formulated a hydrogel incorporating a 2% methanolic licorice extract that exhibited strong baseline antioxidant capacity and satisfactory anti-inflammatory outcomes within an exploratory model of rosacea-associated markers [16]. However, those conventional formulations relied heavily on volatile organic solvents like methanol, which possess distinct environmental and manufacturing limitations compared to bio-based alternatives.

In contrast, our proposed smart-NADES hydrogel establishes a broad phytochemical profile due to the capability of the deep eutectic matrix to simultaneously dissolve and stabilize both hydrophilic saponins (glycyrrhizin) and hydrophobic polyphenols [8]. This mechanism is further supported by the complete lack of intrinsic macromolecular anti-inflammatory activity in the blank NADES matrix ($IC_{50} \approx 28,000 \mu\text{g/mL}$) compared to the promising potency of the complete extract ($IC_{50} = 63 \pm 5 \mu\text{g/mL}$). Consequently, the developed smart-NADES gel represents a biocompatible topical delivery architecture capable of maximizing the antioxidant and sustained anti-inflammatory performance of the total licorice phytocomplex.

We must acknowledge that the absence of a vehicle-only control group (blank NADES hydrogel without licorice extract) represents a limitation of the present in vivo study. While D-sorbitol and lactic acid are recognized skin permeation enhancers, their primary role within the polymer hydrogel network is technological and kinetic. As recently demonstrated by Hasanpour et al., NADES, when integrated into semi-solid formulations, acts fundamentally as a biocompatible delivery vehicle that optimizes the partitioning and transdermal diffusion of active ingredients into the target tissue, rather than eliciting independent pharmacological activity [52]. Supported by our previous findings regarding the biological neutrality and excellent dermal tolerance of this specific eutectic combination, we conclude that the pronounced anti-inflammatory response is driven specifically by the synergistic licorice phytocomplex, whereas the smart-NADES matrix serves its designated role as a functional delivery matrix.

Another important methodological characteristic of the present study is the exclusive use of female mice in the in vivo anti-inflammatory screening. This design was selected based on established laboratory animal data demonstrating that male mice under group-housing conditions display high territorial aggressiveness and fighting, which frequently

result in mechanical skin trauma and subsequent non-specific inflammatory artifacts on the extremities [53,54]. These baseline housing-induced stressors can severely compromise experimental reproducibility in localized skin models [55]. Utilizing female mice effectively eliminated these behavioral variables, ensuring stable baseline parameters for topical hydrogel evaluation. Furthermore, comprehensive meta-analyses have confirmed that female rodents do not display greater overall variability in physiological parameters than males [27,28]. However, we must acknowledge the exclusive reliance on a single sex as a limitation of this work. Because sex-specific dimorphisms in immune signaling and epidermal barrier kinetics are complex, the data obtained here primarily reflect the functional efficacy of the smart-NADES hydrogel within the female physiological phenotype. Future translational phases of this research will require cross-validation in sex-balanced cohorts to fully characterize the global therapeutic profile of the developed topical system.

4.6. Safety Profile and Future Perspectives

Importantly, no adverse reactions, systemic behavioral anomalies, or localized toxic phenomena were observed across any of the animal cohorts throughout the entire duration of the *in vivo* experiments. The complete absence of visible skin irritation, erythema, or micro-inflammation in the healthy tissue zones confirms the favorable dermatological safety profile of both the raw smart-NADES licorice root extract and the final formulated hydrogels [8].

The hydrogel prepared with a 5% extract payload established the most effective anti-inflammatory therapeutic response, particularly during the terminal tissue-mediated phase of peripheral inflammation. This prolonged, sustained efficacy suggests that the developed formulation acts as a stable passive delivery vehicle, rendering it a promising candidate for subsequent extended preclinical investigations. The *in vitro* anti-inflammatory capacity, which was comparable to that of diclofenac sodium ($IC_{50} = 63 \pm 5 \mu\text{g/mL}$ vs. $70 \pm 4 \mu\text{g/mL}$, respectively), coupled with its prolonged *in vivo* sustained protective efficacy (67.5% vs. 32.9% inhibition at the 24 h mark) highlights the potential therapeutic value of this bio-based green formulation. Consequently, this smart-NADES hydrogel represents a promising, sustainable direction for the localized management of inflammatory pathologies [1].

5. Conclusions

For the first time, a comprehensive, multi-methodological evaluation of the integrated antioxidant and *in vivo* anti-inflammatory potential of a “smart-NADES” licorice root extract was systematically performed. Our findings confirm that the eutectic extract possesses promising total antioxidant capacity (TAC, $IC_{50} = 11.9 \pm 0.6 \mu\text{g/mL}$), which is closely linked with its overall biological efficacy. The *in vitro* screening revealed a tight cross-correlation between the extract’s robust antioxidant profiling and its ability to inhibit thermal protein denaturation ($IC_{50} = 63 \pm 5 \mu\text{g/mL}$), showing a promising protective capacity comparable to the reference drug diclofenac sodium.

The subsequent *in vivo* validation using a formalin-induced peripheral paw edema model confirmed the functional anti-inflammatory efficacy of the developed topical hydrogels. Notably, the optimized formulation containing a 5% (*w/w*) smart-NADES licorice extract suppressed local tissue edema by 67.5% after 24 h, numerically surpassing the commercial reference Emulgel containing 2% diclofenac (32.9%), although this specific pairwise difference did not reach statistical significance ($p = 0.186$) due to the biological variability of the outbred animal cohort. A non-monotonic, U-shaped dose–response relationship was observed, establishing the 5% concentration as the optimal dosage for achieving maximum functional anti-inflammatory outcomes while avoiding the limitations observed at a higher 10% payload.

Taken together, these new results provide a pharmacological rationale for the “smart-NADES” paradigm in green pharmacy. The developed hydrogel with a 5% licorice extract represents a highly effective, bio-based, non-toxic formulation with pronounced topical anti-inflammatory properties, serving as a promising platform for subsequent investigations into the treatment of dermatological and localized inflammatory disorders.

Author Contributions: Conceptualization, A.N.S., O.N.P. and E.V.F.; methodology, A.N.S., V.A.S. and D.Y.I.; formal analysis, A.N.S., D.Y.I. and O.N.P.; investigation, V.A.S. and D.Y.I.; resources, E.V.F.; data curation, A.N.S., V.A.S., O.N.P. and D.Y.I.; writing—original draft preparation, A.N.S., V.A.S. and O.N.P.; writing—review and editing, A.N.S., V.A.S. and O.N.P.; visualization, V.A.S. and O.N.P.; supervision, E.V.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki, and approved by the Bioethical Commission of Saint Petersburg State Chemical and Pharmaceutical University (Protocol No. Mice-01-Inflam/01, dated 1 April 2024).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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

References

1. Sohail, R.; Mathew, M.; Patel, K.K.; Reddy, S.A.; Haider, Z.; Niazi, M.; Khan, R.S.; Reddy, V.A.; Khurshid, H.; Rukh, L.; et al. Effects of non-steroidal anti-inflammatory drugs (NSAIDs) and gastroprotective NSAIDs on the gastrointestinal tract: A narrative review. *Cureus* **2023**, *15*, e37080.
2. Pastorino, G.; Cornara, L.; Soares, S.; Rodrigues, F.; Oliveira, M.B.P.P. Liquorice (*Glycyrrhiza glabra*): A phytochemical and pharmacological review. *Phytother. Res.* **2018**, *32*, 2323–2339.
3. Leite, C.S.; Bonafé, G.A.; Carvalho Santos, J.; Martinez, C.A.R.; Ortega, M.M.; Ribeiro, M.L. The anti-inflammatory properties of licorice (*Glycyrrhiza glabra*)-derived compounds in intestinal disorders. *Int. J. Mol. Sci.* **2022**, *23*, 4121.
4. Klinge, S.A.; Sawyer, G.A. Effectiveness and safety of topical versus oral nonsteroidal anti-inflammatory drugs: A comprehensive review. *Physician Sportsmed.* **2013**, *41*, 64–74.
5. Drosopoulou, K.; Kosheleva, R.I.; Ofrydopoulou, A.; Tsoupras, A.; Mitropoulos, A. Topical and transdermal delivery of non-steroidal anti-inflammatory drugs (NSAIDs) for inflammation and pain: Current trends and future directions in delivery systems. *Processes* **2025**, *13*, 907.
6. Krekhova, F.; Shishov, A.; Meshcheva, D.; Bulatov, A. Deep eutectic solvents: Extraction and derivatization. A review. *TrAC Trends Anal. Chem.* **2026**, *200*, 118851.
7. Shikov, A.N.; Shikova, V.A.; Whaley, A.O.; Pozharitskaya, O.N.; Flisyuk, E.V.; Smirnova, A.I.; Serebryakova, O.G.; Kiselev, D.A.; Shishov, A.Y.; Ivkin, D.Y. The ability of acid-based natural deep eutectic solvents to co-extract elements from the roots of *Glycyrrhiza glabra* L. and associated health risks. *Molecules* **2022**, *27*, 7690.
8. Shikova, V.A.; Pozharitskaya, O.N.; Flisyuk, E.V.; Ivkin, D.Y.; Shikov, A.N. Safety of NADES extract of *Glycyrrhiza* roots after topical application and peroral administration to mice. *Molecules* **2025**, *30*, 4704.
9. Hilali, S.; Van Gheluwe, L.; Yagmur, M.; Fauconnier, M.L.; Chemat, F.; Vandenberghe, M.; Caron, D.; Korb, E.; Simar, M.; Deschamps, A.; et al. NaDES-based biorefinery of *Spirulina* (*Arthrospira platensis*): A new path for sustainable high value-added metabolites. *Sep. Purif. Technol.* **2024**, *329*, 125123.
10. Jitesh, S.; Geetha, R.V. Anti-inflammatory activity of *Glycyrrhiza glabra* extract—An in vitro study. *J. Pharm. Sci. Res.* **2017**, *9*, 451–454.
11. Semwal, D.K.; Kumar, A.; Semwal, R.B.; Dadhich, N.K.; Chauhan, A.; Kumar, V. Glycyrrhizin (glycyrrhizic acid)—Pharmacological applications and associated molecular mechanisms. *Drugs Drug Candidates* **2025**, *4*, 44.

12. Gupta, M.; Karmakar, N.; Sasmal, S.; Chowdhury, S.; Biswas, S. Free radical scavenging activity of aqueous and alcoholic extracts of *Glycyrrhiza glabra* Linn. *Int. J. Pharmacol. Toxicol.* **2016**, *4*, 235–240.
13. Zhang, N.; Fu, J.N.; Chou, T.C. Synergistic combination of microtubule targeting anticancer fludelone with cytoprotective panaxytriol derived from *Panax ginseng* against MX-1 cells in vitro: Experimental design and data analysis using the combination index method. *Am. J. Cancer Res.* **2015**, *6*, 97–104.
14. Pozharitskaya, O.N.; Obluchinskaya, E.D.; Shikov, A.N. Mechanisms of bioactivities of fucoidan from the brown seaweed *Fucus vesiculosus* L. of the Barents Sea. *Mar. Drugs* **2020**, *18*, 275.
15. Fu, K.Y.; Light, A.R.; Maixner, W. Relationship between nociceptor activity, peripheral edema, spinal microglial activation and long-term hyperalgesia induced by formalin. *Neuroscience* **2000**, *101*, 1127–1135.
16. Semenescu, I.; Bora, L.; Dușe, A.O.; Muntean, D.; Simedrea, R.I.; Popa, M.; Tănase, I.; Matea, C.T.; Vlase, L.; Pârvu, A.E. Formulation and biological evaluation of *Glycyrrhiza glabra* L. methanolic extract: An exploratory study in the context of rosacea. *Antioxidants* **2026**, *15*, 158.
17. Dai, Y.; van Spronsen, J.; Witkamp, G.J.; Verpoorte, R.; Choi, Y.H. Natural deep eutectic solvents as new potential media for green technology. *Anal. Chim. Acta* **2013**, *766*, 61–68.
18. Pozharitskaya, O.N.; Obluchinskaya, E.D.; Shikova, V.A.; Flisyuk, E.V.; Vishnyakov, E.V.; Makarevich, E.V.; Shikov, A.N. Physicochemical and antimicrobial properties of lactic acid-based natural deep eutectic solvents as a function of water content. *Appl. Sci.* **2024**, *14*, 10409.
19. Egorov, M.V.; Kurkin, V.A. Qualitative and quantitative analysis of the medicinal product “Licorice Syrup”. *Sovrem. Probl. Nauk. Obraz.* **2015**, *4*, 553. (In Russian)
20. Pozharitskaya, O.N.; Shikov, A.N.; Makarova, M.N.; Kosman, V.M.; Faustova, N.M.; Tesakova, S.V.; Makarov, V.G.; Galambosi, B. Anti-inflammatory activity of a HPLC-fingerprinted aqueous infusion of aerial part of *Bidens tripartita* L. *Phytomedicine* **2010**, *17*, 463–468.
21. Youssef, F.S.; Gamal El-Din, M.I.; El-Beshbishy, H.A.; AbouZid, S.F.; Alqahtani, A.M.; Alqahtani, T.; Ahmed, S.A. *Eremophila purpurascens*: Anti-oxidant, anti-hyperglycemic, and hepatoprotective potential of its polyphenolic rich leaf extract and its LC-ESI-MS/MS chemical characterization and standardization. *ACS Omega* **2023**, *8*, 31928–31940.
22. Prieto, P.; Pineda, M.; Aguilar, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Anal. Biochem.* **1999**, *269*, 337–341.
23. Gorjanović, S.; Komes, D.; Pastor, F.T.; Belščak-Cvitanović, A.; Pezo, L.; Hečimović, I.; Sužnjević, D. Antioxidant capacity of teas and herbal infusions: Polarographic assessment. *J. Agric. Food Chem.* **2012**, *60*, 9573–9580.
24. Zatlá, A.T.; Hammoudi, A. Phytochemical screening and inflammatory activity evaluation of hydroalcoholic extract of *Glycyrrhiza glabra* root. *Chem. Proc.* **2024**, *16*, 5.
25. Church, D.; Elsayed, S.; Reid, O.; Winston, B.; Lindsay, R. Burn wound infections. *Clin. Microbiol. Rev.* **2006**, *19*, 403–434.
26. Reichert, M.N.; Koewler, N.J.; Hargis, A.M.; Felgenhauer, J.L.; Impelluso, L.C. Effects of depilatory cream formulation and contact time on mouse skin. *J. Am. Assoc. Lab. Anim. Sci.* **2023**, *62*, 153–162.
27. Smarr, B.; Kriegsfeld, L.J. Female mice exhibit less overall variance, with a higher proportion of structured variance, than males at multiple timescales of continuous body temperature and locomotive activity records. *Biol. Sex Differ.* **2022**, *13*, 41.
28. Becker, J.B.; Prendergast, B.J.; Liang, J.W. Female rats are not more variable than male rats: A meta-analysis of neuroscience studies. *Biol. Sex Differ.* **2016**, *7*, 34.
29. European Council. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. Available online: <https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF> (accessed on 23 July 2026).
30. Karkishenko, N.N.; Grachev, C.V. (Eds.) *Manual for Laboratory Animals and Alternative Models in Biomedical Research*; Pro-file-2C: Moscow, Russia, 2010.
31. Wheeler-Aceto, H.; Cowan, A. Neurogenic and tissue-mediated components of formalin-induced edema: Evidence for supraspinal regulation. *Agents Actions* **1991**, *34*, 264–269.
32. Kuvaeva, E.V.; Kolesnik, D.A.; Levshukova, P.O.; Kirillova, E.N.; Ivkin, D.Y. Evaluation of the anti-inflammatory activity of a new 1,6-dihydropyrimidine derivative. *Farmatsiya* **2021**, *70*, 44–47. (In Russian)
33. Benzie, I.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.
34. Sohail, M.; Rakha, A.; Butt, M.S.; Asghar, M. Investigating the antioxidant potential of licorice extracts obtained through different extraction modes. *J. Food Biochem.* **2018**, *42*, e12466.

35. Tian, M.; Yan, H.; Row, K.H. Extraction of glycyrrhizic acid and glabridin from licorice. *Int. J. Mol. Sci.* **2008**, *9*, 571–577.
36. Yakubova, M.R.; Genkina, G.L.; Shakirov, T.T. UV-spectrophotometric determination of glycyrrhizic acid in *Glycyrrhiza glabra*. *Chem. Nat. Compd.* **1977**, *13*, 676–679.
37. Kurkin, V.A.; Egorov, M.V. Standardization of licorice roots and the medicinal product “Liquid licorice extract”. *Fundam. Issled.* **2014**, *6*, 1232–1236. (In Russian)
38. Aly, S.H.; Elissawy, A.M.; Mahmoud, A.M.A.; El-Shazly, M.; Mostafa, N.M.; Singab, A.N.B. Synergistic effect of *Sophora japonica* and *Glycyrrhiza glabra* flavonoid-rich fractions on wound healing: In vivo and molecular docking studies. *Molecules* **2023**, *28*, 2994.
39. Sun, T.; Tanumihardjo, S.A. An integrated approach to evaluate food antioxidant capacity. *J. Food Sci.* **2007**, *72*, R159–R165.
40. Matsuoka, K.; Miyajima, R.; Ishida, Y.; Karasawa, S.; Yoshimura, T. Aggregate formation of glycyrrhizic acid. *Colloids Surf. A Physicochem. Eng. Asp.* **2016**, *500*, 112–117.
41. Liu, F.; Chen, L.; Yin, K.; Fan, T.; Yan, Z. Partitioning behavior of rutin in novel liquid–liquid biphasic systems formed by choline chloride/maltose natural deep eutectic solvents and n-propanol. *Sep. Purif. Technol.* **2023**, *311*, 123271.
42. Huang, Y.; Feng, F.; Jiang, J.; Qiao, Y.; Wu, T.; Voglmeir, J.; Chen, Z.G. Green and efficient extraction of rutin from tartary buckwheat hull by using natural deep eutectic solvents. *Food Chem.* **2017**, *221*, 1400–1405.
43. Wasilewski, T.; Hordyjewicz-Baran, Z.; Malorna, K.; Dresler, E.; Sabura, E.; Zegarski, M.; Stanek-Wandzel, N. Studies on the use of loan extraction to produce natural shower gels (cosmetic) based on grape pomace extracts—The effect of the type of surfactant borrowed. *Molecules* **2025**, *30*, 3709.
44. Yoshino, M.; Murakami, K. Interaction of iron with polyphenolic compounds: Application to antioxidant characterization. *Anal. Biochem.* **1998**, *257*, 40–44.
45. Kejlik, Z.; Kaplánek, R.; Masařík, M.; Babula, P.; Matkowski, A.; Filipenský, P.; Veselá, K.; Gburek, J.; Sýkora, D.; Martásek, P.; et al. Iron complexes of flavonoids—Antioxidant capacity and beyond. *Int. J. Mol. Sci.* **2021**, *22*, 646.
46. Shahidi, F.; Samarasinghe, A. How to assess antioxidant activity? Advances, limitations, and applications of in vitro, in vivo, and ex vivo approaches. *Food Prod. Process. Nutr.* **2025**, *7*, 50.
47. Sadowska-Bartos, I.; Bartosz, G. ORAC: The method of choice for determining antioxidant capacity of food products? *Int. J. Mol. Sci.* **2026**, *27*, 4825.
48. Knez, E.; Kadac-Czapska, K.; Grembecka, M. Evaluation of spectrophotometric methods for assessing antioxidant potential in plant food samples—A critical approach. *Appl. Sci.* **2025**, *15*, 5925.
49. Asan-Ozusaglam, M.; Karakoca, K. Evaluation of biological activity and antioxidant capacity of Turkish licorice root extracts. *Rom. Biotechnol. Lett.* **2014**, *19*, 8994–9005.
50. Makhmudov, L.U.; Aripov, T.F.; Vypova, N.L.; Khadzheva, M.U.; Khushbaktova, Z.A.; Syrov, V.N. Antioxidant and hepatoprotective properties of complexes of the monoammonium salt of glycyrrhizic acid with amino acids and phenolic compounds. *Eksp. Klin. Farmakol.* **2023**, *86*, 23–28. (In Russian)
51. Ciganović, P.; Jakimiuk, K.; Tomczyk, M.; Zovko Končić, M. Glycerolic licorice extracts as active cosmeceutical ingredients: Extraction optimization, chemical characterization, and biological activity. *Antioxidants* **2019**, *8*, 445.
52. Hasanpour, F.; Budai-Szűcs, M.; Kovács, A.; Ambrus, R.; Jójárt-Laczkovich, O.; Szalai, B.; Pavlić, B.; Simon, P.; Törteli, L.; Berkó, S. The role of natural deep eutectic solvents in a hydrogel formulation containing lidocaine. *Pharmaceutics* **2025**, *17*, 324.
53. MacLellan, A.; Adcock, A.; Mason, G. Behavioral biology of mice. In *Behavioral Biology of Laboratory Animals*; CRC Press: Boca Raton, FL, USA, 2021; pp. 89–111.
54. Lidster, K.; Owen, K.; Browne, W.J.; Prescott, M.J. Cage aggression in group-housed laboratory male mice: An international data crowdsourcing project. *Sci. Rep.* **2019**, *9*, 15211.
55. Hylander, B.L.; Repasky, E.A.; Sexton, S. Using mice to model human disease: Understanding the roles of baseline housing-induced and experimentally imposed stresses in animal welfare and experimental reproducibility. *Animals* **2022**, *12*, 371.

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.